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Cloning and expression analysis of alcohol dehydrogenase (*Adh*) hybrid promoter isolated from *Zea mays*

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Hybrid promoters are created by shuffling of DNA fragments while keeping intact regulatory regions crucial of promoter activity. Two fragments of alcohol dehydrogenase (*Adh*) promoter from *Zea mays* were selected to generate hybrid promoter. Sequence analysis of both alcohol dehydrogenase promoter fragments through bioinformatics tools identified several crucial cis regulatory elements and transcription factors binding sites. Both fragments were separately cloned in the TA vector (pTZ57R/T) and fused to get the complete hybrid promoter (*Adh*-H). Alcohol dehydrogenase hybrid promoter was further cloned in expression vector pGR1 through adaptor ligation. Transient *β*-glucuronidase (GUS) assay revealed that hybrid promoter exhibited high expression under anaerobic conditions in wheat tissues. From the study it is concluded that hybrid promoter (*Adh*-H) may be used to derive gene expression in monocots during anaerobic conditions. The present work also provides an important insight in the designing of hybrid monocot promoters to improve multiple traits in crops without facing intellectual property rights (IPRs) issues.

Key words: Hybrid promoter, histochemical β-glucuronidase *(GUS)* assay staining, *cis* regulatory elements, alcohol dehydrogenase, *Zea mays.*

INTRODUCTION

Promoters are regulatory elements that control transcription and the level of gene expression (Hernandez-Garcia et al., 2014). Several promoters isolated from viral, bacterial and plant origin have been characterized and used extensively in transgene expression system (Yoshida and Shinmyo, 2000; Muller and 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. The strength and expression behavior of promoter depends upon interaction promoter cis

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regulatory elements with transcription factors (Atchison, 1988). Analyzing promoter sequence through available databases like plantCARE and plantPAN, we can predict promoter expression and strength (Lescot et al., 2001). 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. These promoters may be used to generate transgenic plants transformed with multi genes where single promoter may lead to gene silencing due to post transcriptional gene silencing (Mol et al., 1989). There have several hybrid promoters been synthesized including E4/E8 promoter (De Boer et al., 1983) and tacI/tacII promoter (Bestwick and Kellogg, 2000). The expression analysis of these promoters was conducted in monocots and dicots (Lee et al., 2007). In the present study, we have generated a hybrid alcohol dehydrogenase promoter and analyzed its cis regulatory elements and expression behavior in monocot system.

Alcohol dehydrogenase (*Adh*, EC 1.1.1.1) is an enzyme that catalyzes interconversion of aldehydes and alcohols (Arnold et al., 2013) and detoxification of acetaldehydes (Garabagi et al., 2005). It maintains cellular level of NAD^+ which is constantly required in several crucial biochemical reactions. Its activity has been detected in a vast number of higher plants including *Arabidopsis*, maize, wheat, rice, tomato, potato and pea (Batut et al., 2013; Mardanova et al., 2007). *Adh* is essential for the survival of plants during prolonged anaerobic conditions, fruit ripening and seedling development (Thompson et al., 2010). Two *Adh* genes have been reported in maize, named *Adh1* and *Adh2*, which are located on distinct chromosomes. *Adh1* is located on chromosome 1 and *Adh2* on chromosome 4 (Calo et al., 2013). The 5' untranslated region of the *Adh* mRNAs showed a conserved sequence (G-TCNGGAGTGG) at about 45 base pairs upstream from the translation start site. This conserved sequence was located in both *Adh1* and *Adh2* genes and supposed to be important in an-aerobiosis. Several regulatory elements associated with anaerobic induction have been identified in *Adh1* promoter. These anaerobic response elements (AREs) of *Adh*1 have two copies of GC-element (59-GCC[G/C]C-39) and two copies of GT-elements (59-[T/C]GGTTT-39). GC-regulatory elements are required for the expression of *Adh*1 while GT motif is involved in general anaerobic induction (Petolino and Davies, 2013). The 5´UTR region of the tobacco *Adh* gene was reported to be an efficient translational enhancer in *Arabidopsis* and tobacco (Satoh et al., 2004). *Adh* promoter also showed expression in aerobic conditions. However, the expression level is very low in aerobic conditions as compared to in anaerobic conditions. Hundreds of polypeptides are synthesized in roots under aerobic conditions including *Adh*, but the expression level of *Adh* increases several folds during anaerobic conditions (Chung and Ferl, 1999).

The present work was designed to construct a hybrid promoter and to evaluate the efficiency of this promoter by transient expression using *GUS* reporter gene. The main objectives of the study aimed at identification of promoter regions of *Adh* gene variants from HTGS database available at NCBI. These promoter fragments were separately cloned in TA vector and fused to generate hybrid promoter. Finally, transient expression analysis of hybrid promoter was analyzed in monocot plant wheat. The novel hybrid promoter may be part of expression cassette to improve cereals crops without facing IPR issues.

MATERIALS AND METHODS

For generation of alcohol dehydrogenase promoter, two fragments located on distinct chromosomes were picked from High through put Genomic Sequences (HTGS). Fragment-I *(AdhI*) of 1.1 Kb was retrieved from HTGS sequence of *Zea mays* chromosome 1 (AC190915.3). The second fragment (*Adh-II)* of 390 pb was isolated from HTGS sequence of *Z. mays* chromosome 4 (AC213880.3). Various bioinformatics tools were used to predict regulatory regions in both fragments. Fragment-I was to be ligated upstream of fragment-II in TA cloning vector though directional cloning. For amplification of alcohol dehydrogenase promoter fragments, multiple sets of primer pairs were designed with specific restriction sites to facilitate cloning. The fragment-I contained *Sac*I restriction site in forward primers and *Apa*I site the reverse primers. The forward primers for fragment-II contained *Apa*I and reverse primers had *Hind*III site (Table 1).

