Volume 53(1):9-15, 2009
Acta Biologica Szegediensis
http://www.sci.u-szeged.hu/ABS

ARTICLE

PCR based isolation and cloning of HMW glutenin gene(s) from wheat (*T. aestivum* var. PBW343) and its fusion with kafirin gene promoter of sorghum

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ABSTRACT With the aim of grain sorghum improvement to enhance its economic value, HMW glutenin gene subunit(s) were isolated from wheat (*T. aestivum* var. PBW343) using allele specific primers (AS-PCR) designed on the basis of allelic data available in NCBI Gene Bank. Three glutenin genes *viz.* Ax1, Dx5 and Dy10 were isolated, cloned, sequenced and analyzed using on line bioinformatics tools. These sequences showed significant homology with the HMW glutenin gene sequences available in the NCBI gene bank. To facilitate transformation of these genes to sorghum, these genes were transferred to a pUC based vector under the regulation of kafirin promoter of sorghum which makes it specific for expression in the endosperm of sorghum. HMW glutenin gene cloned in pGEM vector was excised using restriction enzymes SacI and SacII and directional cloning was performed successfully to clone under the control of kafirin gene promoter of sorghum. Transfer of these gene(s) could be useful for improving the bread making quality of sorghum.

Acta Biol Szeged 53(1):9-15 (2009)

KEY WORDS

cloning
gene cassette
glutenin
kafirin
promoter
sorghum
wheat

Sorghum (*Sorghum bicolor* L.) is a major food crop in the semi-arid tropics of Asia and Africa. In these areas where the people are poor and food resources are limited, sorghum is a major source of protein. Traditionally, in the developing countries, sorghum is consumed as porridge or flat bread. It contains similar levels of starch and protein as in other cereals and hence is considered nutritious. However, some factors like poor dough making quality, low digestibility and presence of tannin make it less palatable. Although sorghum is widely used and consumed but poor shelf life and inability to make soft, fluffy, palatable and easily digestible roti or bread limits its role in the economic empowerment of sorghum producers and end users.

For the regular use of sorghum it is important that it must have good bread making quality as that of wheat. Recombinant DNA technology has tremendous potential to enhance the bread making quality of sorghum grain by transferring the bread making character of bread wheat into sorghum. After a very long investigation, it was found that the glu D1 encoded HMW glutenin subunit pair 5+10 and glu 1Ax1 is associated with greater dough strength of the wheat (Shewry and Halford, 2002). Ovidio and Anderson (1994) confirmed the role of glu D1 (Dx5- Dy10) in bread making quality of wheat. They analyzed that the y- type subunits are the main components responsible for dough making quality of the flour while x-type subunit have only minor effect.

Altpeter et al. (2004) generated and characterized transgenic rye synthesizing substantial amounts of high-molecular-weight glutenin subunits (HMW-GS) from wheat. They reported that the amount of polymerized glutelins was significantly increased in transgenic rye, more than triple as compared to the wild type. The expression of wheat HMW-GS in rye leads to a high degree of polymerization of transgenic and native storage proteins, probably by formation of intermolecular disulfide bonds. This is an important step towards improving bread-making properties of economically important grains.

HMW-GS significantly contributes towards dough improvement and for these, glutenin alleles of wheat are important genetic resources for quality improvement. For the better use and quality improvement, it is important to use the indigenous sources of the genes. Indian resource of the genes is well acclimatized in Indian condition and there will be zero risk of the gene flow, as the genes are well established. Exotic genes may disturb our gene pools emerging and also by drifting native genes. Biosafety regime also encouraged the use of indigenous resources. Indian cultivated wheat var. PBW 343 was a good choice for the glutenin genes. Compared to other wheat cultivars, it has Dx5-10 and 1Ax1, glu gene alleles. With the aim of improvement of grain sorghum to enhance its marginal value for poor consumers and producers, the pres-

