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CLONING AND EXPRESSION ANALYSIS OF *D-HORDEIN* HYBRID PROMOTER ISOLATED FROM BARLEY (*HORDEUM VULGARE L.)*

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Abstract

Two variants of *D-hordein* promoter from barley (*Hordeum vulgare* L.) were selected to generate hybrid promoter by using bioinformatics tools. Sequence analysis of both *D-hordein* promoter variants revealed several crucial *cis* regulatory elements and transcription factors binding sites within the promoter region. Various regulatory motifs related to endosperm specific expression were located in D-*hordein* promoter fragments. Both variants were separately cloned in TA vector (pTZ57R/T) and then joined to get the complete hybrid promoter (D horH). Hybrid *D-hordein* promoter was further cloned in expression vector pGR1through adaptor ligation. Transient *GUS* assay revealed that hybrid promoter exhibited endosperm specific expression in wheat. From the study it is demonstrated that hybrid promoter (D horH) may be used to derive endosperm specific expression in monocots. The present work provides an important insight in the designing of hybrid monocot promoters to improve multiple traits in crops without facing IPR issues.

Key words: *D-hordein*, *Hordeum vulgare*, Hybrid promoter, Histochemical *gus* staining.

Introduction

Promoters are regulatory elements that control transcription and the level of gene expression (Ricarda, 2002). Several promoters isolated from viral, bacterial and plant origin have been characterized and used extensively in transgene expression system (Shinwari *et al*., 1998; Yoshida & Shinmyo, 2000; Lessard *et al*., 2002; Muller & Wasseneger, 2004). A variety of plant promoters are being used in different genetic engineering strategies for gene expression studies as well as introduction of transgene for crop improvement and bio-pharmaceutical applications (Potenza *et al*., 2004).

Many constitutive as well as seed specific promoters have been identified from monocots. However, the major problem regarding the use of these promoters is the intellectual property right (IPR) issues. The generation of hybrid promoters by recombination of promoter sequences and functional domains is of great interest. Hybrid promoter development is one of the strategies which can be used to combat the patency issues in transgenic plant development. The modification of the promoters using the core-upstream regions from HTGS, combined with other important regulatory regions on different chromosomes of a genome generates a hybrid promoter. Comai & colleagues (1990) reported that by doubling the enhancer elements in a chimeric 35S promoter, expression of the gene may increases 2-folds. Another hybrid promoter was generated by joining the enhancer elements of CaMV with *cis*- elements of alpha amylase, the new hybrid generated by this fusion showed highest activity in rice protoplast (Omirulleh *et al*., 1993).

In the present research, two fragments of barley *D-hordein* promoter were used for generation of hybrid *D-hordein* promoter. Upstream regions of *D-hordein* gene located at distinct chromosomes were identified and retrieved from HTGS for evaluation of promoter activity. *D-hordein* is alcohol soluble seed storage protein mainly expressed in central starchy endosperm, while B-*hordein*s expresses mainly in peripheral endosperm (Radchuk *et al*., 2006; Shewry *et al*., 1996). The genes for B-, C- and *D-hordein*s have been isolated and characterized, but their regulation and expression have been studied only in transient expression analysis in some monocots and in stably transformed tobacco (Brandt *et al*., 1985; Forde *et al*., 1985; Marris *et al*., 1988; Sørensen *et al*., 1996).

Promoters of *hordein* genes including B, C and *D-hordein*revealed that *D-hordein*promoter exhibited 3-5 folds higher expression in developing endosperm (Sorensen *et al*., 1996).Expression of *D-hordein*promoter remains stable during *lys3A* (high lysine mutation) assay, while that of B-*hordein* and C-*hordein* was reduced to 2-fold and 10-folds respectively. Promoter of *D-hordein* shows transcriptional activation even during hypermethylation because it has CpG Island that prevents the promoter from methylation. *D-hordein* promoter is reported to be more stable in inheritance pattern than rice action or maize ubiquitin promoter (Myeong *et al*., 2002). Structural motif analysis showed that barley *D-hordein* is homologues to wheat glutenins. Qiang & Colleagues (2010) reported that arrangement of *cis*-acting elements and their interaction with other elements is very important in the functioning of *hordein* promoter. However, HTGS data indicates that multiple copies of *D-hordein* genes are present in barley. The comparison of *D-hordein* regulatory regions indicated that not only their nucleotide sequences are variable but these variants also have different motifs for regulation.

Using various databases like plant CARE and plant PAN, various motifs that can modulate rate of transcription can be identified (Lescot, 2002). These conserved *cis*-regulatory elements (CREs) are involved in constitutive, tissue specific and inducible gene expression in plants (Nakashima *et al*., 2000). In monocots seed storage proteins promoters, GCN4 and prolamin box are two main motifs of endosperm box. The GCN4 and prolamin-box also have important role in nitrogen regulation, when they have synergistic interaction with

one another and positioned properly with other upstream elements of *hordein* promoter (Muller & Knudsen, 1993).

The present study was conducted to develop *D-hordein* hybrid promoter between the regulatory sequence variants of single gene homologue and then looking into its expression pattern. Sequence analysis of both promoter fragments identified several motifs related to light, stress responsiveness and endosperm specific expression. Both promoter fragments were fused and cloned in plant expression vector upstream of *GUS* gene. Transient *GUS* assay demonstrated that hybrid *D-hordein* promoter (D horH) exhibited endosperm specific expression in monocot plant wheat.

Many monocot promoters have been identified from highly expressed genes and are widely used in genetic engineering. These promoters are used as part of single as well as multiple expression cassettes for genetic modification of multiple traits in crops. However, the utilization of those promoters for developing commercial crop varieties requires overcoming the IPR policies. In order to overcome the IPR issues and have a free hand in the production and commercialization of transgenic crops, the basic research on identification of alternative promoters is essentially required. Therefore, hybrid *D-hordein* promoter may be used for genetic modification of monocots.

Materials and Methods

Bioinformatics approach to characterize the hybrid promoter: HTGS data indicated that barley *D-hordein* gene has two variants regulated by two dissimilar promoter sequences. A few of the *D-hordein* promoters have already been characterized but the database search indicated that some of them still remain to be characterized. One of the promoter sequences (AR906101.1) has already been reported, while the second promoter reported in this work was not studied previously. In this research work, hybrid promoters were generated by fusing the dissimilar regions of the uncharacterized promoter with the characterized promoter fragments. The hybrid promoter was then evaluated for expression of a reporter gene (*GUS*).

The reported patented 1.5 Kb *D-hordein* promoter has been shown to exhibit endosperm specific expression, initial 1100 bases of this promoter carry the *cis*-acting regulatory motifs that play important role in regulation and enhancement of gene expression, and remaining 400 bases have motifs that play role in endosperm specific expression. In HTGS we found a 437 bp upstream *D – hordein* that also have endosperm specific motifs (Sorensen *et al.,* 1996). These 437 base pairs were found to be much similar to those 400 bases, while the differences were observed in the first 1000 base pairs (bp). These 1000 bp of newly identified promoter variant were fused with the 400bp of reported promoter to generate a hybrid promoter. Translation start codon and exact coding frame were identified through Expasy and Justbio. Motifs in these promoter fragments were located through bioinformatics tools such as Plant-CARE (cis acting regulatory elements), and PLACE (plant cis-acting regulatory DNA elements). BDGP (Berkeley Drosophila Genome Project) and TSSP (plant promoter identification program) were used to locate the transcription initiation site (TiS). Patent BLAST of complete *D-hordein* hybrid promoter was also performed, to find similarity with already reported sequence in database.