Cloning of alcohol dehydrogenase promoter in TA cloning vector

The alcohol dehydrogenase hybrid promoter was generated by joining the two fragments from each variant. Both *Adh* promoter fragments were amplified using *Zeamays* DNA as template. Both promoter fragments were PCR amplified using selected primers and cloned independently in TA cloning vector. Annealing temperatures, genomic DNA and Mg^{+2} concentrations were optimized prior to cloning.

Generation of alcohol dehydrogenase hybrid promoter

PTZ vector having promoter fragment-II was digested with *Apa*I and *Hind*III to generate sticky ends complementary to fragment-I. Promoter fragment-I was also digested with *Sac*I and *Apa*I to join upstream of promoter fragment II cloned in TA vector. Both fragments-I and II were ligated at *Apa*I site and transformed. Hybrid promoter clone was confirmed through restriction with *Sac*I and *Hind*III. Alcohol dehydrogenase hybrid promoter clone (*Adh*-H) was then used for further cloning in the plant expression vector pGR1.

Cloning of alcohol dehydrogenase hybrid promoter in pGR1

A Plant expression vector pGR1 (provided by gene isolation group, NIBGE) 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. Hybrid alcohol dehydrogenas promoter was picked from TA vector and cloned into pGR1 by replacing 35S promoter. The resultant clone containing hybrid promoter was

Table 1. Primers used for cloning of alcohol dehydrogenase hybrid promoter.

Primer	Name of primer	Sequence
Forward primers for *Adh*-fragment I	HAdhZmzV1F-1	5'AGTGAGCTCGATCCTAGGAGCTAAA 3'
	HAdhZmzV1F2	**5'AGTGAGCTCGATCCTAGGAGCTAAAGC 3'**
	HAdhZmzV1F-3	5'AGCGAGCTCCACTTAGCAAACCATTCTAGT 3
Reverse primers for *Adh*-fragment I	HAdhZmzV1R-1	5'TAAGGGCCCTCGGATGCGCCGC 3'
	HAdhZmzV1R2	**5'TAAGGGCCCTCGGATGCGCCGCG 3'**
	HAdhZmzV1R-3	5'TAAGGGCCCCGCTAGCTCGGATCTG 3
Forward primers for *Adh*-fragment II	**HAdhZmzV2-F**	**5'GCAGGGCCCGGAAAACGGTAAACAAGAAA 3'**
	HAdhZmzV2F-2	5'GCAGGGCCCGGAAAACGGTAAACAAGAAAC 3'
Reverse primers for *Adh*-fragment II	**HAdhZmzV2R1**	**5'ATCAAGCTT TGCTTGCTCTCTCTCTCTC 3'**
	HAdhZmzV2R-2	5'ATCAAGCTT TGCTTGCTCTCTCTCTCTCTC 3'

named pGR*Adh*-H.

Transient *GUS* assay

Transient expression studies were carried out to evaluate the activity of the alcohol dehydrogenase 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 promoterless construct was used as negative control. Vector pGR1 with *GUS* gene downstream of 35S promoter was used as positive control. To monitor any false positive result, gold particles without any coating were also bombarded. Wheat leaf, spike, root and endosperm were used as explants for the bombardment experiments.

A 1 µg/µl of plasmid DNA of each construct was used for coating of 1 µm diameter sterile gold particles. Leaves, roots and spikes were taken from wheat plants grown in pots from green house. Wheat seeds were soaked for 2 to 3 days in Petri plates containing sterile distilled water and cut with sterile blade longitudinally to expose endosperm. All tissues were placed on Petri plates containing ½ MS medium (Murashige and Skoog, 1962) in a way to expose maximum surface area for bombardment. All wheat explants were bombarded at 27 mmHg vacuum using1100-psi rupture disks and 9 cm target distance. Same conditions were used to bombard plasmids having 35S promoter coated and negative control coated gold particles. Petri plates were placed at 25±2ºC for 24 h and then submerged in *GUS* staining buffer containing 0.1M X-Gluc. All tissues were incubated in dark at 37ºC for overnight till appearance of blue color and washed with 70% ethanol 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

A maize alcohol dehydrogenase gene was selected for generation of hybrid promoter. Two upstream regions of alcohol dehydrogenase gene located at distinct chromosomes were retrieved and analyzed through bioinformatics tools. Fragment-I was retrieved from HTGS sequence of *Zea mays* chromosome 1 of clone CH201-528P20 at position 9934-8810 (AC190915.3). The *Adh-II* fragment was isolated from HTGS sequence of *Zea mays* chromosome 4; clone CH201-465N3 at position 70445-70056 under AC213880.3. BLASTp results of both upstream sequences confirmed there was no coding region. Patent BLAST results revealed that novel promoter is 26% dissimilar to already patented sequence (Accession No. 220526.1). Sequence analysis of both fragments revealed several cis acting motifs and transcription factor binding sites as identified through PlantCARE. Nucleotide sequence and motifs of complete hybrid are shown (Table 2 and Figure 1). Core promoter elements including TATA box and CAAT box were present in hybrid promoter. There were several light responsive motifs including ACE motif, Sp1 motif and TCT motifs were detected in *Adh-H* promoter. A 5´UTR with consensus sequence TTTCTCTCTCT was also detected in *Adh-H* promoter. An anaerobic response element ARE (TGGTTT) was also observed in *Adh-H* promoter. Several other crucial motifs including LTR, MBS, TC-rich stretch and AuxRR-core were located in hybrid promoter.

