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Identification and promoter analysis of some important storage protein genes from wheat (*Triticum aestivum* L.)

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Abstract

A cDNA library was constructed using wheat seeds from a cultivar PH82-2-2 with superior bread-making quality, and differential hybridization was conducted by using RNA probes of unpollinated ovary, embryo and endosperm with 10, 000 clones from the cDNA library. 99 out of 800 clones, representing 22 unique genes, were identified as storage protein genes through sequencing blast. These 22 genes encode low molecular weight glutenin subunits (LMW-GS), high molecular weight glutenin subunits (HMW-GS), gliadin and avenins. Three clones 6A4, 6H11 and 8G17 encoding storage proteins were confirmed to express specifically in endosperm via Northern blot analysis. Southern blot result indicated there are at least three copies of 6A4 in wheat genome. A 2-kb promoter of 8G17 was isolated by Universal GenomeWalker^{TM} Kit. The analysis of *cis*-acting elements revealed 8G17 promoter contained endosperm related transcriptional elements. The functional genomics data in this paper will provide valuable information for improvement of wheat flour quality.

Keywords: *Triticum aestivum* endosperm avenin-like proteins low molecular weight glutenin subunit High molecular weight glutenin subunit gliadin promoter.
Abbreviations: HMW-GS, high molecular-weight glutenin subunit; LMW-GS, low molecular-weight glutenin subunit; EM, endosperm motif; GLM, GCN-4 like motif; ZSV, Zeleny sedimentation value; CaMV, cauliflower mosaic virus; DAP, days after pollination.

Introduction

Wheat is the dominant cereal field crop in the world-wide. It is a staple food source for making bread, noodles, cakes and etc. The seed proteins are mainly storage proteins, and unique visco-elastic properties conferred by storage proteins deposited in the endosperm determine wheat flour's suitability for making a variety of food products (Shewry et al., 1997). Two major groups of storage proteins are monomeric gliadins and polymeric glutenins, which form >90% of the total protein content of cereal. Wheat glutenins include high molecular-weight glutenin subunits (HMW-GS) and low molecular-weight glutenin subunits (LMW-GS) (Payne, 1987). Tanaka et al. (2005) demonstrated that LMW-GS affected dough strength significantly in common wheat. LMW-GS genes were classified into 12 groups in Japanese soft wheat Norin 61 (Ikeda et al., 2006) and hexaploid wheat (Huang & Cloutier, 2008). Recently, Dong et al. (2010) analyzed the organization, expressing and functional mechanism of 14 LMW-GS genes in a bread wheat cultivar Xiaoyan 54 and demonstrated these genes contribute quantitatively to bread-making quality. Another class of storage protein related to gliadins and glutenins is avenin, which has two types of a and b. A-type avenin corresponds to the LMW gliadins (Anderson et al., 2001), and b-type avenin was also identified in wheat (Chen et al., 2008). A large scale identification of some endosperm specific storage protein genes will provide important information to improve the bread-making quality of wheat. The synthesis of storage proteins are regulated in temporal and spatial manner primarily in transcriptional level (Sorensen et al., 1989). Several elements involved in the regulation of seed specific expression have been identified. The most conserved element is termed the bifactorial endosperm box or –300 element, which consists of the prolamin box (P-) or endosperm motif (EM) (Weiher et al., 1983) and the GCN4-like motif (GLM) (Hill et al., 1986). G-box motif (Norre et al., 2002) and ACGT motif (Washida et al., 1999) were reported to be also involved in endosperm expression. In addition, although the transformation systems for three most important cereal species wheat, maize and rice have been successful (Vail, 1999), lack of strong endosperm specific promoters is still a limitation for obtaining the required level and pattern of expression. Therefore, the isolation and characterization of seed specific promoters are important for the understanding of the regulation mechanism on the storage proteins and providing an important tool for cereal quality improvement.In this paper, we constructed a cDNA library of wheat seeds from a wheat cultivar with superior bread-making quality. Through differential hybridization, we identified 22 storage protein genes, among which several genes have high repeat numbers and were confirmed to express specifically in endosperm by Northern blot analysis. These storage protein genes potentially are good target genes for improving wheat bread making quality. In addition, an endosperm specific

promoter of 8G17 was cloned, which will play important roles for driving specific genes' expression in endosperm.