Cloning of *D-hordein* promoter in TA cloning vector: The *D-hordein* hybrid promoter was generated by joining the two fragments from each variant of the respective promoter. To obtain complete *D-hordein* hybrid, directional cloning of *D-hordein* fragments was performed using pTZ57R/Tas cloning vector. Both *D-hordein* promoter fragments were amplified using barley DNA as template. Fragment I comprised of 1236 bp and fragment II was of 500bp. various restriction sites were added on primers of both promoter fragments. A *Sac*I and *Apa*I sites were respectively introduced in forward and reverse primers of fragment I. The forward primers for fragment II contained *Apa*I and reverse primers had *Hind*III site. Both promoter fragments were PCR amplified using barley genomic DNA and purified through salt precipitation. There was an internal *Hind*III site in *D-hordein* fragment II. Therefore, it was partially digested with *Apa*I and *Hind*III to avoid complete digestion. Both fragments were cloned in TA cloning vector. Multiple sets of primer pairs were designed from which best primer pairs with sharp band and no primer dimers were selected (Table 3). Annealing temperature, genomic DNA and Mg^{+2} concentrations were optimized prior to cloning.

Generation of hybrid *D-hordein* promoter: PTZ vector having promoter fragment II was digested with *Apa*I and *Hind*III to generated sticky ends complementary to fragment I. Promoter fragment I was also digested with *Apa*I and *Hind*III to join down stream of promoter fragment II cloned in TA vector. Both fragments were ligated and transformed. Hybrid promoter clone was confirmed through restriction with *Sac*I and *hind*III. *D-hordein* hybrid clone (D-horH) was then used for further cloning in the plant expression vector pGR1.

Cloning of hybrid *D-hordein* promoter in pGR1: A plant expression vector pGR1 (provided by plant biotechnology group, Agriculture biotechnology division, NIBGE, Faisalabad) had 35S promoter fused to *GUS* gene followed by CaMV terminator. From pGR1 vector, 35S promoter was excised using *Sac*I and *Hind*III enzymes. Full length *D-hordein* promoter was excised and cloned into pGR1 by replacing 35S promoter. Due to presence of internal *Hind*III site in Fragment II, it was quite difficult to clone hybrid, promoter (D-horH) in pGR1 under *Sac*I and *Hind*III. Therefore, a cloning strategy was designed to insert full length *D-hordein* hybrid in pGR1 (upstream of*GUS*) with the help of an adapter sequence.

Adapter ligation in pGR1: An adapter sequence of 177 base pair having multiple rare cutting sites was already designed in gene isolation group lab, NIBGE. Adapter sequence and pGR1 were double digested with *Sac*I and *Hind*III separately and ligated to obtain adaptor ligated vector PGR177.The *Asc*I and *Not*I sites in pGR177 MCS

(multiple cloning sites) were selected for cloning of *D-hordein* hybrid promoter. *D-hordein* hybrid promoter was amplified by promoter specific primers containing *Asc*I and *Not*I sites in the forward and reverse primers respectively.

The resultant clone containing hybrid promoter was named pGRdhor-H.

Transient *GUS* assay: Transient expression studies were carried out to evaluate the activity of the *D-hordein* hybrid promoter using reporter gene (*GUS*) expression in the monocot plant like wheat. Biolistic Particle Delivery System (PDS1000 He) was used for the bombardment of vector constructs in wheat explants.For comparative analysis, a construct without promoter was also generated to be used as negative control. Vector pGR1 with *GUS* gene downstream of 35S promoter was used as positive control in bombardment experiments. To avoid any false positive result, gold part without coating were also bombarded. Wheat leaf, spike, root and endosperm were used as explants for the bombardment experiments.

A 1µg/µl of Plasmid DNA of each construct was used for coating 500µl sterile gold particles. Leaves, roots and spikes were taken from *In vitro* wheat plants grown in pots from green house. Wheat seed were soaked for 2-3 days in Petri plates containing sterile distilled water and were longitudinally cut to expose endosperm. All tissues were placed on Petri plates containing ½ MS medium (Murashige & Skoog, 1974) in a way to expose maximum surface area for bombardment. All wheat explants were bombarded at 27 mmHg vacuum with 1100 psi rupture disks and 9 cm target distance. Same experimental conditions were used to bombard plasmids having 35S promoter uncoated gold particles and promoter less construct. Petri plates were placed at 25ºC±2 for 24 hrs and submerged in *GUS* staining buffer containing 0.1M X-Gluc. All tissues were incubated in dark at 37ºC for till appearance of blue color and washed with 70% ethanol was added to stop reaction as well as to bleach chlorophyll from green tissues. A digital camera attached with microscope was used to photograph all tissues.

Results

Promoter fragment sequence analysis on plant CARE indicated the basal regulatory elements. Transcription start site (TSS) was 40Base pairs upstream of translation start cod on and was determined through BDGP. In addition our results shows strong expression in endosperm that might be associated with the regulatory element's that are present upstream in promoter region.

A barley *D-hordein* gene was selected for generation of hybrid promoter. Two upstream regions of D- *hordein* gene located at distinct chromosomes were retrieved and analyzed through bioinformatics tools. BLASTp results of both upstream sequences confirmed there was no coding region. Both promoter fragments were cloned separately in TA vector and fused to synthesize hybrid promoter. For functional characterization *D-hordein* hybrid promoter was cloned in an expression vector and analyzed through transient GUS assay in wheat.

Nucleotide sequence of *D-hordein* gene matched in HTGS database at two distinct positions. Both fragments were identified from HTGS sequence of *Hordeum vulgare* BAC 184G9 (AC239006.1) at position of (61285-60052) and (128850-128354) from HTGS. Patent BLAST results revealed that this new promoter is 20% dissimilar to already patented oneTranscription initiation site started at T with 90% probability. The promoter fragment upstream to the transcription initiation site was named *D-hordein*I fragment and the downstream region including the transcription start site was named *D-hordein*II fragment.

Sequence analysis of both fragments revealed several cis acting motifs and transcription factor binding sites as identified through Plant CARE and palnt PAN. Core promoter elements including TATA box and CAAT box were present in both promoter fragments (Fig. 1a & b). An ABRE motif with consensus sequence TACGTG related to abscisic acid induced response was also present in both *D-hordein* fragments. There were several motifs related to light inducibility including G-Box (AACATAATC), I-Box (ACGATAATC), Box-4 ATTAAT and GAGA motif GTAGAGA Hybrid *D-hordein* promoter also contained MBS (TAACTG), RY element CATGCATG and a HSE (GCTTAAAAGA). Both fragments of hybrid promoter contained crucial regulatory motifs related to endosperm specific expression including Skn-1 motif(TAGCTG), GCN4 motif (TGAGTCA), and Prolamine box (TGCTTTGCAAAGCTCCA).

