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CLONING AND TRANSCRIPTIONAL ANALYSIS OF RuBisCO HYBRID PROMOTER

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Abstract

Two variants of RuBisCO promoter from zea mays were selected to generate hybrid promoter by using bioinformatics tools. Sequence analysis of both RuBisCO promoter variants revealed several critical cis regulatory elements and transcription factors binding sites within the promoter region. Various regulatory motifs related to constitutive expression were located in RuBisCO promoter fragments. Analysis of *cis*-regulatory regions has paved way to design synthetic promoters. Both variants were separately cloned in TA vector (pTZ57R/T) and then joined to get the complete hybrid promoter (Rub-H). Hybrid RuBisCO promoter was further cloned in expression vector pGR1. Transient GUS assay revealed that hybrid promoter exhibited endosperm specific expression in wheat. From the study it is demonstrated that hybrid promoter (Rub-H) may be used to derive constitutive expression in monocots. The present work provides an important insight in the designing of hybrid monocot promoters to improve various traits in crops without facing IPR issues. 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.

Key words: RuBisCO, Hybrid, Plant promoter, Cloning, Cis-regulatory elements, Histochemical GUS staining

Introduction

Genetic engineering can be used to introduce different traits into plants. The studies to discover system biology in coordination with bioinformatics has yielded enormous biological data and insight into plant metabolism, transcriptional gene expression. Ribulose-1,5-bisphosphate carboxylase/oxygenase, commonly known by the abbreviations RuBisCO, is an enzyme (4.1.1.39) involved in the first important step of carbon fixation. It catalyzes the carboxylation of ribulose-1,5-bisphosphate and helps in converting atmospheric carbon dioxide and other photosynthetic organisms to energy-rich molecules. It is probably the most abundant enzyme on Earth (Yinon *et al.,* 2019). RuBisCO is the most abundant protein in leaves, accounting for 50% of soluble leaf protein in C_3 plants, and 30% of soluble leaf protein in C_4 plants. Given its important role in the biosphere, the genetic engineering of RuBisCO in crops is of continuing interest especially in monocots crops like zea mays (Parry *et al.,* 2013). RuBisCOs can be partitioned into three types: I form exists in higher plants and many prokaryotes, consisting of eight large subunits (50~60 kD) and eight small subunits (12~18 kD), presenting square symmetry structure. The RuBisCO large subunit (rbcL) gene of higher plants sets in the chloroplast DNA, and it is translated by chloroplast ribosome (Zhang *et al*., 2011: Mukharjee *et al.,* 2015). Structurally, the RuBisCO enzyme consists of eight large subunits (LSU) and eight small subunits (SSU). The SSU proteins are encoded by several genes, which are located in the nuclear genome of plants, while LSU genes are found in plastid genomes. The number of RuBisCO SSU genes in different plants varies from four copies up to fifteen copies or more in some polyploid genomes. At least four copies of RuBisCO SSU genes are known to be present in Arabidobsis thaliana and ten or even more copies per polyploid genome are known in wheat.

Promoters are regulatory elements that control transcription and the level of gene expression (Masood *et al*., 2017). Designing a transformation-cassette that could enable the precise control of transgene activity is one of the major challenges in a plant genetic engineering program. The choice of promoter is the key determinants used in plant biotechnology applications to confer regulated gene expression. Different plant promoters are used in different genetic engineering strategies for gene expression studies as well as introduction of transgene for crop improvement (Masood *et al.,* 2016). Many constitutive as well as seed specific promoters have been identified from monocots plants which include glutelin, actin, oleosin etc. Maize promoters such as Gos-2, Enolase and Actin-2 have been reported for constitutive expression in embryos and leaves (Xiao *et al.,* 2005, Naqvi *et al.,* 2017). The developing countries like Pakistan face critical problems to use such patented promoters for developing commercial transgenic crops for their people. The creation 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 cis-regulatory regions on different chromosomes of a genome generates a hybrid promoter.

In the present study, promoter sequence was selected from High Throughput Genomic Sequences (HTGS) at NCBI. HTGS have unfinished genomic sequences and these sequences are readily available for scientific community (public database). The sequence analysis of these HTGS data may be utilized for the identification of several new promoter sequences in different organisms and applications in biotechnology (Masood *et al.,* 2016; Iqbal *et al.,* 2017). Functional studies on hybrid promoters have resulted in the identification of a number of *cis*-

acting elements and their respective trans-acting DNA-binding factors as they both plays a crucial role in cell signaling (Masood *et al*., 2017). A number of *cis*-regulatory elements are known such as, prolamine box, ABRE, F-box, I-box, G-box, CAAT box, ELI-box, HSE, E-Box etc. (Masood *et al.,* 2020). In the present research, *RuBisCO* promoter was identified, cloned and retrieved from HTGS for evaluation of their activity.