Accepted June 10, 2009
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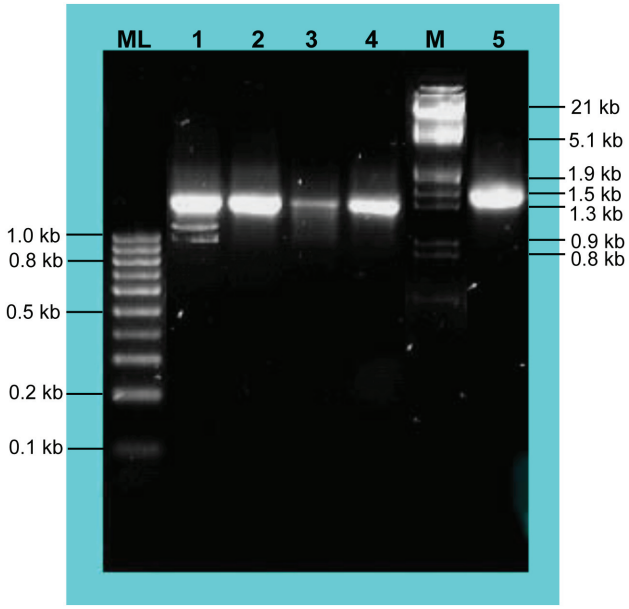

Figure 1. Amplification of HMW glu gene(s), using allele specific primer. Lane-ML : Marker 100 bp ladder, Lane- 1 : glu Dy10 (3 band app. Size 2.0, 1.8 & 1.0 KB), Lane-2 & 5: glu Dx5 (band app. Size 2.0 kb), Lane-3 & 4: glu 1Ax1 (band app. Size 1.8 kb), Lane- M : Marker Lambda DNA dd Hind III/ EcoR I.

ent study was done, in which HMW glutenin genes, 1Dx5, 1Dy10 and 1Ax1 were amplified using PCR with designed gene specific primers and fused with the kafirin gene promoter of sorghum which is previously isolated and characterized (Mishra et al. 2008).

Material and Methods

Isolation of genomic DNA

Seeds of wheat (*Triricum aestivum*) variety PBW-343 were procured from Crop Research Center G.B. Pant University, Pantnagar, India. The variety was selected as it shares alleles x1(Glu-A1), x5+y10 (Glu-D1), x7+y9 (Glu-B1). One gram of etiolated leaves were taken and weighed. These were cut into small pieces and then frozen in liquid nitrogen. The frozen leaves were grinded into fine powder in a pre- cooled mortar with the help of a chilled pestle. Powder was transferred into 50 ml centrifuge tube (Oakridge tube, Labware, USA) containing 15 ml DNA extraction buffer (0.1 M Tris, 0.05 M EDTA, NaCl, pH 8.0) and 1 ml of 20% SDS. After proper mixing, the tube was incubated in a water bath (Hoefer, USA) at 65°C for 10 minutes. 10 ml of 5 M Potassium acetate was added and mixed the content of the tube gently and further incubated on ice for 20 minutes. Tube was centrifuged at 10,000 rpm for 25 min at 4°C in Kendro Biofuge refrigerated centrifuge. Supernatant was collected in a fresh oakridge tube and 15 ml isopropanol was added and mixed gently. The tube was incubated at -20°C for 1 hr and then centrifuged at 10,000 rpm at 4°C for 15 min to collect the DNA pellet. Gently poured off the supernatant and dried the pellet. Pellet was dissolved in 1 ml TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). RNase treatment was given to genomic DNA as RNase stock (10 mg/ ml) was added to give final concentration of 10µg/ ml and incubated for 1 hr at 37°C. The DNA was extracted with phenol, chloroform and isoamyl alcohol (25:24:1). To the supernatant, collected in a fresh tube, 0.1 volume 3 M sodium acetate (pH 4.8) was added followed by 0.6 volume cold isopropanol. DNA was precipitated by keeping the tube

Table 1. Restriction digestion analysis of the pGEMT-HMW plasmids.

Details	Restriction enzymes		Expected No. of bands and band size		Observed No. of bands and band size	
	Enzymes used	Enzymes which have restriction sites	No. of bands	Band size	No. of bands	Band size
HMW glutenin gene Dx5	SmaI,ClaI, DraI, XmaI, SmaI, XbaI, SacI, SacII, SalI, EcoRI, EcoRV, BamHI, BglI, KpnI, PstI, NotI,SphI, SpoI	HindIII	2	500 bp 1.5 kb	2	500 bp 1.5 kb
		BglI	2	163 bp 1.8 kb	2	100p 1.8 kb
		SphI	2	254 bp 1.7 kb	2	200p 1.7 kb
HMW Glutenin gene Dy10	— Do —	Bam HI	2	129 bp 1.3 kb	2	100 bp 1.3 kb
		Hind III	2	265 bp 1.2 b	2	200 bp 1.2 kb
HMW Glutenin gene Ax1	— Do —	SphI	2	134 bp 1.7 kb	2	100 bp 1.6 kb
		SpoI	2	205 bp 1.6 kb	2	200 bp 1.6 kb
		HindIII	2	320 bp 1.5 kb	2	300 bp 1.5 kb