For amplification of *D hordein* promoter fragments, a gradient PCR was run initially to optimize the annealing temperature and for the selection of best primer pair. Selected primer pairs for amplification of fragment I were H1d-horHVV1F-2 as forward primer and H1d-horHVV1R-1 as reverse primer respectively. On the other hand for amplification of fragment, selected primer pairs included H1d-horHVV2F-1 as forward primer and H1d-horHVV2R-1as reverse primer (underlined in tables 1 and 2). Selected annealing temperatures for amplification of *D-hordein* fragment I and II were 48.4°C and 57.1°C as given in Fig. 2A and B.

PCR products representing both fragments were gel purified and separately cloned TA cloning vector (pTZ57R/T). Clones of both promoter fragments were confirmed through digestion with *Sac*I and *Apa*I as shown in Fig. 3A and B. The final confirmation of the clones was performed by DNA sequencing on an ABI 3100 Genetic Analyzer and comparing the sequences of inserts to the HTGS retrieved sequences.

Promoter fragment II cloned in TA vector was ligated with fragment I using *Apa*I and *Hind*III. Hybrid 1.7 kb promoter clone was confirmed through restriction with *Sac*I and *hind*III (Fig. 4A). *D-hordein* hybrid (D-horH) cloned in TA vector was sub cloned in the plant expression vector pGR1. Meanwhile an adapter containing various restriction sites was ligated in the *Sac*I and *Hind*III sites of pGR1 resulting the plasmid pGR177 (Fig. 5A). The 1.7Kb fragment of *D-hordein* hybrid promoter was amplified using forward (HordFLAsc) and reverse (437Hord2R) primers that had *Asc*I and *Not*I sites respectively (Table 1). The resultant vector construct having hybrid *D hordein* promoter in pGR177 was named pGRdhor-H (Fig. 5B).

Table 1. Cis acting motifs in *D-hordein* promoter fragment I.

***Cis* regulatory element**	**Sequence of motif**	**Function of motif**
A-Box	CCGTCC	*Cis*-acting regulatory element
ABRE	TACGTG	*Cis*-acting element involved in ab*cis*ic acid responsiveness.
AE-Box	AGAAACAT	*Cis*-acting element that is part of light responsive element.
Box4	ATTAAT	Part of conserved DNA module involved in light responsiveness.
CAAT-box	TCTAACCGG	Common *cis*-acting element in promoter and enhancer region
CCAAT-box	CAACGG	MYBHvI binding site
CCGTCC-box	CCTGCC	*Cis*-acting element related to meristem specific activity.
G-Box	AACACATGGAA	*Cis*-acting element involved in light responsive
G-box	C/A-CACGTGGCA	*Cis*-acting element that is part of light responsive element.
GAG motif	GTAGAGA	part of light responsive element.
GARE motif	AA/GACAG/AA	Gibberelin responsive element
GC motif	GCCCCC	*Cis*-acting element involved in anoxic specific inducibility.
GCN4_motif	TGAGTCA	*Cis*-acting element involved in endosperm expression.
HSE	GCTTAAAAGA	*Cis*-acting element involved in heat stress responsiveness
MBS	TAACTG	MYB binding site involved in drought inducibility.
O2-site	GATGAAATGG	*Cis*-acting element involved in zein metabolism.
RY-element	CATGCATG	*Cis*-acting element involved in seed specific regulation.
Skn-1_motif	TACTG	*Cis*-acting element required for endosperm expression
TATA box	TATAT/CATAT	Core promoter element around -30 of transcription start site.
TC-rich repeats	T/CT/CTTCC/TC/T	*Cis*-acting element involved in stress and defense responsiveness

Table 2. Cis acting motifs in *D-hordein* promoter fragment II.

***Cis*-regulatory element**	**Sequence of motif**	**Function of motif**
A-Box	CCTGCC	*Cis*-acting regulatory element
ABRE	TACGTG	*Cis*-acting element involved in ab*cis*ic acid responsiveness.
CAAT-box	TCTAACCGG	Common *cis*-acting element in promoter and enhancer region
CCAAT-box	CAACGG	MYBHvI binding site
G-Box	AACACATGGAA	*Cis*-acting element involved in light responsive
G-box	C/A-CACGTGGCA	*Cis*-acting element that is part of light responsive element.
I-box	ACGATAATC	Part of light responsive element
TATA-box	TATAT/CATAT	Core promoter element around -30 of transcription start site.
Prolamin-box	TGCTTTGCAAAGCTCCA	Involved in endosperm specific expression

Table 3. List of primers designed for amplification and cloning of hybrid *D-hordein* promoter.

	Name of primer	**Sequence**
Forward primers for *D-hordein*-fragment I	H1d-horHVV1F-1	5'AGTGAGCTC ATTTGCAAAAGCAATGAC3'
	H1d-horHVV1F-2	5'AGTGAGCTC ATTTGCAAAAGCAATGACT3'
	H1d-horHVV1F-3	5'AGTGAGCTCGCAAAAGCAATGACTAACAGATAC 3'
Reverse primers for *D-hordein*-fragment I	H1d-horHVV1R-1	5' TAAGGGCCCTTCCAAAAGCTCATTTGTT 3'
	H1d-horHVV1R-2	5' TAAGGGCCCGCTCATTTGTTGCTTGTCATG 3'
Forward primers for *D-hordein*-fragment II	H1d-horHVV2F-1	5'TAAGGGCCCAGATGATTTATCAGTTTACTTGTTC 3'
	H1d-horHVV2F-2	5' TAAGGGCCCCAGTTTACTTGTTCCATGCAA 3'
	H1d-horHVV2F-3	5' TAAGGGCCCCAGTTTACTTGTTCCATGCAA 3'
Reverse primers for *D-hordein*-fragment II	H1d-horHVV2R-1	5' TGAAAGCTT TTGTTCCATGCAAGCTACCTTC 3'
	H1d-horHVV2R-2	5'TGAAAGCTT CTCAGAGGACTGTCAATGAATTG 3'
Primers for pGR1 cloning	HordFLAsc	5'CTGGGCGCGCCGCAATGACTAACAGATACATATATTG
	437Hord2R	5' TATGCGGCCGCTCTCAGAGGACTGTCAATG 3'