Both promoter fragments were cloned separately in TA vector and ligated directionally to synthesize hybrid promoter. For functional characterization alcohol dehydrogenase hybrid promoter was cloned in an expression vector and analyzed through transient *GUS* assay in wheat. For amplification of *Adh* promoter fragments I and II selected annealing temperature through gradient PCR were 53.7 and 53.1°C respectively (Figure 2A and B). *Adh* promoter fragment-I was amplified using forward primer H*Adh*ZmzV1F-2 having *Sac*I restriction site, and reverse primer H*Adh*ZmzV1R-2 having *Apa*I restriction site. *Adh* promoter fragment-II was amplified at 53.1ºC with forward primer H*Adh*ZmzV2F-1 having *Apa*I restriction site and reverse primer H*Adh*ZmzV2R-1 having *Hind*III restriction site (Table 1). Clones of both promoter fragments in TA vector were confirmed through digestion with *Sac*I and *Apa*I (Figure 3A and B). Clones were also confirmed by DNA sequencing on an ABI 3100 Genetic Analyzer. Promoter fragment II cloned in TA vector was ligated with fragment I using *Apa*I and *Hind*III. Hybrid 1.5 kb promoter clone

Table 2. Cis-*regulatory* elements in alcohol dehydrogenase hybrid promoter.

Motif	Sequence	Function
5´ UTR Py-Rich Stretch	TTTCTCTCTCTCTC	*Cis*-acting element conferring high transcription level.
ABRE	TACGTG	*Cis*-acting element involved in abscisic acid responsiveness.
ACE	C/GT/CA/GACGTATT/C	*Cis*-acting element involved in light responsiveness.
ARE	TGGTTT	*Cis*-acting element essential for the anaerobic induction.
ATGCAAAT Motif	ATACAAAT	*Cis* regulatory element associated to TGAGTCA motif
AuxRR-core	GGTCGAT	*Cis*-acting element involved in auxin responsiveness.
CAAT-box	TCTAACCGG	Common *cis*-acting element in promoter and enhancer region
CAT-box	GCCACT	*Cis*-acting element related to meristem expression.
ELI BOX-3	AAACCAATT	Elicitor responsive elements
GARE motif	AAACAGA	Gibberelin responsive element
LTR	CCGAAA	*Cis*-acting element involved in low temperature response
MBS	TAACTG	MYB binding site involved in drought inducibility.
Sp1	CCC/G/A	Light responsive element
TATA-box	TATAT/CATAT	Core promoter element around -30 of transcription start site.
TC-rich repeats	ATTTTCTTCA	*Cis*-acting element involved in stress and defense responsiveness
TCT-motif	TCTTAC	Part of light responsive element
Circadian	CAAAGATATC	*Cis*-acting element involved in circadian control

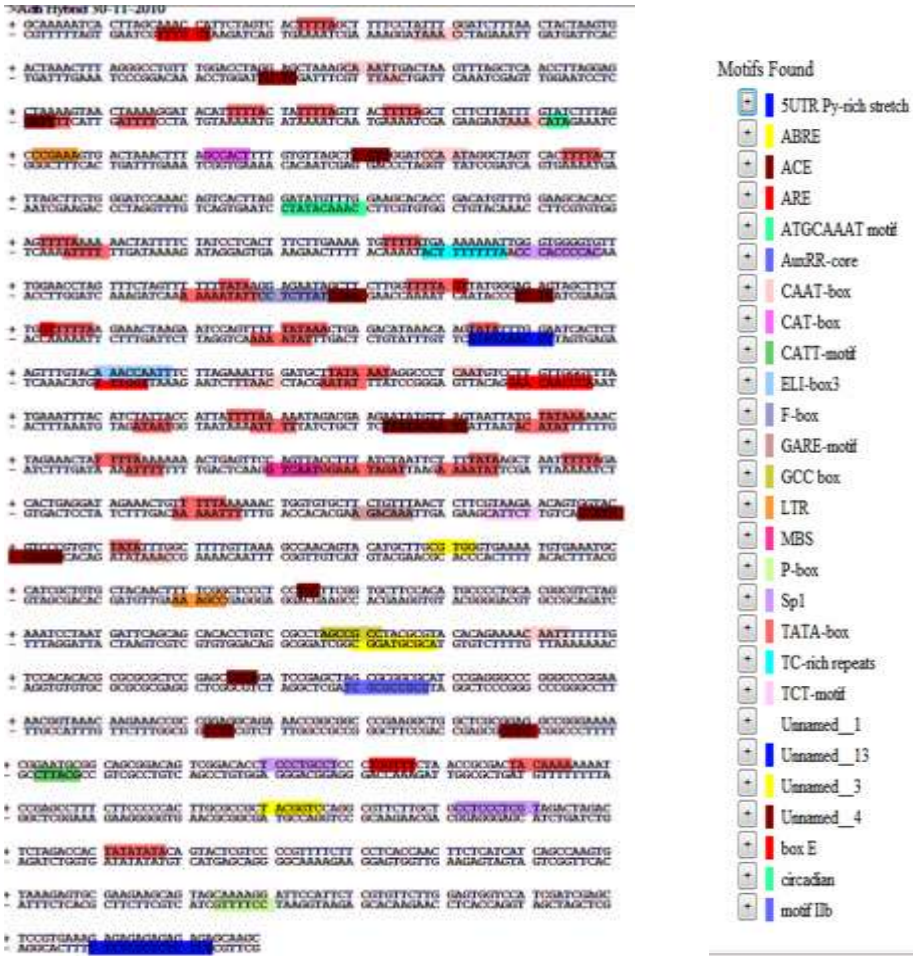

Figure 1. Complete nucleotide sequence of alcohol dehydrogenase hybrid promoter (*Adh-H*) showing cis acting regulatory motifs (PlantCARE analysis). Arrow indicates joining region of promoter fragment I and promoter fragment II.

