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ARTICLE

Construction of gene cassette harboring HMW glutenin gene of wheat driven by γ-kafirin promoter of sorghum

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ABSTRACT Modern biological tools of genetic engineering and biotechnology can allow transfer of gene(s) across crop species. The r-DNA technology has tremendous potential to transfer bread making character of bread wheat into sorghum by transferring glutenin gene(s), which can improve the visico-elastic property of the sorghum flour/dough. These genes in addition to improving quality can significantly contribute to improve the nutritional status by the addition of more protein fractions also. In the simplest approach, new HMW gluten loci may be created via transformation to bioengineer sorghum quality. For this, amplified γ-kafirin promoter (574 bp) was subcloned in pCAMBIA1304 by replacing CaMV35S promoter (ca. 800 bp) of the *gus* reporter gene resulting in vector pkaf-gus, where the expression of *gus* reporter gene is under the control of γ-kafirin promoter. In order to construct a gene cassette where HMW glu gene(s) will be under the control of γ-kafirin gene promoter, kafirin promoter was first cloned in pUC19 and then HMW gene(s) were excised from their respective vectors and cloned under the control of promoter. Finally, two gene cassettes were developed as pKaf-Dx5 and pKaf-Dy10 where expression of the HMW glu gene Dx5 (8.7 kb) and Dy10 (6.4 kb) was driven by the γ-kafirin gene promoter. Both gene cassettes are ready to clone in any vector to bioengineer sorghum by genetic transformation
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KEY WORDS

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Sorghum improvement can be a multidirectional program. There could be different strategies for the enhancement of economic value of the sorghum. However, lysine content enhancement and dough making quality improvement are the major areas that need urgent attention. The scope to improve the nutritional and dough quality of sorghum grain protein by employing classical plant breeding seems to be limited as only low level of variations are available in sorghum gene pool for crossing. Only two mutant high lysine genes are currently available. These are spontaneous mutant gene *h1*, which was initially identified in an Ethiopian line (Singh and Axtell 1973) and *P 721 opaque* gene which was induced with ethyl methane sulphonate (EMS) (Axtell et al. 1979). Both of these lines can be defined as "low prolamin" mutants with pleiotropic effects on other grain characteristics. Hence, it has proved difficult to incorporate the high lysine phenotype into varieties with high yield and acceptable agronomic performance and processing properties.

Of all cereal grains, wheat is unique because wheat flour has the ability to form dough that exhibits the rheological properties required for the production of bread and for the wider diversity of foods. The unique properties of the wheat grain reside primarily in the gluten forming storage proteins of its endosperm. Glutenin are among the largest protein molecules in nature (Wrigley 1996) and classified as prolamins (Shewry and Halford 2002). Wheat prolamins are characterized as HMW prolamins (High molecular weight glutenin subunits, HMW-GS), S-rich prolamins (γ-gliadin, α-gliadin and B & C type of LMW-GS) and S-poor prolamins (D type of LMW glutenin). HMW-GS have been closely associated with bread making quality. After 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; Altpeter et al. 2004). D'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. Two hypotheses have been proposed to explain the superior bread/ dough-making quality of wheat cultivars possessing subunits Dx5+Dy10 compared with those possessing subunits Dx2+Dy12. The first hypothesis considers that the additional cysteine residue present in the N-terminal domain of the Dx5 subunit plays an important role in influencing the disulphide cross-linked

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glutenin network necessary for dough properties (Greene et al. 1988). The second hypothesis proposes that the higher proportion of consensus-type repeats in the Dy10 subunit gives rise to a more regular pattern of β-turns in the central repetitive domain, which is responsible for conferring better elastic properties on this subunit and consequently on the dough (Flavell et al. 1989).

Genetic engineering offers an opportunity to overcome the limitations of plant breeding and has opened up new avenues to improve the physical and nutritional quality of grain sorghum. Gene manipulation includes two major approaches. First is a gene alteration in which expression of existing seed storage protein gene(s) is enhanced by addition, deletion or substitution of certain sequences by site directed mutagenesis (Mutational breeding). Second approach is the addition of a foreign gene with novel or improved characters (Transgenic technology). Transgenic technology is worldwide used technique to enhance economic value of the crops.