ATTTGCAAAAGCAATGACTAACAGATACATATATTGCAAAAAAAAACAGAGGATAATCACTTTTATTAGAT
GAAATAAACAGATCAATTTACATAAGTCCTCACTTCTCCAAACAGTATTCAGGACCATGATAAAACCGATT
ACGTAGCTCTGTTTTGGAAGATCCAAATCCTCAAGTTGAGTTTCATTAATTGGAATCGATTGTATGCTAAAC
ACGATGAACAAATGGTGGGTTACGTGGCATAGCATACAACTATTCCCCTATTATTCTGCATGCATGATCTC
AATCGGACTCCTTCCTAGTTCCTAGTTGGCTCTGCTTTGAACTTTCATCCACATCTCTTTGAGTTATTATTAA
CAGACGCAAGAAACATTTTTTTGCGCTAAGCCAAGGTGAGGCAAGGTCGCATTGGAGGACTGATGGACTG
GCTTCGATGGATTATGATATACTCGGTTTTGCCTGTTTGACTGTTACGTTTTTCTAATTTTGTGGTTAGGAAT
TTTTCGCCGCAGAGTATAGAATAACTAAGCTCAACACAAACAATTTAGCAAGCACATTAAACTGGGATCGT
AGGAGCGCACCTGGATTTTGTTGGTTGATGGTGGATGAAATGGGTGAATTTAATAACTGATATAGTGTCAG
TGCAACGGAAGCCCATTTTTCATACAAGTTATTAATATTGTCAACATTTGTCAACAAACAAATGTTTAACTC
AGGTTTGCAATTATGAAGCCCCAATTATAAGAAGGGGATATTATGATGGCGTGAGCAAGTGATAAGGCCA
AGGGGAGAAGAAGTGCAGCATCTACGCAGCCCAGTGAAAGATAGTGAAAATACAGAGAGGCAGGGACGG
GGGAGCAACACATGGAAATCATAGAAGAACAAAAGAGTTTAAACATAGGAGGCAGATATAATGGACAGC
TAAATCTGCATTATCTCATTTGGGAAATGAAAAAAATAATCCTATTCTTGTGTAAATCAAAACTATTTGCC
GCGAATTTTCTTCGAAGATCCTGTGTTAATTTTAGACACGGCTGACCAAAGGTTTTCAATTAGTTGAGTTTT
GTCACGGAAAGGTGTTTCCATACATCCAAAAATTCTAAAAACTTTTTGATACGGCGCGTTCGTAGCATAGC
TAGATGTTGTGAGTCACTGGATAGATATTGTGAGTCATATCGTGGATTTGTGTTGCCTGCAAATCCAACTA
CATGACAAGCAACAAATGAGCTTTTGGAAAGATGATTTATCAGTTTACTTGTTCCATGCAAGCTACCTTCC
ACTACTCGACATGCTTAGAAGCTTCGAGTGCCCGCGGATTTGCCAAAGCAATGGCTAACAGACGCATATTC
TGCCAAAAACCCAGAACGATAATCACTTCTCGTAGATGAAGAGAACAGACCAAGATACAAACGTCCACAC
TTCTGCAAACAGTACCCCAGAACTAGGATTAAGCCGATTACGTGGCTTTAGCAGACCGTCCAAAAAAACT
GCTTTGCAAAGCTCCAATTCCTCCTTGCTTATCCAATTTCTTTTGTGTTGGCAAACTGCACTTTTTCCAACCG
ATTCTGTTCTTCCCGTGTTTCTTCTTAGGCTAGCTAACATAGCCGTGCACACAGCCATGGTCCGGAACCTTC
ACCTCGTCCCTATAAAAGCCCAGCCAATCTCCACAATCTCTTCATCACCGAGAACACCGAGCACCACGAAA
CTAGAGATCAATTCATTGACAGTCCTCTGAG

Fig. 1(a). Complete nucleotide sequence of *D-hordein* hybrid promoter.

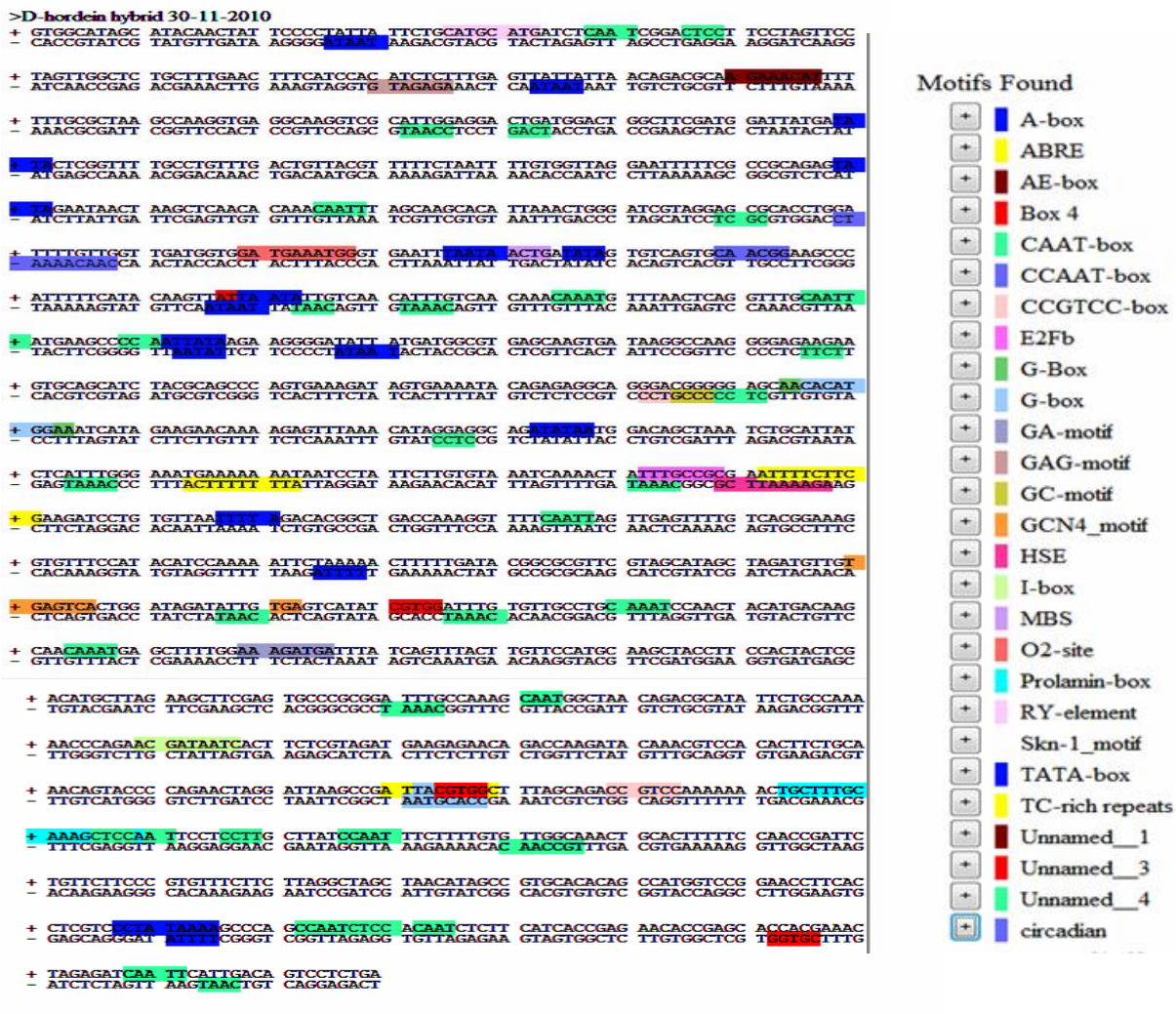

Fig. 1(b). Motifs present in promoter sequence of *D-hordein*.