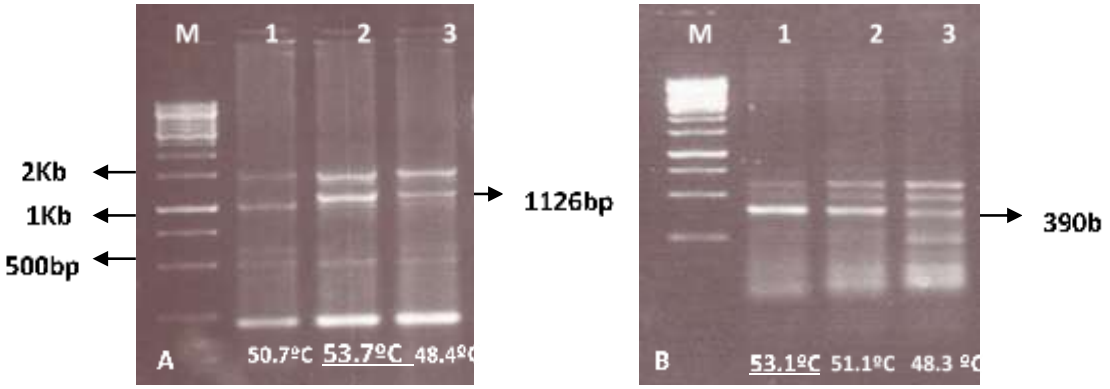

Figure 2. Gradient PCR of *Adh-I* and *Adh-II* promoter fragments: (A); PCR amplification *Adh-I* fragment at different annealing temperatures. M; 1 Kb ladder, Lanes 1-3 show PCR at 50.7, 53.7 and 48.4ºC respectively. (B); Gradient PCR of *Adh-II*. M; 1 Kb ladder, Lanes 1-3 represent PCR of *Adh-II* at 53.1, 51.1 and 48.3ºC respectively (underlined were temperatures selected for PCR amplification).

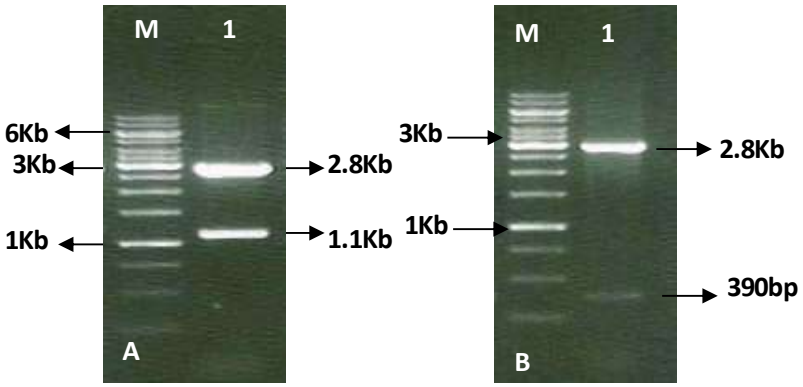

Figure 3. Cloning of *Adh-I* and *Adh-II* promoter fragments in TA cloning vector (A), Restriction digestion of *Adh-I* fragment with *Sac*I and *Apa*I. M; 1 Kb ladder, Lane 1 represent *Adh-I* clone (1126bp). (B), Restriction digestion of *Adh-II* fragment with *Apa*I *and Hind*III. M; 1 Kb ladder, Lane 1 represents *Adh-II* clone (390 bp).

was confirmed through restriction with *Sac*I and *hind*III (Figure 4A). *Adh* hybrid (*Adh*-H) cloned in TA vector was subcloned in the plant expression vector pGR1. The resultant vector construct having alcohol dehydrogenase hybrid promoter was named pGR*Adh*H and confirmed through digestion of resultant vector with *Sac*I and *Hind*III (Figure 4B). PCR also confirmed the cloning of 1.5 Kb complete hybrids in pGR1 (Figure 4C). The pGR*Adh*, therefore, represents a transient expression vector having *Adh* promoter instead of 35S promoter to control expression of *GUS* reporter gene (Figure 5).

To evaluate activity of hybrid promoter, transient *GUS* assay was performed in wheat tissues. Wheat leaves were bombarded with pGR1 under both 35S and *Adh*-H showed *GUS* expression (Figure 6). Wheat spikes bombarded with 35s and *Adh-H* promoters showed *GUS* expression in pedicel attachment region (Figure 7). The roots bombarded with *Adh-H* promoter exhibited high *GUS* expression. The microscopic view revealed that instead of blue spots a diffused kind of blue staining was observed in roots (Figure 8). The results revealed that *Adh* promoter expressed *GUS* in leave tissues and the staining intensity was comparable to the control plasmid (pGR1). In wheat seeds, the *GUS* activity was not observed under *Adh-H* promoter as detected under 35S promoter. However, the aleurone cells indicated the *GUS* expression (Figure 9). No GUS stain was detected in tissues bombarded with promoter-less constructs and unbombarded negative controls.