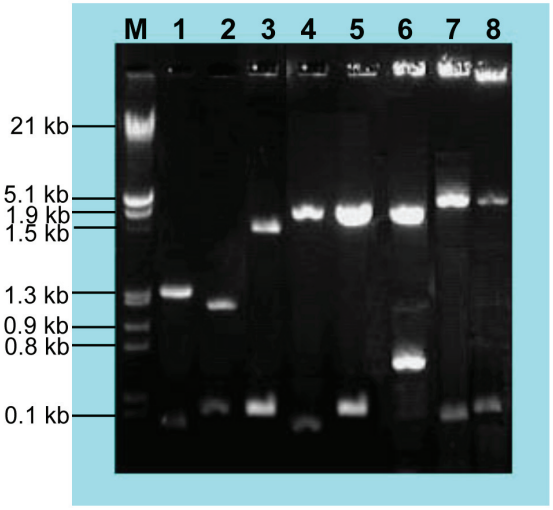

Figure 2. Restriction digestion of putative HMW glutenin gene(s) clones. Lane- M: Marker, Lane- 1: RD by Bam HI of glu Dy10 (2 band app. Size 0.1 & 1.3 kb), Lane- 2: RD by Hind III of glu Dy10 (2 band app. Size 0.2 & 1.2 kb), Lane- 3: RD by Hind III of glu 1Ax1 (2 band app. Size 0.3 & 1.5 kb), Lane- 4: RD by Sph I of glu 1Ax1 (2 band app. Size 0.1 & 1.6 kb), Lane- 5: RD by Spe I of glu 1Ax1 (2 band app. Size 0.2 & 1.6 kb), Lane- 6 : RD by Hind III of glu Dx5 (2 band app. Size 0.5 & 1.5 kb) Lane- 7 : RD by Bgl I of glu Dx5 (2 band app. Size 0.15 & 1.7 kb) Lane- 8: RD by Sph I of glu Dx5 (2 band app. Size 0.25 & 1.7 kb).

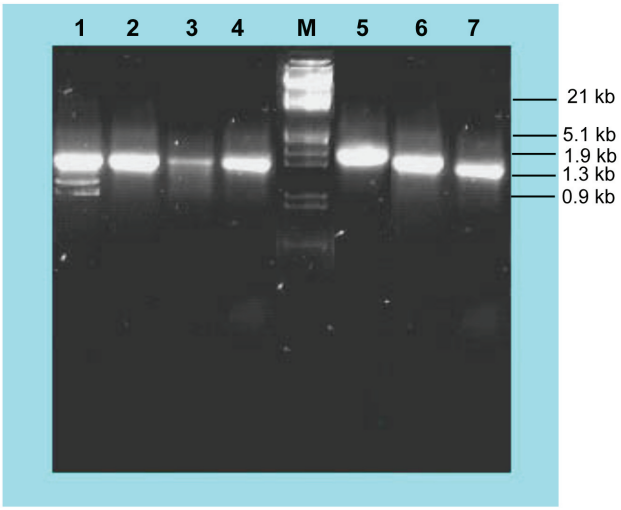

Figure 3. PCR of putative clones using allele specific primer. Lane- 1: PCR on gDNA, glu Dy10 (3 band app. Size 2.0, 1.8 & 1.0 KB), Lane-2: PCR on gDNA, glu Dx5 (band app. Size 2.0 kb), Lane-3 & 4: PCR on gDNA, glu 1Ax1 (band app. Size 1.8 kb), Lane- M: Marker Lambda DNA dd Hind III/ EcoR I, Lane- 5: PCR on putative plasmid, glu Dx5 (band size 2.0 kb), Lane- 6: PCR on putative plasmid, glu 1Ax1 (band size 1.8 kb), Lane- 7: PCR on putative plasmid, glu Dy10 (band size 1.4 kb), (g DNA- genomic DNA).

at -20°C for overnight and then pelleted by centrifuging at 10,000 rpm at 4°C for 12 min. DNA pellet was dried after washing with 70% ethanol and re-dissolved in 100 µl of TE buffer and then stored at -20°C.