Proper regulation of the expression of introgressed seed storage proteins gene is necessary for the success of a transgenic. Seed protein gene expression is not only developmentally but also metabolically regulated. Promoters including several upstream sequences are crucial and required to regulate gene expression both quantitatively and qualitatively. The regulatory sequences of promoters that define the qualitative specificity of gene expression in plants have been studied and much information has accumulated in recent years (Mishra et al. 2008). Attempts were made to express different genes in sorghum by using either constitutive promoters or gamma zein promoter however expression level was not reported (Grootboom and O'Kennedy 2003). It was therefore concluded that if the dough making quality of sorghum is to be improved by introgressing a glutenin gene, a seed specific promoter from sorghum will be better.

Alpha kafirin promoter of sorghum expresses α- kafirin proteins in abundant and it is located in the core of the protein body. β-kafirin promoter expresses protein in the interior of the protein body (Shull et al. 1992). These two promoters will be poor choice for the expression of the HMW glu gene of the wheat as the basic protein body of the sorghum gets disrupted and resultant is the alteration of basic property of the sorghum proteins. γ-kafirin proteins are found periphery of the protein body and it is expected that γ-kafirin gene promoter will allow expression of desired HMW glu gene at periphery of the protein body by replacing the γ-kafirin protein or as the floating protein bodies so that we will get better dough making quality without alteration of the basic property of the sorghum flour (Mishra et al. 2008; Bansal et al. 2008). Therefore γ-kafirin gene promoter with minimum regulatory essential motifs was isolated and its temporal and spatial expression was studied previously (Mishra et al. 2008). Proper regulation of transformed seed storage protein gene is necessary for the success of transgenic plants. Endospermal expression of native γ-kafirin gene is prerequisite for development of bioengineered grain sorghum, hence it has also been studied previously (Bansal et al. 2008). In the present study a suitable ready to use gene construct, harboring HMW glutenin gene driven by γ-kafirin promoter, was prepared for transformation, using previously isolated and characterized γ-kafirin gene promoter of sorghum (Mishra et al. 2008) and HMW glutenin gene of wheat (Pandey et al. 2008).

Materials and Methods

Cloning and sequencing of the amplified gamma kafirin gene promoter

PCR based amplified γ-kafirin gene promoter (574bp) (gene accession AJ 629151; Mishra et al. 2008) was purified using MinElute PCR Purification kit (Qiagen, Germany), according to manufacturer's instructions, cloned in pGEM *T*-easy vector (Promega, USA) and sequenced using T-7 and SP-6 primers. Sequence was analyzed using bioinformatics tools and submitted to NCBI (www.ncbi.nlm.nih.gov) data bank.

Construction of vector pKaf-gus

pCAMBIA 1304 and pGEM-kaf were isolated and subjected to restriction digestion by *Nco I* and *Sac I* (MBI fermentas, Canada) to release kafirin promoter (574 bp) from pGEM *T*-easy and CaMV35S promoter (size 800 bp) from pCAMBIA 1304. *Gamma* kafirin promoter and pCAMBIA lacking CaMV35S promoter (pCAMBIA1304△CaMV35S) were purified from gel using SNAP Gel purification kit (Invitrogen, Life Tech, USA). *Gamma* kafirin promoter was cloned in pCAMBIA1304△CaMV35S, in place of CaMV35S promoter, the resultant plasmid was named pKaf-gus and positional cloning was confirmed by restriction digestions and PCR.

Construction of vector pKaf

Plasmid pUC 19 and gene construct pkaf-gus were isolated and digested with enzyme *Pst 1* (MBI fermentas, Canada). After purification of digested plasmids, linearized pUC 19 was again digested with enzyme *BamH I* while pkaf-gus was digested with *Bgl II*. Double digested pUC 19 and γ-kafirin promoter were eluted from the agarose gel using MinElute Gel extraction kit (Qiagen, Germany) following manufacturer's instructions and *gamma*-Kafirin promoter was sub-cloned in pUC 19 vector. Subcloned kafirin promoter was transformed to *E. coli* DH 5 α strain and positive recombinants were selected and named as p*kaf*. Ligation was confirmed by restriction digestion analysis.