DISCUSSION

The object of present study is to synthesize hybrid

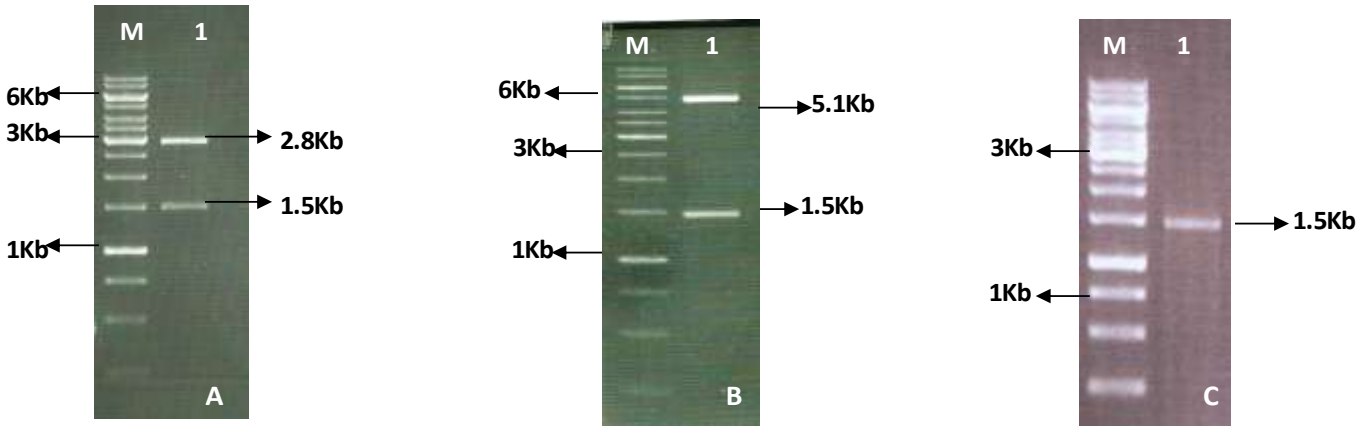

Figure 4. Cloning of *Adh* hybrid promoter in TA cloning vector and expression vector pGR1. (A) Double digestion of *Adh* complete hybrid with *Sac*I and *Hind*III showing 2.8 kb vector backbone along with 1.5 kb promoter clone. M; 1 Kb ladder, Lane 1; Hybrid *Adh* promoter (*Adh*-H) in TA cloning vector. (B) Confirmation of clone containing *Adh*-H promoter in pGR1: M; I Kb ladder (A); Double digestion with *Sac*I and *Hind*III releasing 5.i pGR1 vector backbone and 1.5 Kb Hybrid *Adh* promoter (*Adh*-H). (C) Confirmation of cloning through PCR amplification. M; I Kb ladder (A); PCR analysis of *Adh*-H promoter using promoter specific primers.

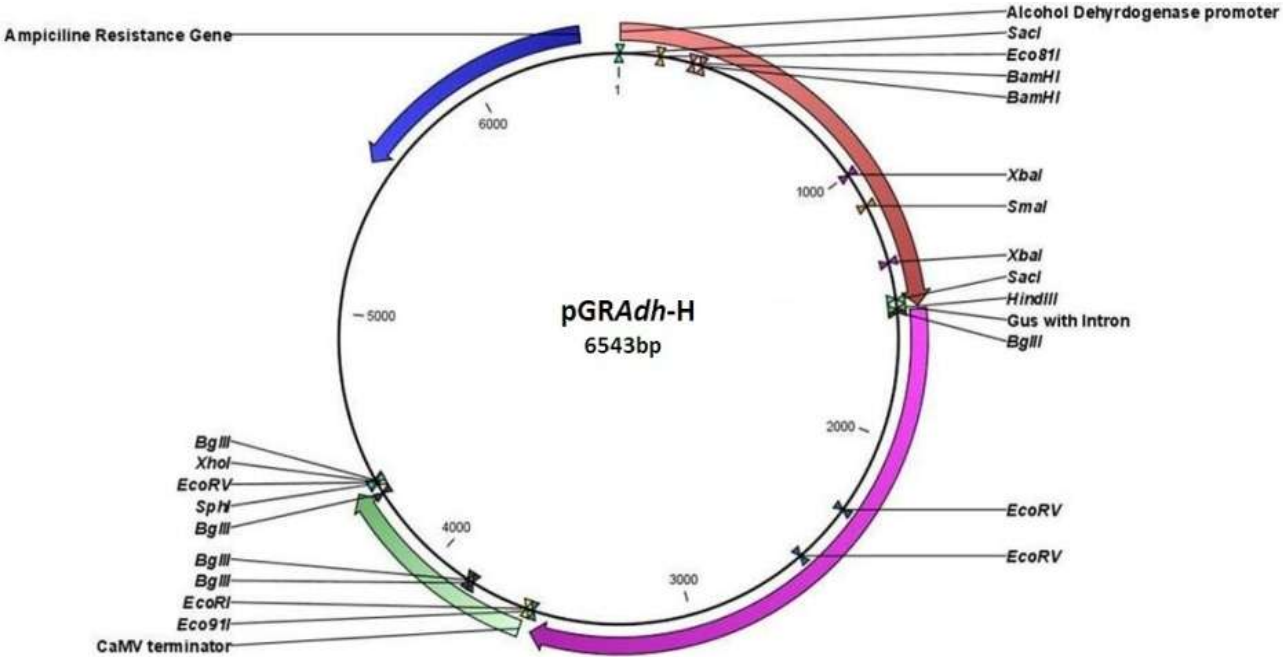

Figure 5. Physical map of pGRAdhH vector.

promoter to develop novel regulatory sequence in order to control gene expression. A variety of promoters are available for introduction and expression of transgenes in plants. However, these promoters cannot be used freely due to IPR policy. Along with isolation of novel promoters, synthesis of hybrid promoter is also carried out for gene expression. In the present study, two fragments of *Adh* promoter located no different chromosomes of maize were fused to generate hybrid promoter. Although, nucleotide sequences of both fragment-1 and fragemt-2 were already patented but hybrid promoter had only 74% similarity with patented sequences. In hybrid *Adh* promoter, along with core promoter elements most of the crucial regulatory motif were remained intact and functional. Essential *cis*-acting regulatory elements of *Adh-H* promoter (TATA box and CAAT box) are often conserved in many species and localized at 50 to 100 bp upstream of the transcription start site (TSS), while the other *cis*-regulatory motifs around them are variably placed. The most common *cis*-regulatory element in