PCR based amplification of HMW glutenin gene(s)

Allele specific primers (F: 5' – GAC AGT CCA CCG AGA TGG - 3', R: 5' – GCA AGC TGC AGA GAG TTC - 3' for Dy10; F: 5' – CAT GGT CCT GAA CCT TCA CC - 3', R: 5' – CAG AGA GTT CTA TCA CTG GC - 3' for Dx5 and F: 5' – CCG AGA TGA CTA AGC GG - 3', R: 5' – GCT AA CAT GGT ATG GGC T - 3' for 1Ax1) were designed for the isolation of HMW- glu gene(s) allele on the basis of HMW glutenin gene sequence available on NCBI data bank (gene accession no. X13928, X12928 and X12929 for the HMW glutenin gene Ax2, Dx5, Dy10 respectively). A master mix (25 µl) was prepared, containing 40 ng of the template DNA, 100 ng of each primer (F & R), 200 µM of each dNTPs, 1 x assay buffer and 2.0 unit of Taq DNA polymerase in a 0.2 ml of sterile, thin walled PCR tubes (Axygen). PCR was carried out in Biometra thermal cycler with initial denaturation temperature of 94°C for 5 min, subsequent 35 cycles of 94°C denaturation for 1 min, 54°C annealing for 2 min and 2 min of the extension at 72°C. Final extension was done at 72°C for 5 min. During PCR, lid temperature of the cycler was fixed at 104°C (to prevent evaporation). After the completion of PCR cycles, a sub sample (usually 10 µl) of the amplicons was analyzed on 1.8% agarose gel by electrophoresis and remaining were stored at -20°C for subsequent experiments.

Cloning of PCR amplicons

PCR amplified putative HMW glutenin gene(s) were purified using MinElute PCR Purification System (Qiagen, Germany), following manufacturer's instructions. An aliquot of the purified putative glutenin gene(s) was analyzed on agarose gel before using it in the ligation reaction to confirm its elution from the MinElute column (Qiagen, Germany). For the ligation, pGEM-T Easy vector (Promega, USA) and insert DNA were briefly centrifuged to collect contents at the bottom of the tube. A Ligation reaction (10 µl), containing 1x ligase buffer (300 mM Tris pH 7.8, 100 mM $MgCl_2$, 100 mM DDT, 5 mM ATP), 1 µl (50 ng) pGEM vector, 1 µl (3 unit) T4 DNA ligase and appropriate amount of HMW glutenin gene (insert 75 ng for Dy10, 100 ng for Dx5 and 90 ng for 1Ax1), was set up in 0.5 ml sterile tube. Ligation mixture was mixed gently and then incubated overnight at 4°C in a refrigerated water bath (Hoefer, USA). Ligation mix (5 µl) was transformed to competent cells (100 µl) of *E. coli* strain DH 5α. All putative positive clones were selected and confirmed by PCR using gene specific primers, universal T7 & SP6 primers and restriction digestion. Reselected clones were sequenced by automated DNA sequencer. Sequences of the clones were subjected for the blast and studied using online available bioinformatics tools and submitted to NCBI (www.ncbi.nlm.nih.gov) gene data bank.