Construction of vector pKaf-Dx5 and pKaf-Dy10

Both glutenin genes, Dx 5 and Dy 10, cloned in pBLUSCRIPT SK^-, and vector pKaf were excised by double digestion us-

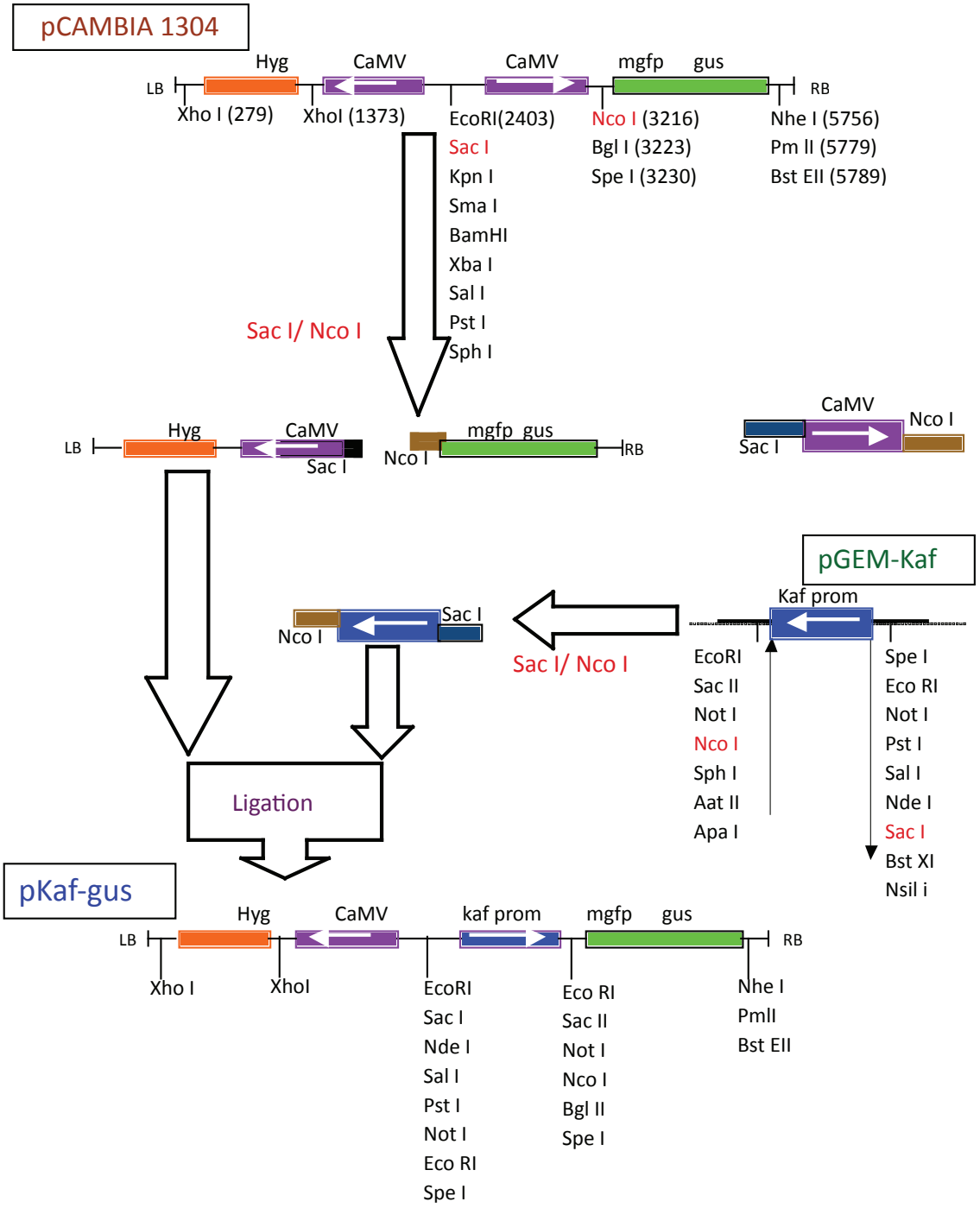

Figure 1. Strategy for construction of gene cassette pKaf-gus.

ing enzymes *Kpn I* and *Sac I* and purified using MinElute Gel extraction kit (Qiagen, Germany). HMW glu genes Dx 5 and Dy 10 were cloned at *Kpn I* and *Sac I* restriction site. Ligated product was transformed to *E. coli* DH 5 α strain and all transformed colonies were picked and recombinant clones/ plasmids were named as pKaf-Dx5 and pKaf-Dy10. Randomly selected putative colonies were inoculated in 3 ml Luria- Bertani (LB) media (1% Tryptone, 0.5% Yeast extract and 0.5% NaCl; pH 7.0) and plasmids were isolated, electrophoresed and then cloning was confirmed by restriction digestion analysis.