Results

Characterization of storage protein genes by differential hybridization

A cDNA library of wheat (*Triticum aestivum* L.cv. PH82-2-2 with superior bread-making quality) seeds at 12 DAP was constructed, which contained $5.0x10^5$ clones. 10,000 clones (2% of total clones) were randomly picked up for plasmid DNA isolation and further hybridized with three RNA probes derived from ovary, embryo and endosperm. Sequencing results of 800 clones showed that they represent 216 unique genes. Fig. 1 showed a flow-chart of diagram depicting various steps of differential hybridization with three probes from ovary, embryo and endosperm. We observed 99 out of 800 clones belonged to genes encoding storage proteins, which represent 22 unique genes, indicating storage protein genes were expressed abundantly in the wheat seeds at 12 DAP (Table 1). The 22 storage protein genes contain 11 LMW-GS genes, 2 HMW-GS genes, 6 gliadin genes and 3 avenin genes. It is obvious that we obtained more LMW-GS clones (58 clones representing 11 genes) than HMW-GS clones (7 clones representing 2 genes), suggesting that more LMW-GS genes were expressed in developing wheat seeds than HMW-GS genes. One interesting finding is that some of the storage protein genes have high number of repeats, for example, 13 cDNA clones have 100% nucleotide sequence similarity with 6H11, although we only displayed the clone 6H11 which has the largest cDNA insert in the cDNA library in comparison of the other 13 clones. Some of these storage protein genes were expressed only in endosperm, and others were expressed in both embryo and endosperm, but not in ovary, indicating pollination induced their expression. Another interesting finding is that we detected 3 avenin-like genes derived from 10 cDNA clones, especially, the clone 6A4 has 7 repeat clones, indicating these clones may play important roles for bread-making quality. Blastn results of the three avenin-like genes showed that they represent three different genes. 6A4 (AF469489) has no significant similarity with the cDNA clones BU607255 and BU607262, between which there has only 59-bp out of 608-bp sharing the same sequences (Table 1).

Identification of the three genes encoding storage proteins

Two clones named 6H11 and 8G17 were hybridized with only endosperm probes only; the third clone 6A4 had hybridization signals with endosperm and embryo probes (Table 1). Two clones were registered in Genbank with Accession No. AF469489 for 6A4, and HQ833341 for 8G17. Sequencing results showed that 6A4 has 95% similarity with a putative avenin-like b precursor (AM087941); 6H11 has 91% similarity with LMW-GS GluA3-13 (FJ549930) (Wang et al. 2010); 8G17 has 100% similarity with LMW-GS group 5 type III (AB062867) (Supplementary material). Clones 6A4 and 6H11 had 7 and 13 identical clones among the clones we have sequenced, indicating these two clones had abundant expression in the developing wheat seeds.

The expression pattern of the three storage protein genes

To investigate whether the three storage protein genes selected were expressed in endosperm-specific manner, Northern hybridization was performed. As Fig. 2 shows, the expression of 6A4, 6H11 and 8G17 transcripts were detected in endosperm of 12 days after pollination (DAP), but not in leaf, un-pollinated ovary, embryo of 12 DAP, indicating the three clones selected were expressed preferentially in endosperm. The clones 6A4 and 8G17 displayed very strong hybridization signals, suggesting they had abundant expression in the wheat endosperm (Fig. 2). We observed Northern blot signals for the three clones were not a single band, instead, a wide range of smears, suggesting some homologous genes of these three clones were hybridized with these three clones' probes.

Southern blot analysis of 6A4

To investigate the copy number of 6A4 gene in the wheat genome, Southern hybridization was carried out by using a 685-bp fragment of 6A4 as a template for probe synthesis. The hybridization result indicated that at least three bands were detected in the wheat genomic DNA digested with *EcoR*I and *Hind*III, respectively (Fig. 3), suggesting that there may have at least three copies of 6A4 in the wheat genome.