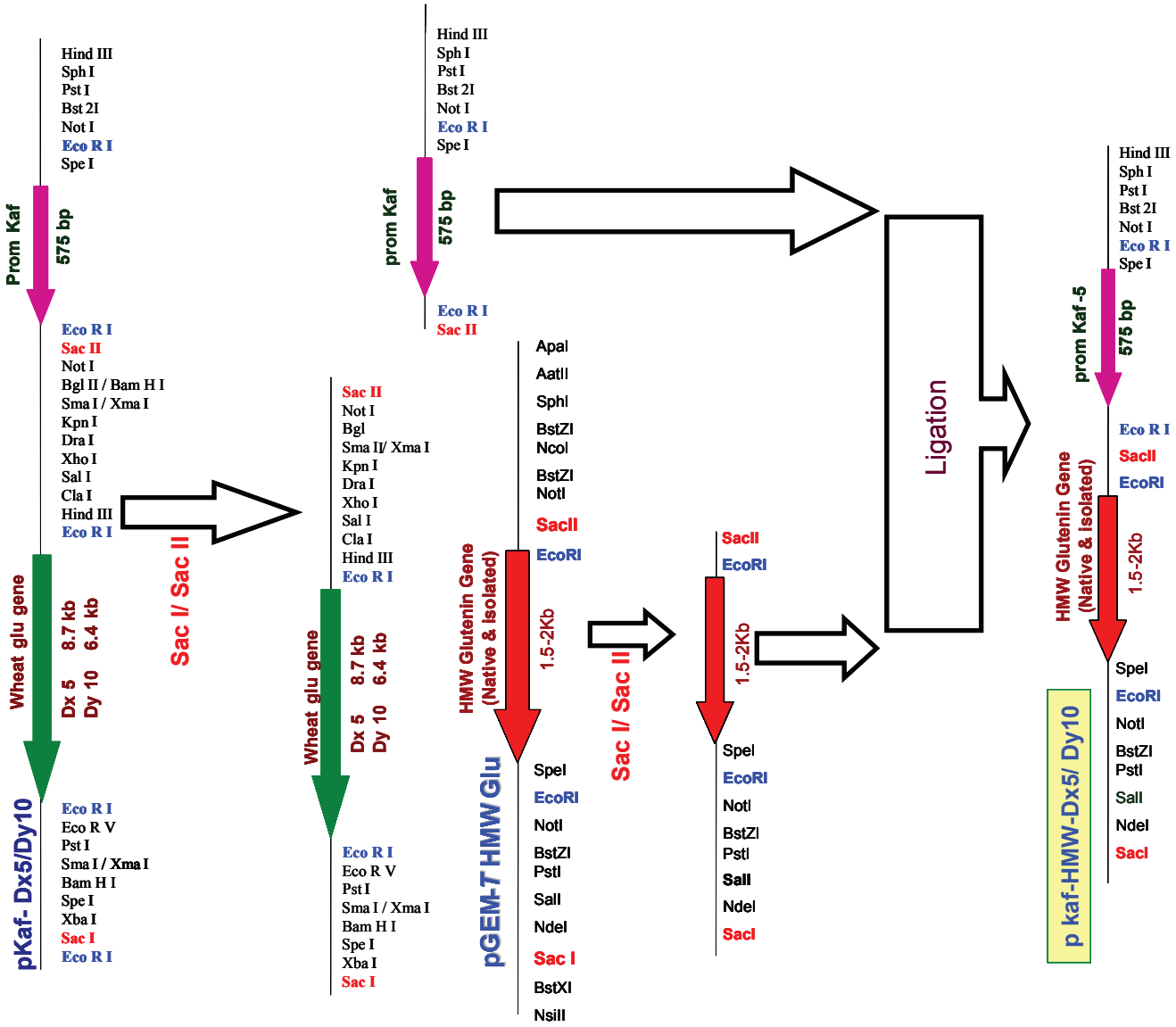

Figure 4. Strategy for construction of gene cassette pKaf-HMW-Dx5/ Dy10 (Indigenous HMW glu gene is driven by gamma kafirin promoter of sorghum).

Construction of a gene cassette (HMW glutenin gene regulated by Kafirin promoter

Restriction digestions were performed by incubating gene cassette pKaf-glu (previously constructed, Mishra et al. 2008b) and pGEM-HMW (HMW glutenin gene cloned in pGEM) with restriction enzymes SacII and SacI. A measure of 20 µl reaction mixture containing 15 µl plasmid DNA sample, 1 unit of enzyme, 2 µl of the respective digestion buffer and 1 x BSA (if needed) was prepared for restriction digestion in a 0.5 ml sterile tube. Tube was incubated at 37°C for overnight in circulating water bath. Restriction digestion was analyzed on 1.2% agarose gel by electrophoresis. Digested indigenous HMW glutenin gene and vector pkaf was gel eluted using same MinElute PCR Purification System (Qiagen, Germany). Vector without gene and indigenous HMW gluten genes both were ready to ligate. Ligation reaction (15 µl) containing, 1 µl Vector(Kafirin promoter vector), 6 µl Insert (Indigenous HMW glutenin gene), 5 unit T4 DNA Ligase and 1 x ligation buffer, was set up and tube incubated at 4°C for 12 hr, then subsequently at 8°C, 16°C and finally 22°C for 1 hr each. 5 µl of each ligated mixture was transformed aseptically in 100 µl E. *coli* DH 5α competent cells. Recombinants were selected