Materials and Methods

Bioinformatics approach to characterize the hybrid promoter: HTGS data indicated that *RuBisCO* gene has two variants regulated by two dissimilar promoter sequences. A few of the *RuBisCO* promoters have already been characterized but the database search indicated that some of them still remained to be characterized. In the present research work, hybrid promoters was made by fusing the dissimilar regions of the uncharacterized promoter with the characterized promoter fragments.

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). TSSP (plant promoter identification program) and BDGP (Berkeley Drosophila Genome Project) were used to locate the transcription initiation site (TiS).

Clo**ning of RuBisCO promoter in TA cloning vector:** The *RuBisCO* hybrid promoter was generated by joining the two fragments from each variant of the respective promoter. To obtain complete hybrid, directional cloning of *RuBisCO* fragments was performed using pTZ57R/T as cloning vector. Both *RuBisCO* promoter fragments were amplified using maize DNA as template. Fragment I comprised of 1037 bp and fragment II was of 501bp. 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. For amplification of RuBisCO promoter fragments, a gradient PCR was run initially to optimize the annealing temperature and for the selection of best primer pair. Both promoter fragments were PCR amplified using barley genomic DNA and purified through salt precipitation. 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 1). Mg^{+2} concentrations, Annealing temperature and genomic DNA were optimized prior to cloning.

Generation of hybrid *RuBisCO* 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 *Sac*I and *Hind*III to join downstream 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. *RuBisCO* hybrid clone (Rub-H) was then used for further cloning in the plant expression vector pGR1.

Cloning of hybrid *RuBisCO* 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 *RuBisCO* promoter was excised and cloned into pGR1 by replacing 35S promoter. The resultant clone containing hybrid promoter was named pRub-H.

Transient expression studies were carried out to evaluate the activity of the RuBisCO hybrid promoter using reporter gene (GUS) expression in a 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 and root 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 in a way to expose maximum surface area for bombardment. All tissues were incubated in dark at 37ºC till the 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.

Table 1. Primers designed for TA cloning of both promoter fragments.

Primer sequence	Length	Tempe-rature	GC content	Promoter fragments
ACTGAGCTCGTGCTTGTTTATGGAAACTGG	30mer	55ºC	43%	Forward primer for fragment I
ACTGAGCTCGGATTCTCGGTTTCTTAAAACC	31mer	56ºC	41%	
ACTGAGCTCGTTTATGTGCTTGTTTATGGAAAC	33mer	55ºC	33%	
ATCGGGCCCTGTTTGGATCTACAGAATTGGG	31mer	54ºC	40.9%	Reverse primer for fragment I
ATCGGGCCCGCCCAATTCTGTAGATCC	27mer	55ºC	29.9%	
CCAGGGCCCTCAGCAGCAGAGGCTATG	27mer	53 ºC	56%	Forward primer for fragment II
CCAGGGCCCAGCAGCAGAGGCTATGAGCA	29mer	58 ºC	55%	
AGTAAGCTTGCTGGCAGGCTGGC	23mer	53 ºC	79%	Reverse primer for fragment II
AGTAAGCTTGCAGGCTGGCTAGTATGTG	28mer	58 ºC	58%	

Results

A maize Rubisco gene was selected for generation of hybrid promoter. Two upstream regions of rubisco gene located at distinct chromosomes were retrieved and analyzed through bioinformatics tools. BLASTp results of both upstream sequences confirmed there was no coding region. Promoter fragment sequence analysis on plant CARE indicated the basal regulatory elements. Transcription start site (TSS) was 38 base pairs upstream of translation start codon and was determined through BDGP. For functional characterization rubisco hybrid promoter was cloned in an expression vector and analyzed through transient GUS assay in wheat. Nucleotide sequence of rubisco gene matched in HTGS database at two distinct positions. Both fragments were identified from HTGS sequence of zea mays chromosome 4 (AC205525.3) at position of (61285-60052) and (5096-5326) from HTGS. The promoter fragment upstream to the transcription initiation site was named Rubisco-I fragment and the downstream region including the transcription start site was named Rubisco II fragment. Sequence analysis of both fragments revealed several cis acting motifs and transcription factor binding sites as identified through Plant CARE and plant PAN (Fig. 14). Both promoter fragments were cloned separately in TA vector and fused to synthesize hybrid promoter.

Selected primer pairs that showed best amplification of fragment I and II were selected (Table 1). The annealing temperatures for amplification of fragment I and II were 55°C and 57.1°C as given in Fig. 4A 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 Figs. 5 and 6 (A&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 ApaI and HindIII. Hybrid 1.5 kb promoter clone was confirmed through restriction with SacI and hindIII (Figs. 7 and 8). RuBisCO hybrid (Rub-H) cloned in TA vector was sub cloned in the plant expression vector pGR1 for expression study (Fig. 9). The resultant vector construct having hybrid RuBisCO promoter was named pGRrub-H (Fig. 13). Nucleotide sequence of RuBisCO hybrid is given in Fig. 12.