Sequence analysis of the clone 8G17 5' –flanking region

A 2-kb 5' flanking region of the clone 8G17 was obtained through Universal GenomeWalker™ Kit (Fig. 4a), which comprises the region from a *Hind*III restriction site at position 1 to the ATG at position 2019 (Fig. 4b). The promoter region of 8G17 contains the TATA box (-79) and CAAT box (-165) necessary for the functions of promoters. Additionally, 8G17 promoter has endosperm specific motifs such as GLM motif (-555), three Prolamin boxes (-112, -569, -1060), ACGT motif (-1522), EM (-298), six E-box motifs (-1932, -1906, -1827, -1689, -587, -235) and one RY like motif (-655) (Table 2). Transgenic Arabidopsis plants transformed with PBI121 containing 8G17 promoter or cauliflower mosaic virus (CaMV) 35S promoter driving *GUS* gene were germinated and stained with X-gluc. Arabidopsis plants transformed with CaMV35S promoter driving *GUS* gene had blue staining for the whole plant, however, plants with 8G17 promoter driving *GUS* gene did not show any blue staining spots for root, leaf, stem and flower (data now shown).

Discussion

In our study, we performed differential hybridization by using endosperm, embryo and ovary probes with 10,000 cDNA clones derived from a cDNA library of wheat seeds of a superior bread-making cultivar PH82-2-2. Based on 800 sequenced cDNA clones, we identified 22 storage protein genes, which encode LMW-GS glutenins, HMW-GS glutenins, gliadins and avenins. This is a pioneer report using cDNA library of wheat seeds to identify storage protein genes. We identified several highly abundant expressing LMW-GS genes, one gliadin gene and one avenin-like gene according to repeat number of these genes (Table 1) and Northern blot results (Fig. 2), which potentially will be good target genes for bread-making quality improvement.

Storage protein genes

Consistent with the report by Huang and Cloutier (2008) that most of LMW-GS genes are located in the D-genome, we obtained 7 LMW-GS genes from *Glu-D3* loci, while only 2 genes were identified from *Glu-A3* and *Glu-B3* loci. The high

Table 1. A list of cDNA clones highly homologous with storage protein genes retrieved from wheat. A total of 800 clones detected with endosperm and/or embryo probes by differential hybridization were sequenced using ABI 377 DNA sequencer. 22 genes were confirmed to be wheat storage protein genes by tblastx in Genbank, and most of them were deposited in Genbank. The clones with same sequences were treated as one gene and the one with longest sequence was shown in this table.

Accession No.	Putative products	Identity (aa)	Tissue	No. Clones
BU607215	LMW glutenin subunit (GluA3-4), FJ549945	261/270 (97%)	Endosperm Embryo	11
BU607218	LMW-m glutenin subunit 0099E23-M (Glu-D3) gene, EU189096	195/197 (99%)	Endosperm	10
BU607202	LMW glutenin subunit B3-4 (LMW-GS) gene, FJ824790	210/219 (96%)	Endosperm Embryo	6
BU607212	LMW-glutenin P3-6 (GluD3-6) gene, DQ457420	225/225 (100%)	Endosperm	5
BU607209	LMW glutenin subunit (GluB3-6) gene, EU369712	265/273 (97%)	Endosperm Embryo	7
HQ833341 (8G17)	LMW glutenin subunit group 5 type III, AB062867	320/320 (100%)	Endosperm	1
BU607165	LMW-GS P-32 (GluD3-3) gene, DQ357058	148/148 (100%)	Endosperm	1
BU607188	LMW-glutenin P3-41 (GluD3-4) gene, DQ457416	230/242 (95%)	Endosperm embryo	1
BU607171	LMW-GS P-12 (GluD3-1) gene, DQ357053	209/216 (97%)	Endosperm Embryo	1
BU607167	LMW glutenin subunit LMW-GS P-21 (GluD3-2) gene, DQ357055	240/247 (98%)	Endosperm	1
6H11	LMW glutenin subunit (GluA3-13) gene, FJ549930	349/382 (91%)	Endosperm	14
BU607180	Alpha-gliadin storage protein, U51303	235/238 (99%)	Endosperm Embryo	18
BU607217	Gamma-gliadin clone 10d11, Af234650	183/185 (99%)	Endosperm	2
BU607230	Gamma-gliadin clone G2656, AF234649	244/246 (99%)	Endosperm	1
BU607194	Alpha-/beta-gliadin class A-II, M10092	173/174 (99%)	Endosperm	2
BU607174	Alpha-/beta-gliadin A-IV, M11075	208/214 (98%)	Endosperm Embryo	1
BU607166	Alpha/beta-gliadin precursor, K03076 X00627	113/141 (80%)	Endosperm	2
AF469489 (6A4)	Putative avenin-like b precursor, AM087941	189/199 (95%)	Endosperm Embryo	8
BU607262	Avenin-like b gene, HM027637	198/204 (97%)	Endosperm Embryo	1
BU607255	Avenin-like precursor, AM087940	165/169 (98%)	Endosperm Embryo	1
BU607197	HMW glutenin subunit 1By15 gene, EU137874	258/290 (90%)	Endosperm Embryo	5
BU607198	HMW glutenin subunit 1Ax1 gene, X61009	190/203 (94%)	Endosperm Embryo	2

Table 2. Putative regulatory elements in the 8G17 promoter.