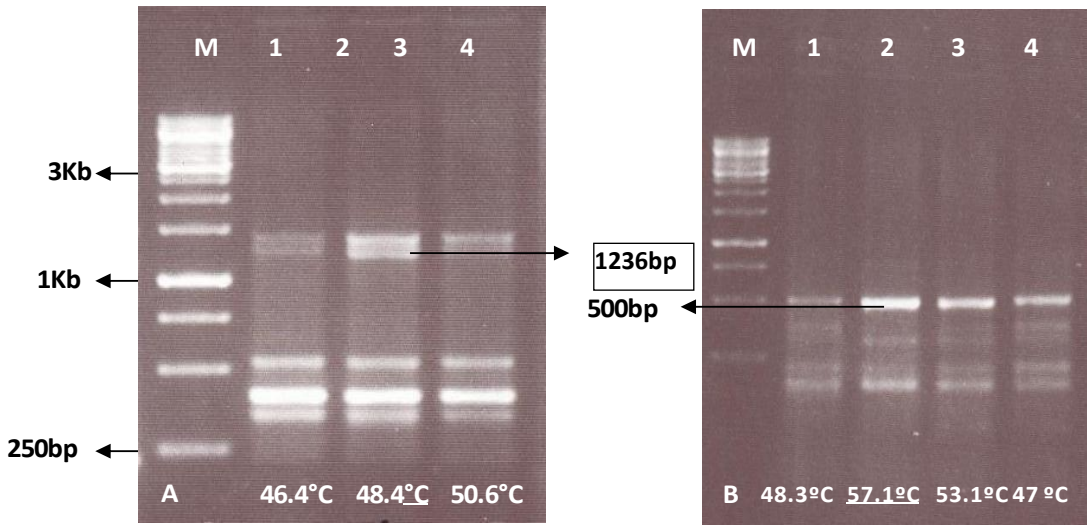

Fig. 2. PCR amplification of *D-hordein*I and *D-hordein*II promoter fragments at different annealing temperatures: A); PCR amplifications at different annealing temperatures. M; 1Kb ladder, Lanes 1-3; Represent PCR of *D-hordein*I fragment at 46.4ºC, 48.4ºC and 50.6 ºC respectively B); Gradient PCR of *D-hordein*II. M; 1Kb ladder, Lanes 1-3; Represent PCR of *D-hordein*II at 48ºC, 57.1ºC, 53.1 ºC and 47 ºC respectively (underlined are selected annealing temperatures)

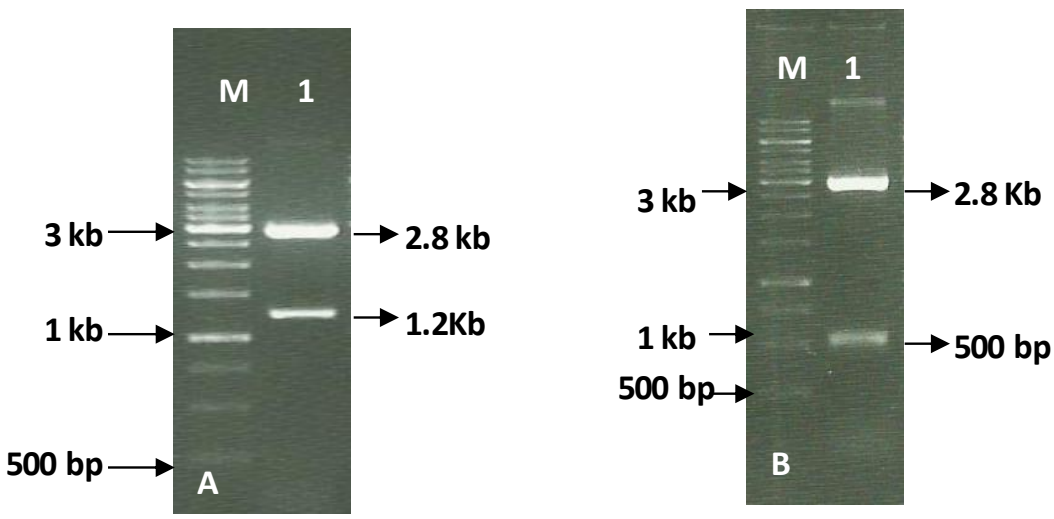

Fig. 3. Cloning of *D-hordein*I and *D-hordein*II promoter fragments in TA cloning vector. A) Confirmation of *D-hordein* fragment I in TA vector by *Sac*I and *Apa*I releasing 1.2 kb promoter fragment I and 2.8 kb vector backbone.B) Confirmation of *D-hordein* fragment II in TA vector restriction with *Sac*I and *Apa*I releasing 500 bp promoter fragment and 2.8 kb vector backbone.

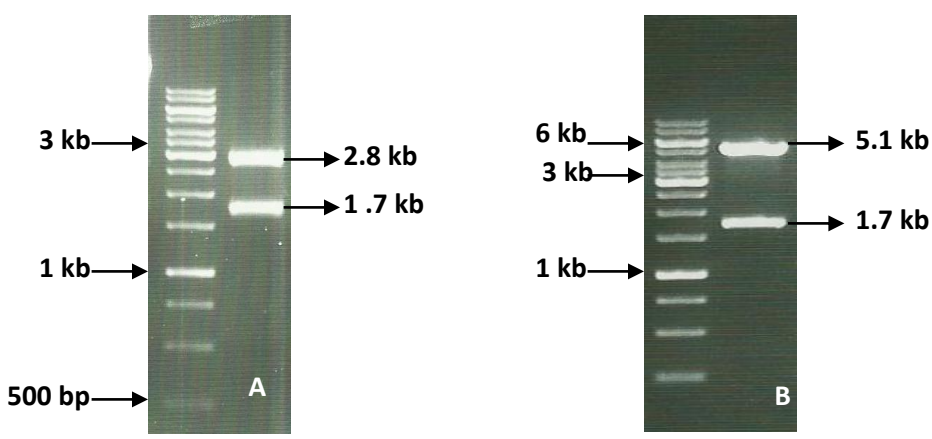

Fig. 4. Cloning of hybrid *D-hordein* promoter A): Confirmation of *D-hordein* hybrid promoter in TA cloning vector by restriction digestion (*Sac*I and *Hind*III) releasing 1.7 kb promoter and 2.8 kb vector backbone. (B):Confirmation of *D-hordein* hybrid in pGR1 by restriction digestion with *Asc*I and *Not*I showing 1.7 kb promoter fragment and 5.1 kb vector backbone.