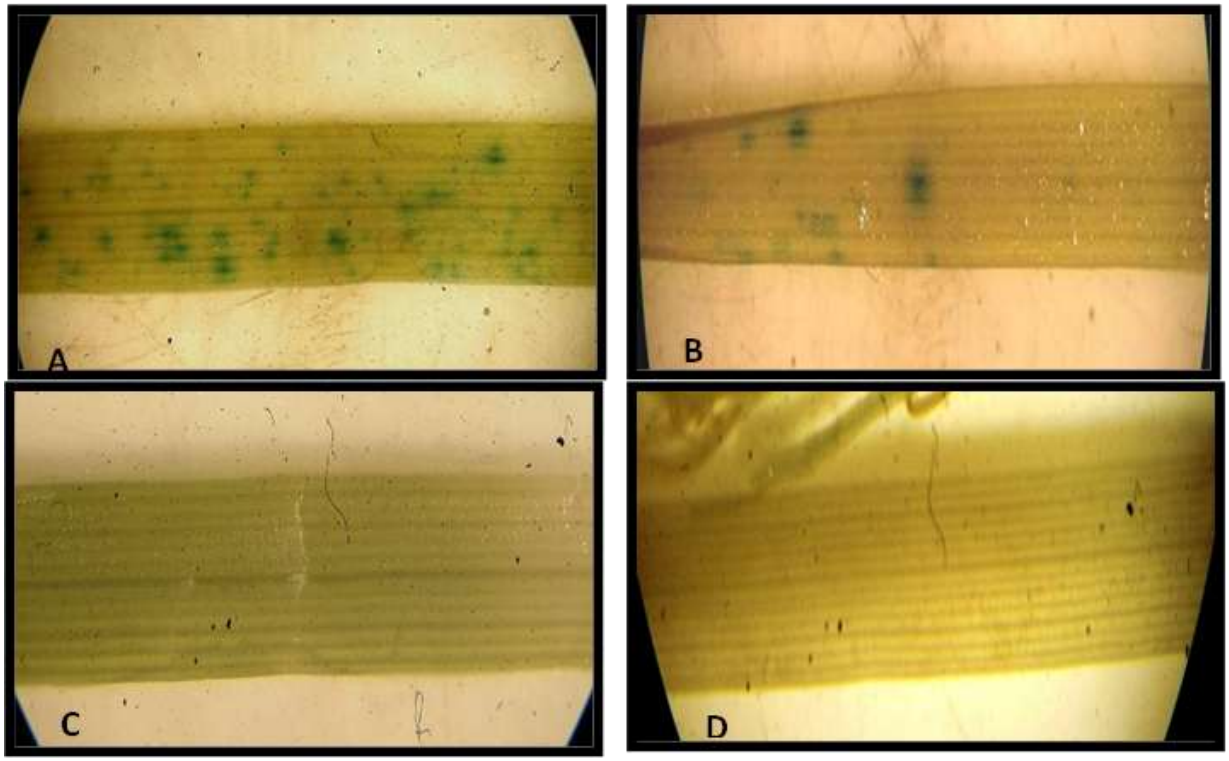

Figure 6. Transient *GUS* expression wheat leaves. (A) Positive control (35S promoter); (B) *Adh*-H promoter showing the localized *GUS* activity as blue spots; (C) Negative control; (D) Un-bombarded *GUS* stained tissues.

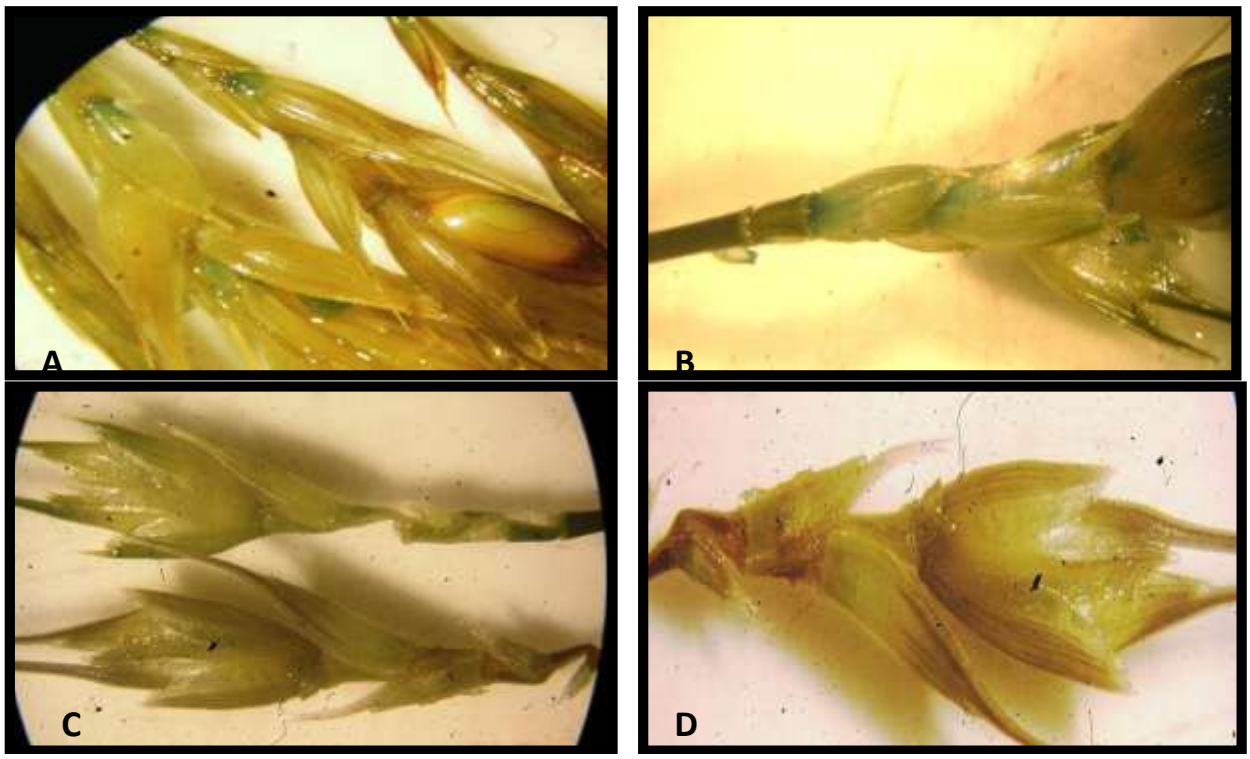

Figure 7. Transient expression in wheat spikes. **(**A) Positive control (35S promoter); (B) *Adh*-H promoter; (C) Negative control; (D) Un-bombarded *GUS* stained tissues.