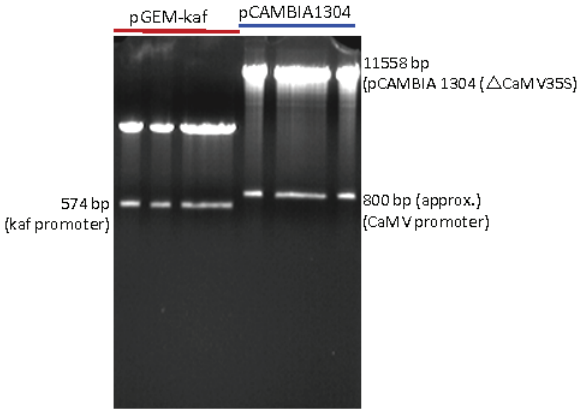

Figure 2. Excision of kafirin promoter from pGEM-kaf and CaMV promoter from pCAMBIA1304 by using restrction enzymes *Nco 1* and *Sac 1*.

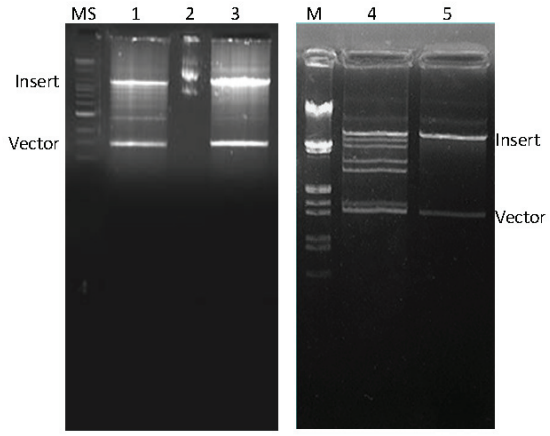

Figure 3. Excision of HMW glutenin Dx5 gene from pK+Dx 5 and Dy10 gene from pK-Dy 10 by restriction digestion with *Kpn I* and *Sac I* .
Lane MS: Marker super mix DNA ladder
Lane 1 : Partialy digested pK+Dx 5
Lane 2 : Undigested pK+Dx 5
Lane 3 : Complete digested pK+Dx 5 (to be eluted)
Lane M : Marker lambda *Hind III/ Eco RI* double digested
Lane 4 : Partial digested pK-Dy 10
Lane 5 : Complete digested pK-Dy 10 (to be eluted)

Result and Discussion

Construction of gene cassette pkaf-gus

Plasmid pCAMBIA 1304 and pCAMBIA 2301, both were available in the laboratory for the sub- cloning of the kafirin promoter(s). Both plasmids were isolated and analyzed for the suitability of sub- cloning by a series of restriction digestions. In pCAMBIA 1304, CaMV 35 S promoter of the reporter gene *gus*/*gfp* is flanked between *Sac I* and *Nco I* restriction enzyme while in pCAMBIA 2301, an extra *Nco I* site is present with the second CaMV 35S promoter of the selection marker gene which limits its selection for subcloning. Thus, pCAMBIA 1304 was selected for the construction of gene cassette on the basis of restriction digestion analysis and compatibility with pGEM-kaf vector. Kafirin promoter, cloned in pGEM- *T* easy vector, has expression direction from *Sac I* to *Nco I* restriction sites. In, pCAMBIA 1304, CaMV 35S promoter is also flanked with these two restriction sites and also has same expression direction (Fig. 1).