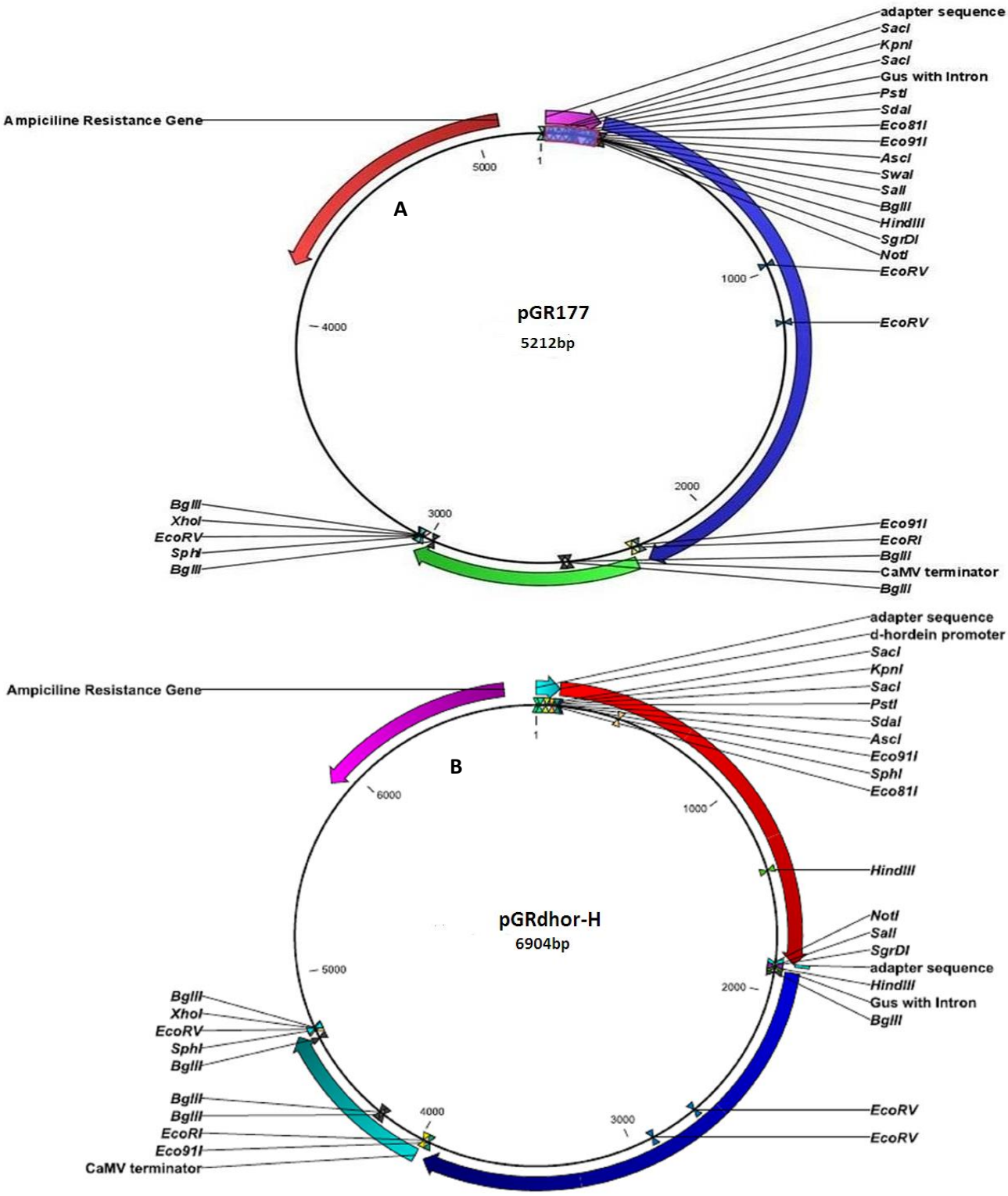

Fig. 5. Plasmid drawings of vector constructs. (A) Physical map of the pGR177 vector containing adapter replaced with 35S promoter in pGR1. (B) Physical map of the pGRdhor-H having *D-hordein* hybrid promoter replaced with 35S promoter in pGR1.

To evaluate activity of hybrid promoter, transient GUS assay was performed in wheat. *GUS* staining of wheat leaves revealed that expression of reporter gene was only observed in leaves bombarded with 35S promoter. No blue stain was detected in leaves bombarded with construct having D-hybrid promoter and negative control (Fig. 6). Similar results were obtained after bombardment of spikes (Fig. 7). Likewise, no expression of Gus was detected in bombarded tissues except 35S promoter (Fig. 7) GUS expression was observed in tissues bombarded with 35s promoter. On the other hand, wheat endosperm bombarded with *D-hordein* promoter showed strong GUS expression as shown in Fig. 8. GUS expression was also detected in endosperm tissues bombarded with 35S promoter (positive control). No blue spots were detected in negative controls (Fig. 9)

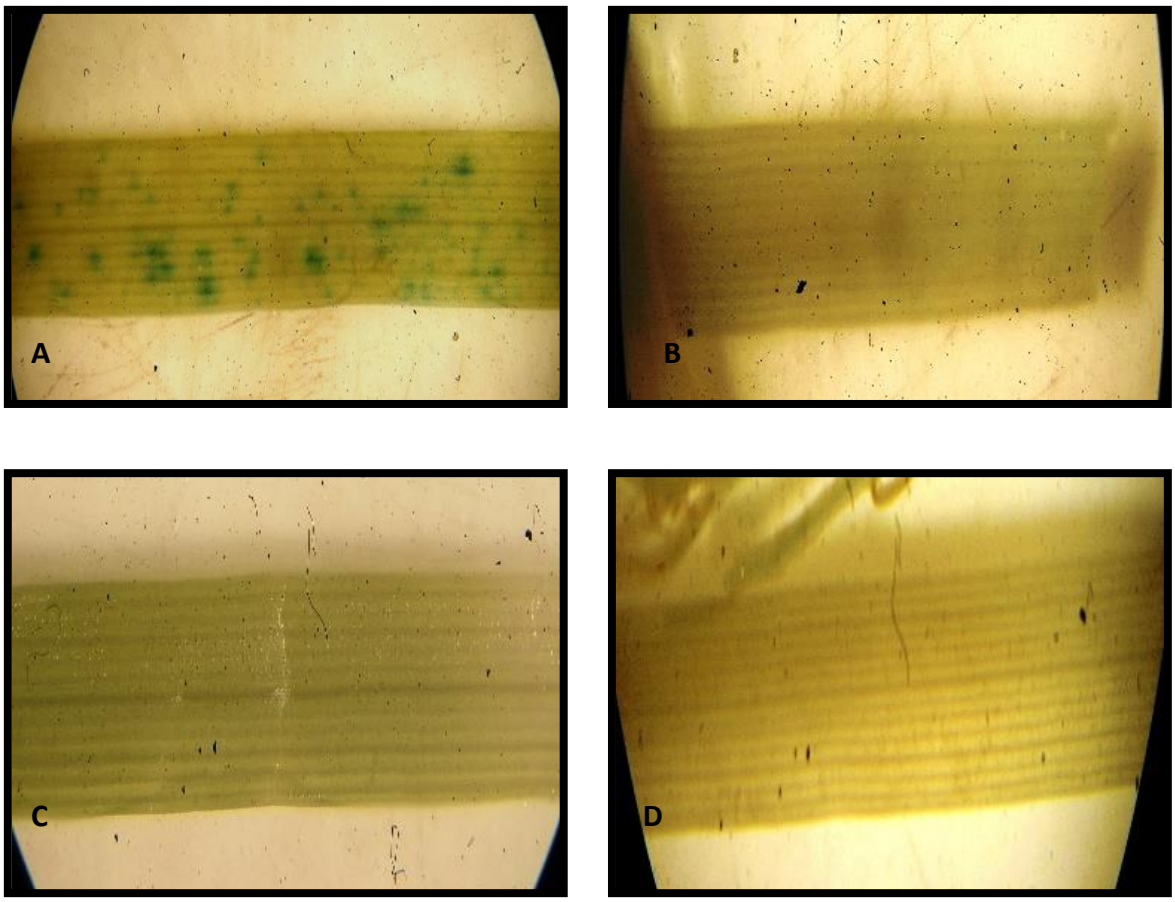

Fig. 6. Transient expression analysis in wheat leaves. A) 35S promoter (positive control). B) Hybrid *D-hordein* promoter (hpGRdhor-H), C) Tissues bombarded with gold particles without DNA coating (negative control). D) Un-bombarded but *GUS* stained wheat seed.