Motif	Position	Sequence in the promoter
TATA box	-79	TATAAAT
CAAT box	-165	CAAT
GCN4 motif	-553	TGAGTCA
GCN4-like motif (GLM)	-555	GATGAGTCAT
Prolamin box	-112,-298, -569, -1060	TG(T/A/C)AAA(G/T)
ACGT motif	-1522	GTACGTG
Endosperm motif (EM)	-298	TGTAAAGT
E-box motif	-1932, -1906, -1827, -1689, -587, -235	CANNTG
RY like motif	-655	CATGCAAG

repeat number of LMW-GS genes and gliadin genes indicated the abundant expression of these genes, separately. This finding is in consistent with the observation of Kawaura et al. (2005) that the expression of LMW-GS genes and gliadin genes was substantially induced at 10 DAP, and gradually decreased as seeds mature. As demonstrated by Dong et al. (2010), the active LMW-GS genes expressed from *Glu-3* loci may largely determine the effect on Zeleny sedimentation value (ZSV), an important indicator of bread-making quality. Therefore, the high abundance of LMW-GS genes expression may contribute to the good bread-making quality of wheat cultivar PH82-2-2. Chen et al. (2008) observed one avenin-like gene was expressed only in endosperm, but not in root, leaf and inflorescence tissues. However, based on the differential hybridization results, we detected two different avenin-like genes (Genbank accession No. BU607262 and BU607255) had expression in embryo besides endosperm. Although 6A4 was detected with embryo and endosperm probes in differential hybridization, Northern blot results showed it only expressed in endosperm. Although Kan et al. (2006) found that the transcripts of avenin-like genes are more abundant in Aegilops species than in bread wheat, in our study, we observed around 10% (10 out of 99 clones) cDNA clones are belonging to avenin-like genes, suggesting that avenin-like genes may also play important roles for determining the bread-making quality of wheat.

Promoter of 8G17 gene

The expression of seed storage protein genes are regulated in a spatial and temporal manner, thus the promoters of storage protein genes will be valuable resources for controlling the expression of transgenes in desired tissues for plant improvement and molecular farming. One good example is that rice as a bioreactor has been used to improve it's nutrient quality by seed specific promoters (Takaiwa, 2007). Several elements have been confirmed to be involved in the seed specific expression of seed storage protein genes, such as GCN4 motif, Prolamin box, ACGT motif, RY repeat motif and E-box (Thomas, 1993). ACGT sequence corresponds to the core sequence of the binding site for basic leucine zipper transcriptional factors (Foster et al., 1994). O2 has been shown to activate genes of storage proteins in wheat seeds through interaction with GCN4 motif (Holdsworth et al., 1995). PlantCARE analysis of 8G17 promoter region indicated that it has all the required elements for endosperm specific expression, such as EM, GCN4 motif, Prolamin box, ACGT motif, RY repeat motif and E-box. Prolamin box repeated many times in the 5' flanking regions of a rice glutenin gene (Washida et al., 1999). Rice glutenin AACA motif together with GCN4 motif and ACGT box are necessary to mediate endosperm expression of the rice *GluB-1* glutenin gene (Washida et al., 1999), however, 8G17 promoter did not have AACA motif. Transgenic Arabidopsis plants transformed with 8G17 promoter driving *GUS* gene showed no blue staining for the vegetable tissues such as root, leaf, stem and flowers compared to the CaMV35S promoter, suggesting 8G17 promoter may drive *GUS* gene expressing specifically in seeds. Although seed staining was not shown blue for Arabidopsis plants transformed with 8G17 promoter driving *GUS* gene, we think the reason may be the endosperm of the Arabidopsis seed is surrounded by the maternal seed coat and is eventually consumed by the developing embryo (Berger, 1999; Brown et al., 1999; Olsen, 2004).