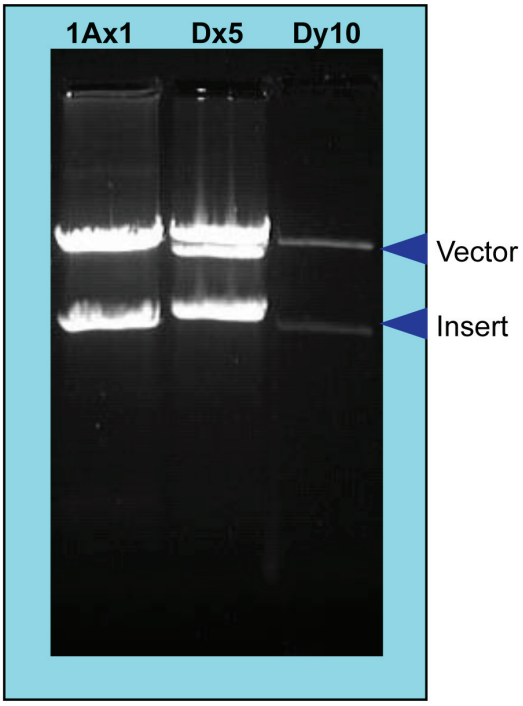

Figure 5. Elution of *glu* gene(s) from vector pGEM-T easy.

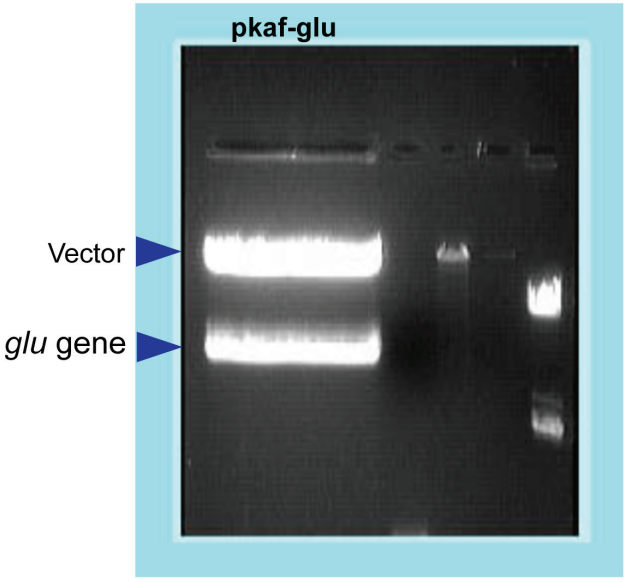

Figure 6. Elution of pkafΔglu (pKaf without *glu* gene).

and analysis of gene construct was done by restriction digestion. Recombinants were named as kaf-HMW (kaf-HMW-A, kaf-HMW-Dx and kaf-HMW-Dy for the gene cassette harboring HMW glutenin gene Ax1, Dx5 and Dy10, respectively with Kafirin promoter).

Result and Discussion

HMW gene amplification and cloning

Sun et al. (2004) developed allele-specific (AS) PCR to isolate and clone HMW-GS genes from cultivated emmer. They showed that single band of strong amplification was obtained through AS-PCR of genomic DNA from emmer. In present study, HMW glutenin gene subunits were amplified using de-

Table 2. Restriction digestion analysis of gene cassette pkaf-HMW-glu (HMW glutenin gene driven by γ- kafirin promoter of sorghum).

Clones	Restriction Enzyme	Expected Results		Observed Results (app.)	
		No. of Bands	Band Size	No. of Bands	Band Size
pkaf-HMW-1Ax (5475 bp)	EcoRI	4	3000 bp 1800 bp 575 bp 100 bp	4	3000 bp 1800 bp 500 bp 100 bp
	PstI	2	3000 bp 2475 bp		3000 bp 2400 bp
pkaf-HMW- Dx 5 (5675 bp)	EcoRI	4	3000 bp 2000 bp 575 bp 100 bp	4	3000 bp 2000 bp 500 bp 100 bp
	PstI	2	3000 bp 2675 bp	2	3000 bp 2500 bp
pkaf-HMW- Dy 10 (5145 bp)	EcoRI	4	3000 bp 1470 bp 575 bp 100 bp	4	3000 bp 1400 bp 500 bp 100 bp
	PstI	2	3000 bp 2145 bp	2	3000 bp 2000 bp