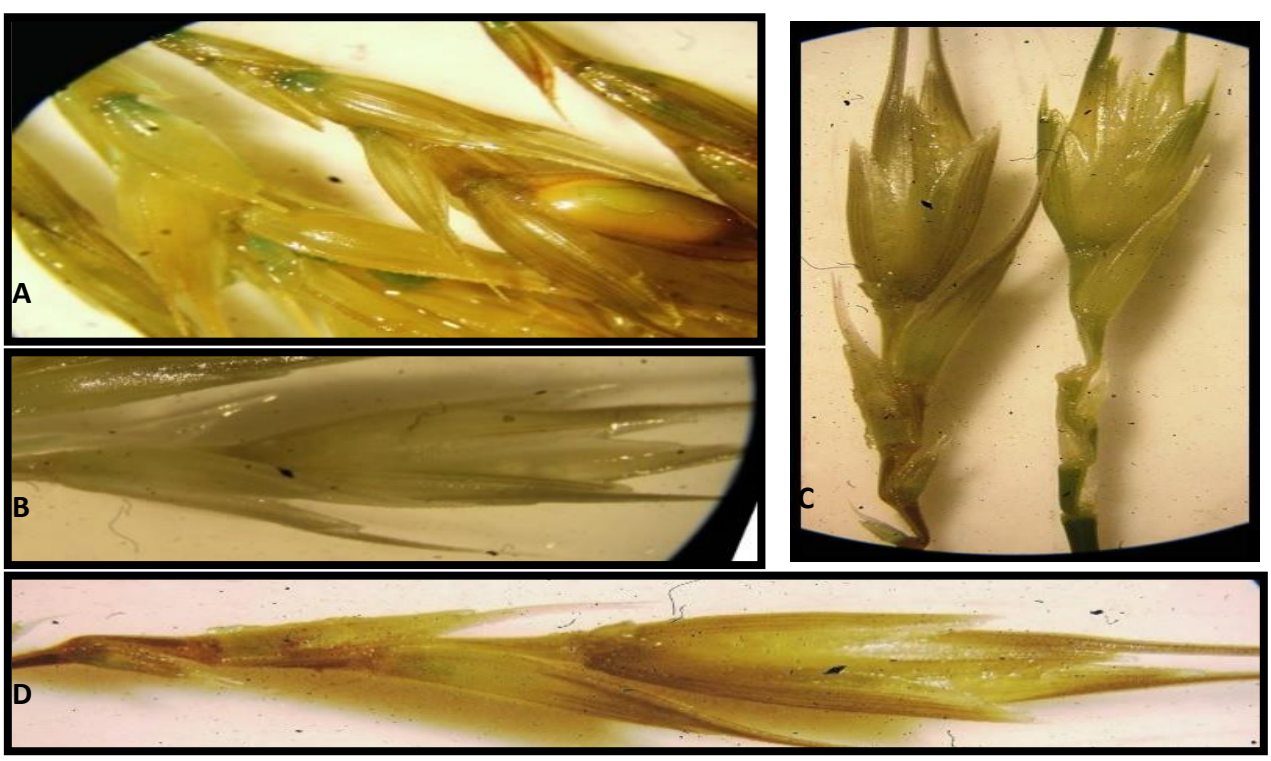

Fig. 7. Transient expression analysis in wheat spikes. Tissues bombarded with A) 35S promoter (positive control), B) Hybrid *D-hordein* promoter (hpGRdhor-H), C) gold particles without DNA coating (negative control), D) Un-bombarded but *GUS* stained wheat spikes.

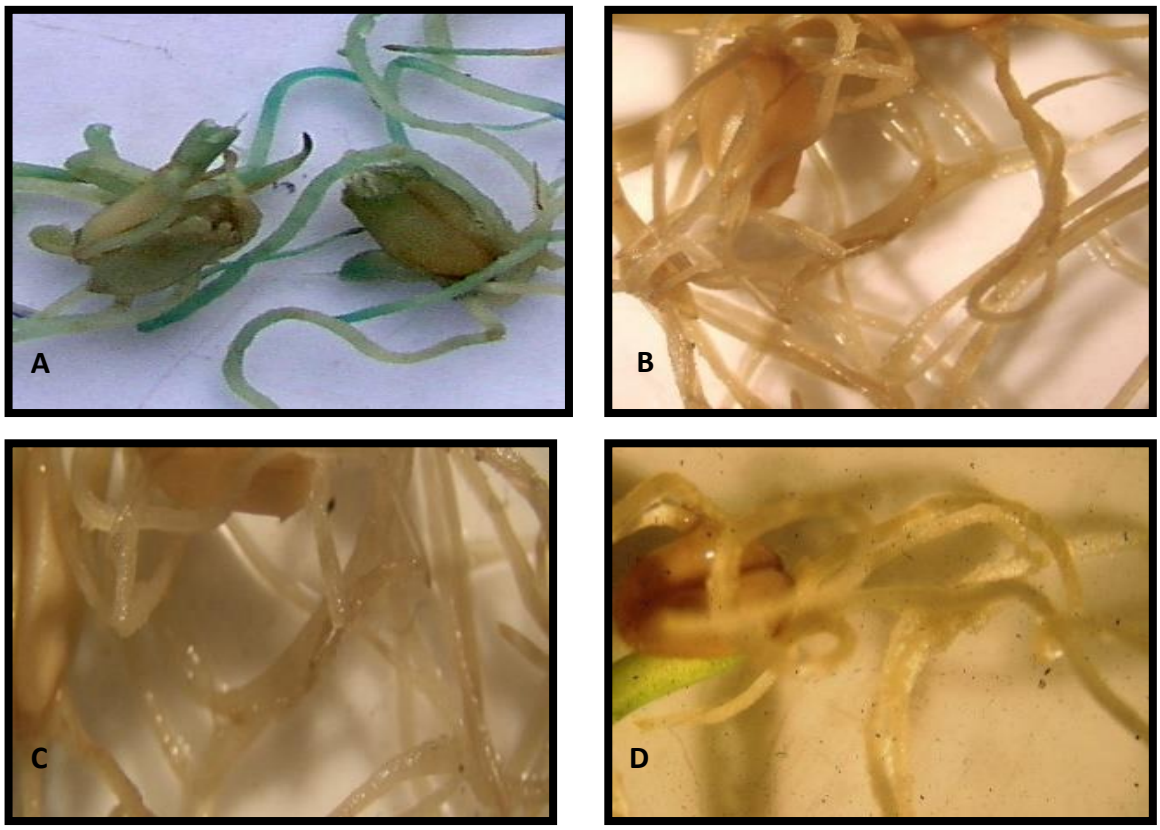

Fig. 8.Transient expression analysis in wheat root. Root tissue bombarded with A) *GUS* activity under 35S promoter, B) Hybrid *D-hordein* promoter (hpGRdhor-H), C) Negative control roots bombarded with gold particles without DNA coating, D) Un-bombarded but *GUS* stained wheat seed.