pCAMBIA 1304 and pGEM-kaf were digested with *Nco I* and after purification, again digested with *Sac I* enzyme. Digested plasmids were electrophoresed on preparative agarose gel for elution. Desired kafirin promoter of size 574 bp (gene accession AJ629151; Mishra et al. 2008) as well as pCAMBIA 1304 (△ CaMV 35 S) of size 11558 bp were eluted from the gel (Fig. 2) and a ligation reaction was set up in different molar ratios of the insert and vector. A measure of 5 µl of the ligation mix was transformed to competent cells of *E. coli* DH 5α and incubated overnight at 37°C and the best result was observed in 1:2 molar ratio. There is a possibility to grow only recombinants as neither pCAMBIA (non- recombinants) nor γ- kafirin promoters could recircularize (as double digested). All colonies were recombinants and 15 representative colonies were picked up and inoculated to LB media containing kanamycin. Plasmid of all representative colonies were isolated and ligation of kafirin promoter to pCAMBIA was confirmed by *Eco RI* restriction digestion. There is only one *Eco RI* site on pCAMBIA whereas in recombinant pCAMBIA 1304, promoter to be cloned on the behalf of CaMV promoter will bring an extra *Eco RI* site, as promoter is flanked with this enzyme site (Fig. 1). Thus, on restriction digestion with *Eco RI* enzyme, insert (cloned kaf promoter) will excise out having the expected size. These recombinant gene cassettes were named as pkaf gus and its efficacy (temporal and spatial expression) was previously checked by transforming to different sorghum tissues (Mishra et al. 2008).

Construction of gene cassettes pKaf 'glu'

The main goal of the research is to develop transgenic sorghum having good rotibread making quality and for this HMW glu gene(s) (viz. Dx5 and Dy10) of wheat were isolated and characterized (Pandey et al. 2008). A fusion of promoter and HMW glu gene(s) in a specific orientation is required.

Subcloning of kaf promoter to pUC 19

Plasmid pUC 19 and gene construct pkaf-gus were isolated in bulk and digested with enzyme *Pst I*, resulting in linearization of the plasmids. After purification of digested plasmids, linearized pUC 19 was again digested with enzyme *Bam HI* while pkaf-gus was digested with *Bgl II*. As a result of the double digestion, pUC 19 plasmid was linearized with *Pst I* and *Bam HI* protruding ends, while kafirin promoter was excised from pkaf-gus vector with *Pst I* and *Bgl II* protruding

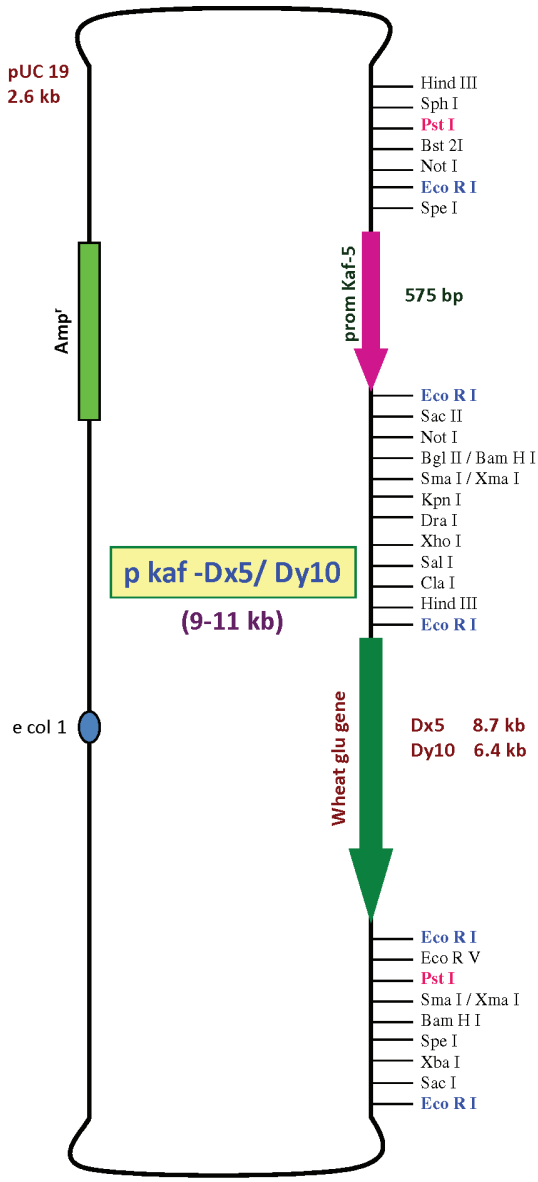

Figure 4. Restriction digestion map of gene construct pKaf -Dx5/ Dy 10.