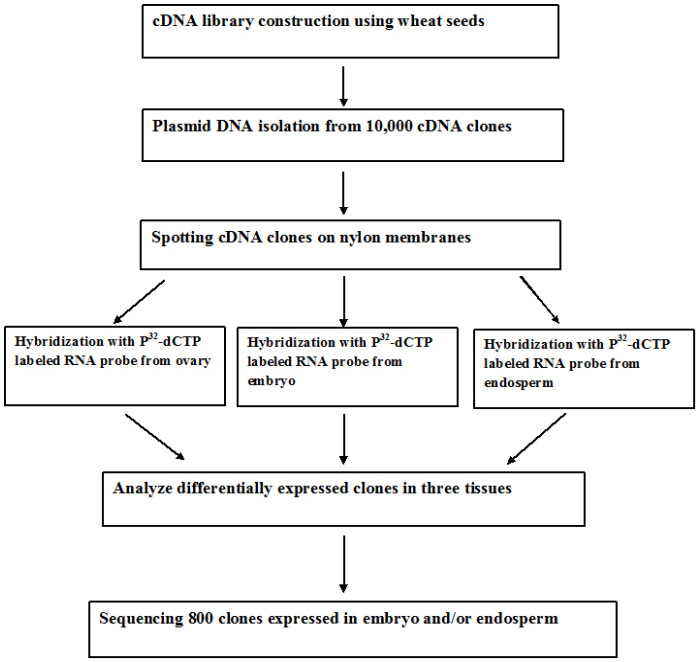

Fig 1. Flow-chart of diagram depicting various steps of differential hybridization. cDNA library was made as follows: wheat seeds at 12d after pollination was used for RNA isolation followed by cDNA synthesis, and cDNAs were ligated to pBluescript Ⅱ SK(+) and transformed into *E.coli* JM109 (TaKaRa, Dalian,China). Three replications of nylon membranes were spotted with 10,000 plasmid DNAs from 10,000 cDNA clones using the Biomek 2000 HDRT system. Three probes were prepared by reverse transcription of 10 μg total RNA of ovaries at the anthesis, embryos and endosperms at 12d after pollination with RAV-2 reverse transcriptase. The selected clones were sequenced with ABI 377 DNA sequencer (PerkinElmer, USA).

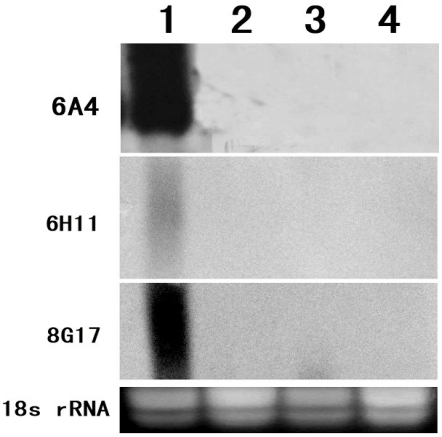

Fig 2. Northern blot analysis of the clones 6H11, 6A4 and 8G17. Twenty micrograms of total RNA were loaded for each sample and hybridized with the three different probes derived from 6A4, 6H11 and 8G17. Lane 1, endosperm at 12 DAP; lane 2, leaf; lane 3, non-pollinated ovary; lane 4, embryo at 12 DAP. 18S rRNA as a control. This result showed that the three clones are specifically expressed in endosperm, and 6A4, 8G17 have strong expression.

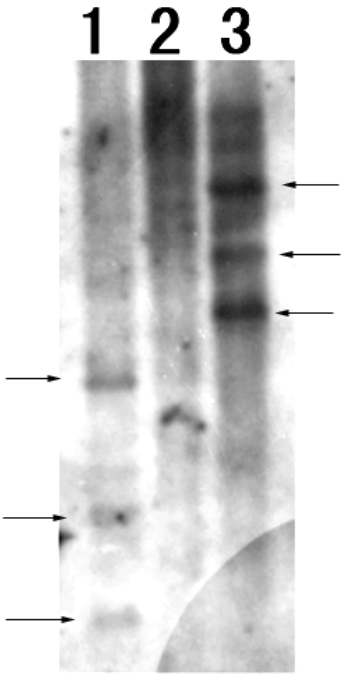

Fig 3. Southern blot analysis of clone 6A4. Genomic DNA (15 µg) isolated from young leaves was digested with *Hind*III (lane 1) and *Xba*I (lane 2) and *Eco*RI (lane 3), respectively, and hybridized with ^{32}P-labeled 685-bp fragment of 6A4. From lane 1 and lane 3, we can see this gene 6A4 has at least 3 copies in wheat genome (arrows show the hybridization bands).