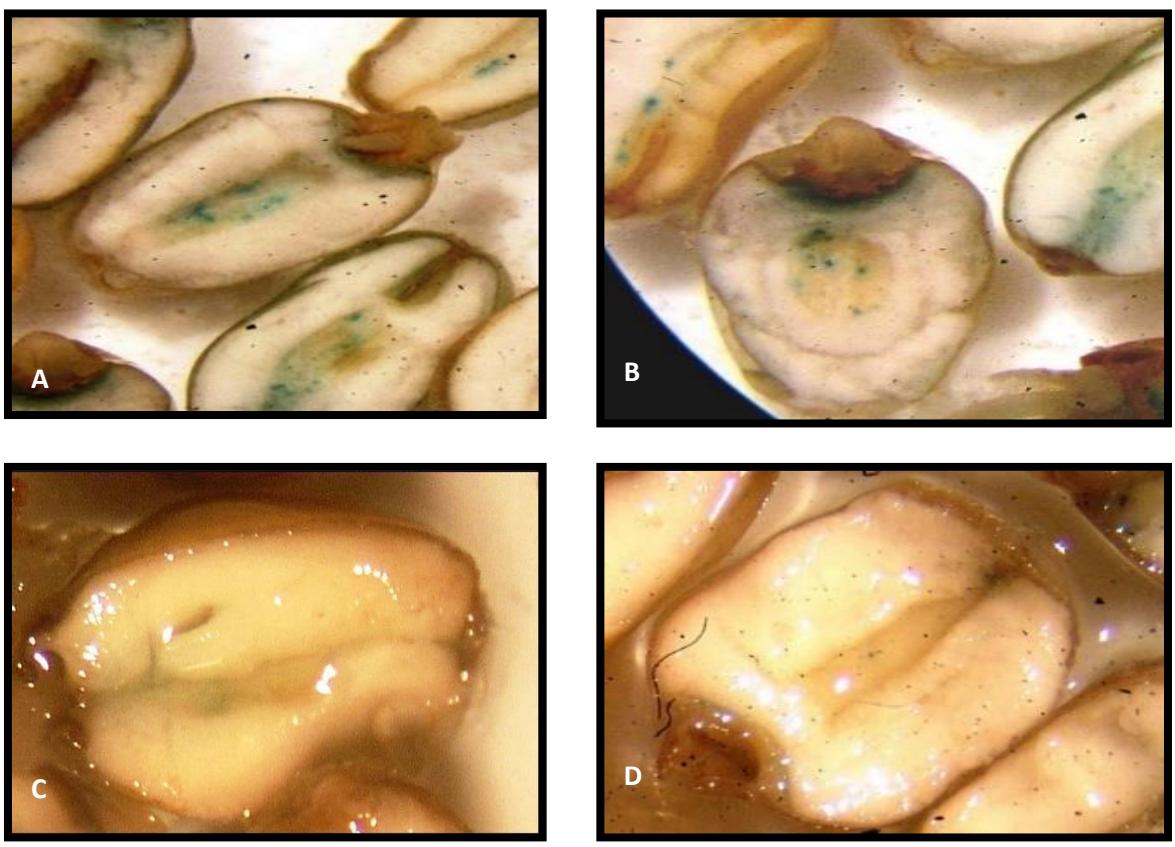

Fig. 9. Transient expression analysis in wheat endosperm. Root tissue bombarded with A) 35S promoter, B) Hybrid *D-hordein* promoter (hpGRdhor-H), showing blue spots, C) Negative control endosperm bombarded with gold particles without DNA coating, D) Un-bombarded but *GUS* stained wheat endosperm.

Discussions

Promoter sequences are very important with respect to the initiation and the duration of transcription (Narusaka *et al*., 1999). Decoding the promoter sequences of genes is one of the most important issues in genome biology. Primary components of promoter are the *cis*-acting regulatory regions. Interpretation of *cis*-regulatory element within the promoter region could facilitate possible prediction of gene expression (Zhang *et al*., 2004). Modification of *cis*-regulatory architecture not only provides the modification in transgene activity and inducibility but also sheds light on functional role of defined *cis*- motifs (Kumlehn, 2006). Variations in the number of regulatory motifs play an important role in the expression studies, particularly, in understanding the binding of *cis*-motifs to particular transcription factors in response to specific stimuli. The localization of the TSS site helps in the restructuring of a promoter, especially the designing of a hybrid promoter. Plant PAN identifies the transcription factors that are key regulators of gene expression (Gurushidze *et al*., 2014).

Motifs conferring endosperm-specific expression reside in the proximal region of the *D-hordein* promoter, upstream to the transcriptional start site. The main regulatory motif that directs the endosperm specific expression in *D-hordein* is "Endosperm box" (Kries & Shewry, 1992). Endosperm box is present in the 5'UTR of many seed storage protein promoters and contains two different *cis*-acting elements named prolamin box (PB: TGTAAAG) and GCN4 like motif (GLM: (G/A) TGA (G/C) TCA (T/C). Full transcriptional activation is achieved by interaction of *cis*-regulatory elements with different transcription factors as they are important part of a regulatory complex. The bZIP transcription factors bind to GCN4 motif (Foster, 1994), while transcription factors (TFs) of DOF (DNA-Binding with One Finger) families bind to the P-box and regulate the endospermic expression. Fine tuning in the *cis*-regulatory motifs and positioning of these elements plays a very important role in promoter architecture. Rushton and colleagues (2002) showed the variations of *cis*-motif copy number, specific order and spacing relative to each other and to the TATA box of CaMV 35S promoter.

The hybrid *D-hordein* promoter was characterized experimentally to determine its expression. Transient *GUS* expression analysis of *D-hordein* hybrid promoter was performed in wheat endosperm, spike, root and leaves. Wheat grains soaked in water overnight were also used for transient expression of *GUS* to evaluate the promoter activity in endosperm. The plasmid pGR1 with 35S promoter showed expression in endosperm along with the other tissues used in these studies (wheat leaves, spike, roots) but the endosperm specific *D-hordein* promoter showed expression only in endosperm. (Holm *et al*., 2006) sequence analysis depicted several motifs related to endosperm specific expressions. Our results suggest that endosperm specific expression of *GUS* gene but hybrid *D-hordein* promoter might be due to prolamine box and Skn-1 motif. The results suggest that hybrid 1756 bp promoter exhibited GUS expression in endosperm tissues. Earlier it was reported that promoters of seed storage proteins exhibit tissue specific expression specially endo- sperm expression (Hovath *et al*., 2000; Wu *et al*., 1998).The reported hybrid promoter had three motifs to control the endosperm specific expression, therefore, no expression for *GUS* was observed in roots, leaves and spikes (Figs. 6-8).Previously barley endosperm was used as explants in transient *GUS* assay but used of heterologous system like wheat was more advantageous (Trifonova *et al*., 2001). This is due to reason that wheat is main cereal crop in Pakistan and this system may directly use to study promoter activity in wheat endosperm. Moreover, there is close sequence similarity in wheat and barley genome (Dubcovsky *et al*., 2001). Therefore, hybrid promoter may be used to engineer wheat plant through transgene technology.

In cereals, endosperm is the most important tissue closely related to productivity and nutritional value of crop. Therefore, endosperm specific promoter may help to express seed storage protein specifically in endosperm. Moreover, hybrid promoters are not only important to achieve higher and specific expression but it also may help to overrule patency issue. This may help to manipulate endosperm for improvement of nutritional value of grain yield of monocots.

Conclusion

Characterization and functional analysis of *cis*-regulatory elements in a promoter sequence through bioinformatics approaches provides an innovative approach in the applications of biotechnology. Analysis of *cis*-regulatory regions has paved way to design hybrid as well as synthetic promoters. It is expected that complete understanding of the regulatory regions and transcription factors in the regulatory regions would help in designing new synthetic/ hybrid promoters for tissue specific or constitutive expression of transgenes. So, the present work also provides an important insight in the designing of hybrid monocot promoters to improve multiple traits in crops without facing IPR issues.

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