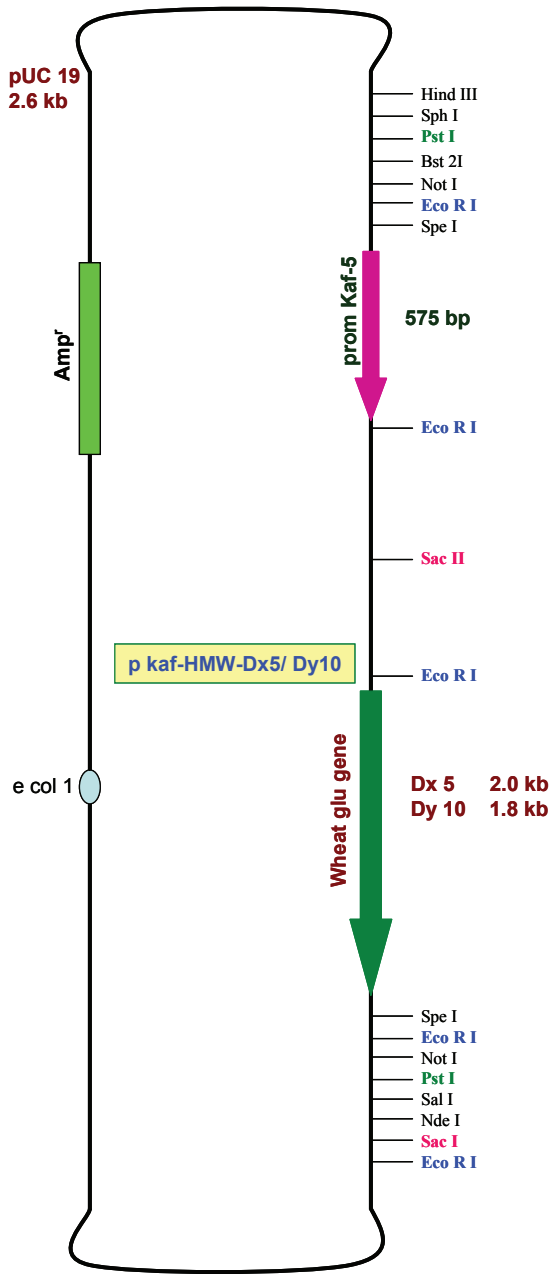

Figure 7. Restriction map of gene construct pKaf-HMW-glu gene(s).

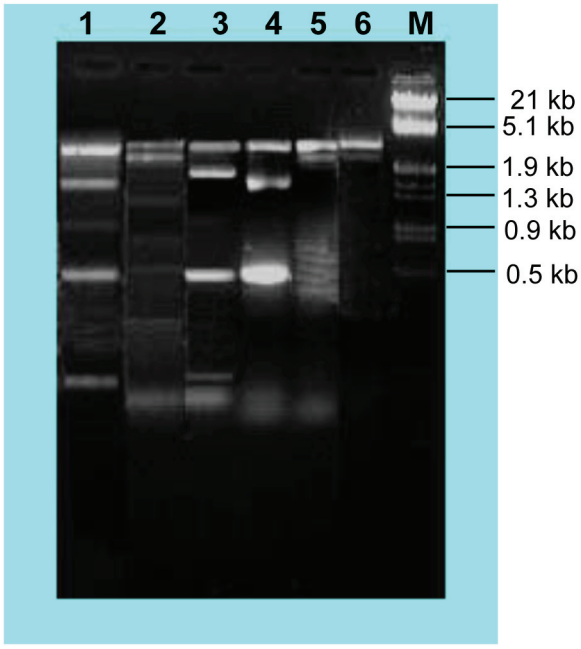

Figure 8. Restriction digestion of putative recombinants pKaf-HMW-glu (Indigenous glu gene driven by kafirin promoter). Lane- 1: RD by Eco RI of glu 1Ax1 (4 band app. Size 3.0, 1.8, 0.5 & 0.1 kb), Lane- 2: RD by Pst I of glu 1Ax1 (2 band app. Size 3.0 & 2.4 kb), Lane- 3 : RD by Eco RI of glu Dx5 (4 band app. Size 3.0, 2.0, 0.5 & 1 kb), Lane- 4: RD by Eco RI of glu Dy10 (4 band app. Size 3.0, 1.4, 0.5 & 0.1 kb), Lane- 5: RD by Pst I of glu Dy10 (2 band app. Size 3.0 & 2.0 kb), Lane- 6: RD by Pst I of glu Dx5 (2 band app. Size 3.0 & 2.5 kb), Lane- M: Molecular wt. marker.