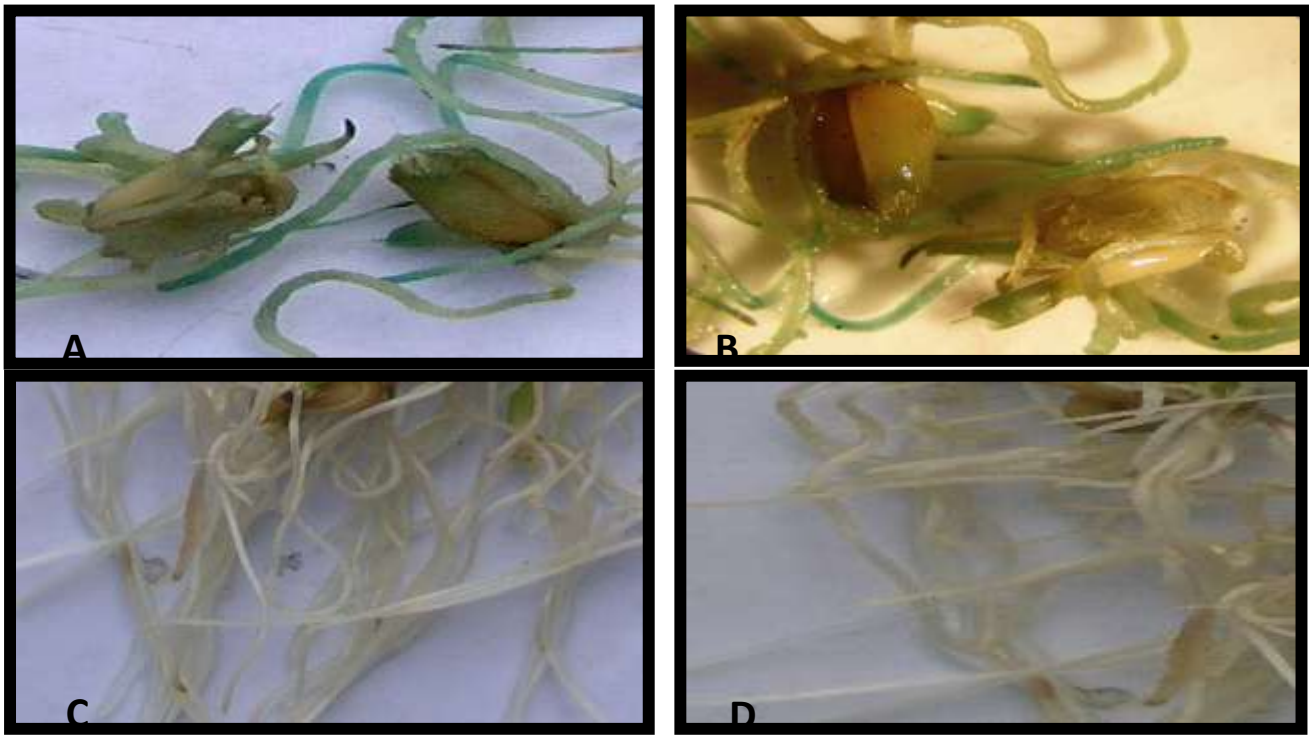

Figure 8. Transient *GUS* expression in wheat roots. (A) Positive control (2X35S promoter); (B) *Adh*-H promoter; (C) Negative control; (D) Un-bombarded *GUS* stained root tissues.