ends. Double digested pUC 19 and kafirin promoter were eluted from the agarose gel. Kafirin promoter was ligated in pUC 19 as both insert and vector having compatible ends (*Bam HI* and *Bgl II* both leaving same protruding ends *GATC*). Subcloned kafirin promoter was transformed to *E. coli* DH 5 α strain and recombinant was selected by blue white color selection and named as pKaf. White colonies were selected, plasmid pKaf was isolated and ligation was confirmed by restriction digestion as in pUC 19 there is only one site of the *Eco RI* while in pKaf, inserted kafirin promoter was flanking within *Eco RI* site and after restriction digestion with *Eco RI*, kafirin promoter of approximate size 574 bp was excised and thus giving positive confirmation of the cloning.

Subcloning of HMW glu gene to pKaf vector

Kafirin promoter was cloned in pUC 19 in such orientation that it's expression direction is *Pst I* to *Bam HI / Bgl II*. Both glu genes Dx 5 and Dy 10 were isolated in bulk and both genes were excised by double digestion using enzyme *Kpn I* and *Sac I* (Fig 3). Excised genes were eluted from the agarose gel and purified. These eluted glu gene(s) have expression direction from *Kpn I* to *Sac I*. Vector pkaf was also double digested with same *Kpn I* and *Sac I* restriction enzymes and purified. HMW glu genes Dx 5 and Dy 10 were cloned at *Kpn I* and *Sac I* restriction sites, which is present just down-stream to kafirin promoter in same expression orientation. Ligated product was transformed to *E. coli* DH 5 α strain and all transformed colonies were picked. All grown colonies were putative recombinant as pKaf and glu gene(s) were double digested and eluted so there is no possibility of any type of self ligation. Randomly selected putative colonies were inoculated in 3 ml LB media for the plasmid isolation. There is no color selection and all colonies were found white as glu gene(s) were cloned in pKaf, which was a recombinant (kaf promoter was cloned at lac z site of the pUC 19). Recombinant clones/ plasmids were named as pKaf-Dx 5 and pKaf-Dy 10. All plasmids were electrophoresed and then digested with *Eco R I* to confirm cloning of the glu gene(s) as pKaf containing *Eco RI* site with kafirin promoter (~600 bp) while pKaf-Dx 5/ Dy 10 containing an extra *Eco RI* site with glu gene(s) (8.7 kb Dx 5 and 6.5 kb Dy 10). The map of the gene construct pKaf prom Dx 5/ Dy 10 is shown in Figure 4.

Finally, two gene cassettes were developed as pKaf-Dx5 and pKaf-Dy10 where, expression of HMW glu gene Dx5 and Dy10 was driven by the kafirin gene promoter. Both gene cassettes are ready to clone in any transformation vector by excising at *Pst I* restriction sites. Like other prolamin genes, kafirins are also subjected to tissue-specific and developmental regulation, being expressed exclusively in the starchy endosperm during mid- and late-development. This control of gene expression is exerted primarily at the transcriptional level (Bartels and Thompson 1986; Sorensen et al. 1989; Mutisya et al. 2006). Freitas et al. (1994) reported a *gus* gene expression driven by γ-kafirin gene promoter while similar type of expression was also observed by Mishra et al. (2008). DeRose et al. (1996) observed *gus* gene expression in transgenic tobacco seeds, developed by *Agrobacterium* mediated transformation, using an α-kafirin gene promoter and *uidA* gene construct. The HMW glutenin protein 1Ax1, Dx5 and Dy10 are successfully expressed in several other plants and also in *E. coli*. In these studies, HMW glutenin gene was expressed in either homologous system (*i.e.* Plant system) with plant promoter (CaMV, HMW or LMW glutenin promoter) or heterologous system (*i.e. E. coli*; Bartels et al. 1985; Galili

1989; Altpeter et al. 2004; Pandey et al. 2008). With the best of our knowledge there is no report on the expression of wheat glutenin gene(s) driven by the kafirin gene promoter and present study may be the first report on the availability of a gene construct (Fig. 4) where HMW glutenin gene is driven by kafirin gene promoter.