Materials and methods

Plant materials

Plants of wheat (*Triticum aestivum* L. cv. PH82-2-2) were grown in the greenhouse at the campus of Shandong Agricultural University, China. The whole immature seeds at 12 DAP for later cDNA library construction and young endosperm, embryo, leaf, root, ovary tissues were harvested into liquid N_2 and stored at – 80 ºC.

Screening of storage protein genes from cDNA library

A wheat cDNA library was constructed using wheat immature seeds of 12 DAP. Plasmid DNAs of 10,000 clones randomly picked out from the library were prepared. Construction of cDNA library, differential screening and sequence analysis were described in our paper published (Li et al., 2004). Breifly, twenty µL 10,000 extrated plasmid DNAs were transferred into the wells of 384-well plates, and denatured with equal volume of 0.4 mol/LNaOH. After that, the denatured DNA was spotted onto nylon membranes using the Biomek 2000 HDRT system. Three probes were prepared by reverse transcription of 10 mg total RNA of ovaries at the anthesis, embryos and endosperms at 12 DAP with RAV-2 reverse transcriptase. Hybridization was carried out at 65℃ for 4 h, in 6×SSC, 5×Denhart's, 6% SDS and 20 mg/mL salmon sperm DNA. A total of 800 clones were chosen from the hybridization signals detected with endosperm and/or

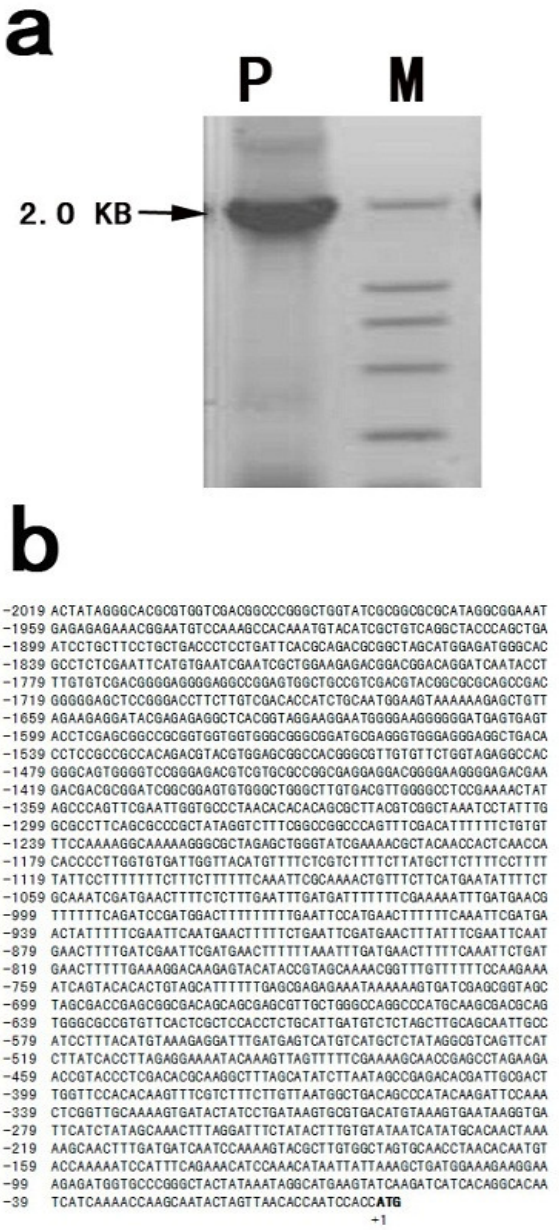

Fig 4. Cloning of 8G17 promoter. a, Cloning of 8G17 promoter by PCR. Genomic DNA of wheat cultivar PH82-2-2 was isolated and digested by *Eco*RV. Digested genomic DNA was ligated to GenomeWalker Adaptors. A fragment (about 2 kb) was amplified from the digested genomic DNA by primers 5'-CATTTGTGCAATGGCACTTGTCGCC-GCA-3' and 5'-TGCCTGTGATGATCTTGATACTTCAT-GC-3' with anckor primer P1 and P2. PCR reaction was performed as follows: 7 cycles: 94 ºC 25 sec, 72ºC 3 min; 32 cycles: 94ºC 25 sec, 67ºC 3 min; 67C for an additional 7 min after the final cycle. P, 8G17 promoter; M, DNA marker DL-2000. b, Nucleotide sequence of 8G17 promoter. The start codon ATG is marked as +1. The number of nucleotides from ATG to the upstream 5' flanking region is 2019. The 8G17 promoter region has been deposited in Genbank (AY436766).