signed gene specific primers. Three amplicons of approx. size 2.0, 1.4 & 1.0 kb were amplified with glu Dy10 primers while one intact band of 2.0 kb and 1.8 kb was found with Dx5 and 1Ax1 glutenin primers, respectively (Fig. 1). Multiple bands in Dy10 could be due to two probable reasons- first is that the primers designed for Dy10 subunits from NCBI data may not be highly specific for Dy10 of PBW 343. Therefore they could fatuously amplify some other HMW glutenin subunit like 'By' subunit from the wheat genomic DNA. Second is that due to chance the primers could anneal anywhere in the genome and amplify some other regions. PCR amplicons contain hanging 'A' (because it has been amplified by Taq DNA polymerase), so vector to be used should contain hanging 'T'. Different cloning vectors are available for the cloning of PCR products. pGEM T- easy vector system (Promega, USA) was used to clone the PCR amplicons (putative HMW glutenin genes). According to their designed primer pair, the expected size of the amplicon was 1.5 kb for the HMW glutenin subunits Dy10 therefore amplicon of size 1.5 kb was eluted from the agarose gel, quantified and cloned. Besides this Dx5 and 1Ax1 were also cloned in same vector. Putative recombinant plasmids (p-GEM-HMW) were isolated from all white colonies and electrophoresed on 1% agarose gel. The amplified PCR products were cloned at the multiple cloning sites (MCS) hence each clone will have a molecular size of approximately 5 kb. The presence of insert was confirmed by a series of restriction digestion analysis (Table-1, Fig. 2) and PCR using gene specific primers (Fig. 3). The selected recombinants for 1Ax1, Dx5 and Dy10 of size 1.8 kb, 2.0 kb and 1.47 kb were sequenced and submitted to NCBI (accession no.DQ211817, DQ211818 and DQ211819, respectively). All the sequences were searched for homology on blast tool of

the NCBI web site. All the clones showed 97-99% homology with HMW glutenin gene of *Triticum aestivum.*

Construction of a gene cassette (Kafirin promoter with indigenous HMW glutenin gene)

The main objective of the research is to develop transgenic sorghum having good bread making quality. For the expression of HMW glutenin gene, it must be driven by γ- kafirin gene promoter of sorghum. Since the glutenin gene(s) are to be transformed to sorghum, pkaf –Dx5/ Dy10, a vector engineered by us and is carrying kafirin promoter (specific for driving expression in endosperm, Mishra et al. 2008a) was selected. A gene cassette was constructed (Fig. 7) by directional cloning of HMW glutenin gene (Fig. 4) isolated HMW glutenin gene of wheat variety PBW 343 in a gene cassette pkaf- Dx5/ Dy10 (Mishra et al. 2008b) constructed earlier.

Sub cloning of indigenous HMW glutenin gene

Plasmid pGEM-HMW and gene construct pkaf- Dx5/ Dy10 (procured HMW glu gene driven by Kafirin promoter) were isolated and subjected for restriction digestion with SacII and SacI, resultant HMW glutenin gene from pGEM as well as from vector pkaf- Dx5/ Dy10 were excised. Isolated indigenous HMW glutenin genes and pkaf glu (kafirin promoter without glutenin gene) were eluted from the gel (Fig. 5 and 6, respectively) and purified. The HMW glutenin gene was cloned downstream to the kafirin gene promoter as Figure 4. Subcloned gene(s) were transformed to competent cells of *E. coli* strain DH 5 α and recombinants were selected on the selection marker ampicillin (50µg/ ml). All colonies were probable recombinants as there is no chance of self ligation (both ends are different hence non-compatible). Recombinants were named as kaf-HMW (kaf-HMW-A, kaf-HMW-Dx, kaf-HMW-Dy for the gene cassette having HMW glutenin gene Ax1, Dx5, Dy10 respectively with Kafirin promoter). Thus by directional subcloning expression vectors pkaf-HMW-Dx5 and pkaf-HMW- Dy10 were constructed (Fig. 7). Plasmid DNA of recombinants (pkaf-HMW-glu) was isolated in bulk and purified and checked by 0.8% agarose gel electrophoresis. Proper cloning was confirmed by restriction digestion with EcoRI and PstI (Fig. 8). The results of the restriction digestion was summarized in Table 2. The recombinants gave the result as per expectation and support the proper directional cloning.

Conclusion

In the present study, it has been possible to clone all the three glutenin genes *viz.* Dx5, Dy10 and Ax1 from wheat (*T. aestivum* var. PBW 343) and significant sequence homology was observed. Wheat variety PBW 343 is known to be good for bread making because of the qualities of its glutenin products. Hence transfer of these gene(s) could be useful in improving the bread making quality of sorghum to facilitate transformation of these genes to sorghum, these genes have been transferred to a pUC based vector under the regulation of kafirin promoter of sorghum which makes it specific for expression in the endosperm of sorghum.

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