References

Altpeter F, Popelka JC, Wieser, H (2004) Stable expression of 1Dx5 and 1Dy10 high-molecular-weight glutenin subunit genes in transgenic rye drastically increases the polymeric glutelin fraction in rye flour. Plant Mol Biol 54:783-792.

Axtell JD, Van-Scoyoc SW, Christensen PJ, Ejeta G (1979) Current status of protein quality improvement in grain sorghum. In Seed Protein Improvement in Cereals and Grain Legumes, Vol. 1, IAEA, Vienna, pp. 357-365.

Bansal S, Mishra A, Sharma S, Khanna VK, Tomar A, Garg GK (2008) Isolation and temporal endospermal expression of *gamma* -kafirin gene of grain sorghum (Sorghum bicolor L. moench) var. M 35-1 for introgression analysis of transgene. J Cereal Sci 48:808-815.

Bartels D, Thompson RD (1986) Synthesis of messenger-RNAs coding for abundant endosperm proteins during wheat grain development. Plant Sci 46:117-125.

Bartels D, Thompson RD, Rothstein S (1985) Synthesis of a wheat storage protein subunit in Escherichia coli using novel expression vectors. Gene 35:159-167.

D'Ovidio R, Anderson OD (1994) PCR analysis to distinguish between alleles of a member of a multigene family correlated with wheat bread making quality. Theor Appl Genet 88:759-763.

De Rose RT, Begum D, Hall TC (1996) Analysis of kafirin promoter activity in transgenic tobacco seeds. Plant Mol Biol 32:1029-1035.

Flavell RB, Goldsborough AP, Robert LS, Schnick D, Thompson RD (1989) Genetic variation in wheat HMW glutenin subunits and their molecular basis of bread making quality. Biotechnology 7:1281-1285.

Freitas F, Yunes JA, Silvia MJda, Arruda P, Leite A (1994) Structural characterization and promoter activity analysis of *gamma* kafirin gene from sorghum. Mol Gen Genet 245:177-186.

Galili G (1989) Heterologous expression of a wheat high molecular weight glutenin gene in *Escherichia coli*. Proc Natl Acad Sci USA 86:7756-7760.

Greene FC, Anderson OD, Yip RE, Halford NG, Malpica-Romero JM, Shewry PR (1988) Analysis of possible quality-related sequence variations in the 1D glutenin high molecular weight subunit genes of wheat. In Miller TE, Koebner RMD, eds., Proceedings of the 7th International Wheat Genetic Symposium, IPSR, Cambridge, 735-740.

Grootboom A, O'Kennedy MM (2003) Genetic enhancement of nutritional quality of grain sorghum In Belton PS, Taylor JRN, eds., Workshop on the proteins of sorghum and millets: Enhancing nutritional and functional properties for Africa, Pretoria, South Africa.

Mishra A, Tomar A, Bansal S, Khanna VK, Garg GK (2008) Temporal and spatial expression analysis of *gamma* kafirin promoter from Sorghum *(Sorghum bicolor* L. moench) var. M 35-1. Mol Biol Rep 35:81-88.

Mutisya J, Sun C, Palmqvist S, Baguma Y, Odhiambo B, Jansson C (2006) Transcriptional regulation of the sbeIIb genes in sorghum (*Sorghum bicolor*) and barley (*Hordeum vulgare*): importance of the barley sbeIIb second intron. J Plant Physiol 163:770-780.

Pandey R, Mishra A, Garg GK, (2008) Plant promoter driven heterologous expression of HMW glutenin gene(s) subunit in *E. coli*. Mol Biol Rep 35:153-162.

Shewry PR, Halford NG (2002) Cereal seed storage proteins: structures, properties and role in grain utilization. J Exp Bot 53:947-958.

Shull JM, Watterson JJ, Kirleis AW (1992) Purification and Immunocytochemical localization of kafirins in *Sorghum bicolor* (L. Moench) endosperm. Protoplasma 171:64-74.

Singh R, Axtell JD (1973) High lysine mutant gene *(h1)* that improves protein quality and biological value of grain sorghum. Crop Sci 13:535-539.

Sorensen MB, Cameron-Mills V, Brandt A (1989) Transcriptional and post-transcriptional regulation of gene expression in developing barley endosperm. Mol Gen Genet 217:195-201

Wrigley CW (1996) Giant proteins with flour power. Nature 381:738.