embryo probes (Fig. 1). Sequencing of 800 clones was performed with ABI 377 DNA sequencer (PerkinElmer, USA). Blastn and tblastx were used for the sequences blast in Genbank.

Northern blot analysis

To verify whether the three clones 6A4, 6H11 and 8G17 were expressed in tissue-specific manner, Northern hybridization was performed as described by Sambrook et al. (1989). Twenty μg of total RNA was fractionated by gel electrophoresis in 1.2 % formaldehyde agarose gels and transferred from agarose gels to nylon membrane. Pre-hybridization was performed at 42 ℃ for 12 h. Hybridization was conducted at 42 ℃ for 48 h. 482-bp fragment of 6A4, 436-bp fragment of 8G17, 380-bp of 6H11 digested by *Not*I and *EcoR*I were used as the template for the synthesis of probe. The position of these templates for synthesizing probes were shown in Supplementary material. Filters were washed subsequently in 2 × SSC with 0.2 % SDS and 0.2 × SSC with 0.2 % SDS. Autoradiography was performed at –70 ℃.

Southern blot analysis

Genomic DNA was extracted from fresh leaf tissues of wheat using CTAB method (Dellaporta et al., 1983). Ten micrograms of genomic DNA were digested with EcoRI, HindIII, XbaI restriction enzymes at 37℃ overnight, respectively, separated on 1% agarose gel and transferred to nitrocellulose membrane. Pre-hybridization was done at 65℃ overnight. The probe was synthesized using the 482-bp fragment of 6A4 as the template. Hybridization was carried out at 65℃ for 24 h. Filters were subsequently washed in 2×SSC with 0.2% SDS and 0.2×SSC with 0.2% SDS. Autoradiography was performed at –70℃.

Isolation and cis-element analysis of the promoter of 8G17

Extraction of wheat genomic DNA was conducted as described by Dellaporta et al. (1983). The promoter of 8G17 was isolated according to Universal GenomeWalker Kit (CLONTECH Laboratories, Inc., CA, USA). Genomic DNA was digested by *EcoR*V and was purified. Digested genomic DNA was ligated to GenomeWalker Adaptors. A fragment (about 2kb) was amplified from the digested genomic DNA (primers: 5'-CATTTGTGCAATGGCACTTGTCGCCGCA-3' and 5'-TGCCTGTGATGATCTTGATACTTCATGC-3'; anckor primer P1 and P2). PCR reaction was performed as follows: 7 cycles: 94℃, 25 sec, 72℃, 3 min; 32 cycles: 94℃, 25 sec, 67 ℃, 3 min; 67℃ for an additional 7 min after the final cycle. A 2-kb 8G17 promoter region has been deposited in Genbank (Accession number AY436766). *Cis*-acting elements of 8G17 were predicted using PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html). The promoter of the clone 8G17 in pGEM-T vector was cut out with *Hind*III and *BamH*I, and subcloned into pBI121 upstream of the *GUS* gene. The 8G17-GUS construct was first introduced into *Agrobacterium tumefaciens* C58c1 and verified by using PCR and sequencing. The empty pBI121 including CaMV35S promoter driving *GUS* gene was transformed to C58c1 as control. The transformation was conducted using floral dip methods (Clough and Bent, 1998). Histochemical GUS assays were performed as described by Jefferson et al. (1987).

Conclusions

Twenty-two unique storage protein genes were identified by differential hybridization of a wheat cDNA library using wheat developing seeds from PH82-2-2 with superior bread-making quality. Out of the 22 storage protein genes, three genes were confirmed to have strong expression in endosperm which should be good target genes for improving wheat bread-making quality. Further, the analysis of *cis-acting* elements revealed 8G17 promoter contained all required endosperm related transcriptional elements, which will be a valuable resource for cereal quality improvement.

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