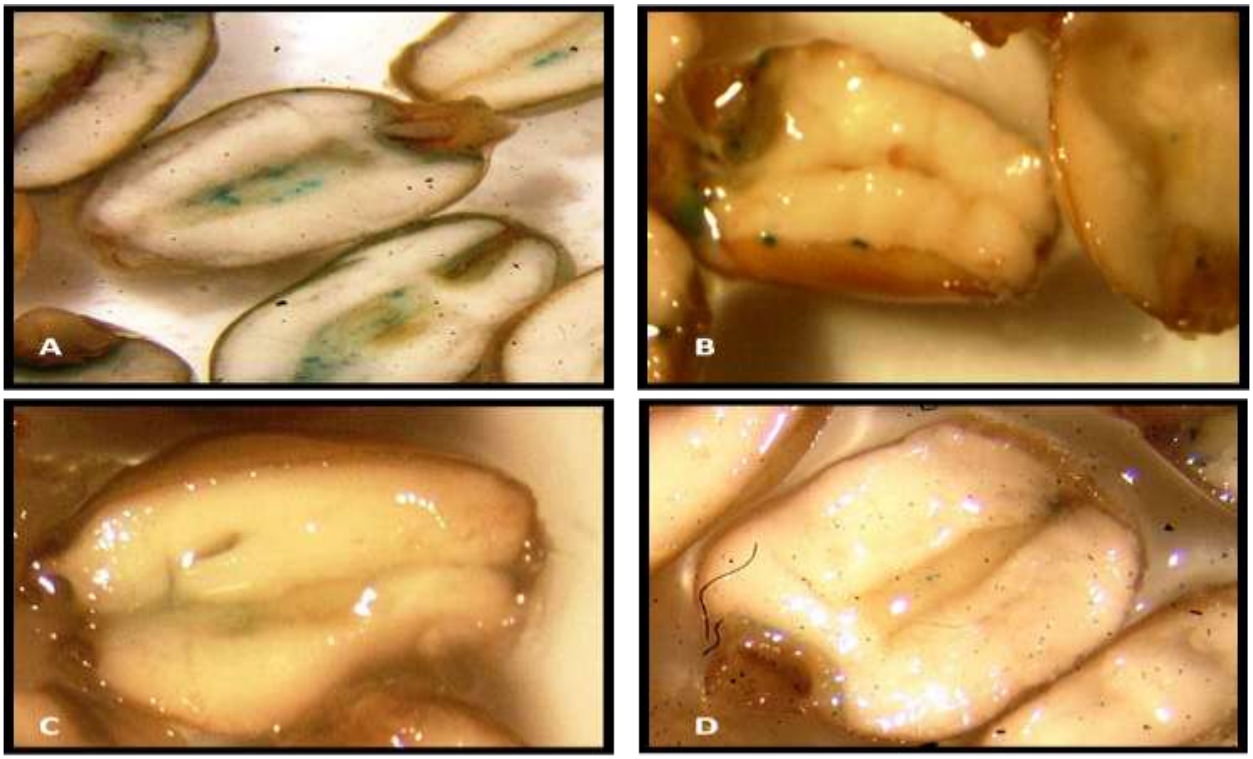

Figure 9. Transient *GUS* expression in wheat seeds. (A) 2X35S promoter Positive control; (B) *Adh*-H promoter Showing the localized *GUS* activity in the form of blue spots confined to the aleurone layer; (C): Negative control; (D) Negative control; Un-bombarded *GUS* stained tissues.

hybrid *Adh* promoter is anaerobic responsive element (ARE), that was first identified in maize and *Arabidopsis Adh1* promoters (Park et al., 2012). ARE motif was present in both fragments of *Adh* promoter. Anaerobic response element (ARE) consists of GT- and GC-motifs, which are both crucial for gene expression especially under anaerobic conditions. These GC- and GT-rich motifs are able to activate transcription in response to hypoxia in wheat protoplasts, maize protoplasts and hypoxic tobacco plants (Deal and Henikoff, 2011). There are different binding sites for all the *cis*-regulatory motifs that specifically bind and activate the particular regulatory element. GCBP-1 is the binding sites of GC motif and it is important in the hypoxic activation of gene expression mediated by the ARE sequence. A conserved sequence (G-TCNGGAGTGG) is located at about 45 bp upstream from the translation start site and has been proposed to be important in anaerobiosis (Hou et al., 2012). Similarly, a drought and ABA induced transcription factor *AtMYB2* binds to the GT-motif as GT-motif site resembling to Myb-transcription factor-binding site.

Analysis of the regulatory sequences in *Adh*-H promoter also showed the presence of some important motifs that may serve as essential regulatory elements in promoter activity. Regulatory motifs within the second fragment of *Adh-H* hybrid showed much variation with that of already reported promoter, although some common motifs were also identified. A 5´ UTR Py rich stretch present at -369 and -373 position of *Adh-II* confers high transcriptional level in *Adh*-II, while this stretch was absent in the reported promoter. The 5´ UTR Py rich stretch was first reported in the HMG2 stretch of tomato promoter, where it helps in advanced transcription (Peremarti et al., 2010). There are three motifs which were found associated with the selected *Adh-II* fragment but have not been reported in the previously characterized *Adh* promoter. The first one named AuxRR-motif is present within the promoter region of *Adh*-II and absent in the reported *Adh* promoter. AuxRR-motif is essential *cis*-element involved in auxin responsiveness (Yang et al., 2013). Abscisic acid regulatory element such as ABRE is the second motif absent in published promoter but found in *Adh*-H at position 179 bp upstream of TSS. This motif is involved in abscisic acid responsiveness (Narusaka et al., 2003). The last identified motifs, CATT and P-box, were not reported in the patented *Adh* promoter but present in the second fragment (*Adh-II*) of the promoter characterized in this studies.

To evaluate the potential strength of *Adh-H* promoter, *GUS* expression level was determined in various wheat tissues, that is, roots, endosperm, leaves and spike. A*dh* hybrid is a constitutive promoter and is expressed at a moderate level in all wheat tissues. However, expression was raised many folds in roots under normal condition. This shows that expression may be increased under hypoxic or anoxic conditions. It is reported that expression in the roots under aerobic conditions also requires all the same GC and GT-rich motifs that are activated in hypoxic conditions. However, the expression under aerobic conditions is several folds less than that of anaerobic conditions (Arnold et al., 2013). On the other hand, expression of *Adh*-H the sectioned germinating wheat seed revealed the expression in aleurone layer but not in the endosperm indicating that *Adh* promoter does not express in wheat endosperm. Although hybrid *Adh*-H promoter exhibited expression in most of wheat tissues but roots showed significantly high expression. The hybrid *Adh*-H promoter may be used to derive specific expression in roots or anaerobic conditions.

Conclusion

Along with exploration of novel promoters, hybrid promoters are synthesized for gene transformation. In the current study, two fragments of maize *Adh* promoter from distinct chromosomes were ligated to synthesize. Along with crucial regulatory elements, *Adh*-H promoter contained a number of anaerobic response elements. Through transient *GUS* assay, hybrid *Adh* promoter showed expression in wheat plants especially in roots. This may be related to presence of anaerobic response elements in Adh-H promoter. From the study, it is concluded that hybrid Adh-H promoter may be used to expression transgenes in monocots especially root related expression.

Conflict of Interests

The authors have not declared any conflict of interests.

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