The major objective of this research work was to perform transient expression analysis of rubisco promoter to analyze promoter functionality. Transient analysis was carried out for evaluation of activity of *Rub* promoter by expressing reporter gene (*GUS*) in various wheat tissues including leaves and roots. Both positive and negative controls were also used for keeping a check on the accuracy of the transient expression system. Wheat leaves were bombarded with pGR1 under both 2X35S and *Rub* promoters (Figs. 10 and 11) respectively. *GUS* stained spots followed by the *GUS* staining reaction were observed using an inverted microscope fitted with Polaroid digital camera. The results revealed that Rubisco promoter expressed *GUS* in leaf tissues and the staining intensity was comparable to the control plasmid (pGR1).

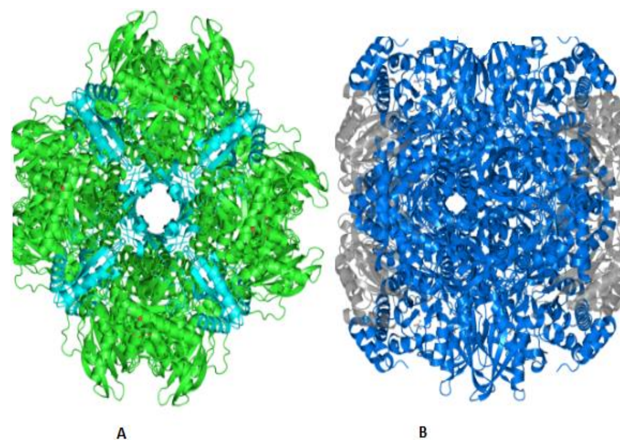

Fig. 1. (A): 16-meric assembly of Rubisco having 8 copies of Ribulose bisphosphate carboxylase large chain, 8 copies of oxygen molecule, 8 copies of Ribulose bisphosphate carboxylase small chain and 16 copies of phosphate ion. (B): 8 copies of Ribulose bisphosphate carboxylase large chain.

Discussion

Monocot crops like wheat, barley, rice, maize, sorghum, oats, rye and millet, represents the major food crops in Pakistan. Improvement of these cereal crops by molecular biological approaches is one of the major goals in plant biotechnology (Frame *et al.,* 2011). These crops need an improvement in productivity as well as increase in the nutritional values. Isolation and characterization of useful promoters that can serve as regulatory regions for expression of the gene of interest are routinely required for genetic manipulation of plants (Parry *et al.,* 2013; Iqbal *et al.,* 2017). RuBisCO promoter from zeamays shows constitutive expression in the monocots and dicots species. The HTGS data indicates that multiple copies of RuBisCO genes are present in zea mays, gene variants of RuBisCO in different monocots is also variable both at the amino acid and nucleotide level. The comparison of regulatory regions of RuBisCO variants indicated that not only their nucleotide sequences are variable but these variants also have different motifs for RuBisCo gene regulation (Masood et al., 2019). This study was focused on generating hybrid RuBisCO promoter by joining the important *cis*-regulatory motifs of the two promoter variants that affect the activity of hybrid promoter.

The steps involved are isolation of retrieval of the promoter regions of *RuBisCO gene* variants from HTGS database available at NCBI then isolation and cloning of promoter fragments from plant chromosomal DNA. RuBisco hybrid promoter was cloned in expression vector and further evaluated using transient *GUS* expression system. Our results show strong expression in endosperm that might be associated with the regulatory element's that are present upstream in promoter region. Hybrid promoter has can use to express forieg gene and help to overcome the IPR issues as well as provide free hand in the production, expression and commercialization of agricultural crops.

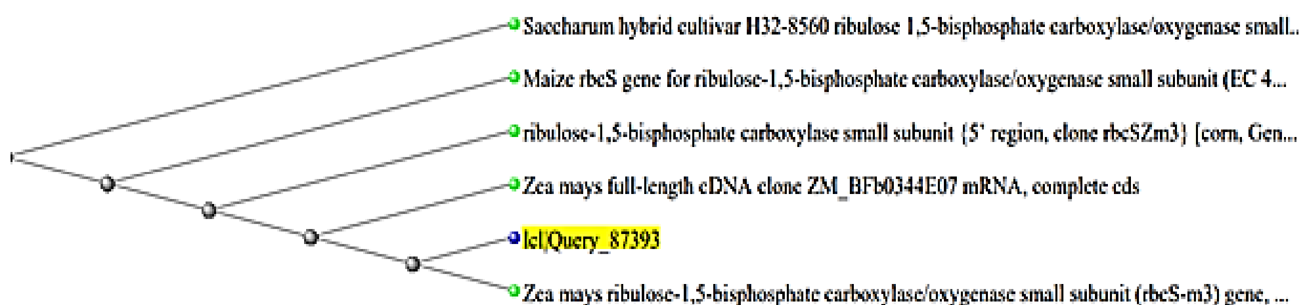

Fig. 2. Phylogenetic analysis of Rubisco gene fragment I.

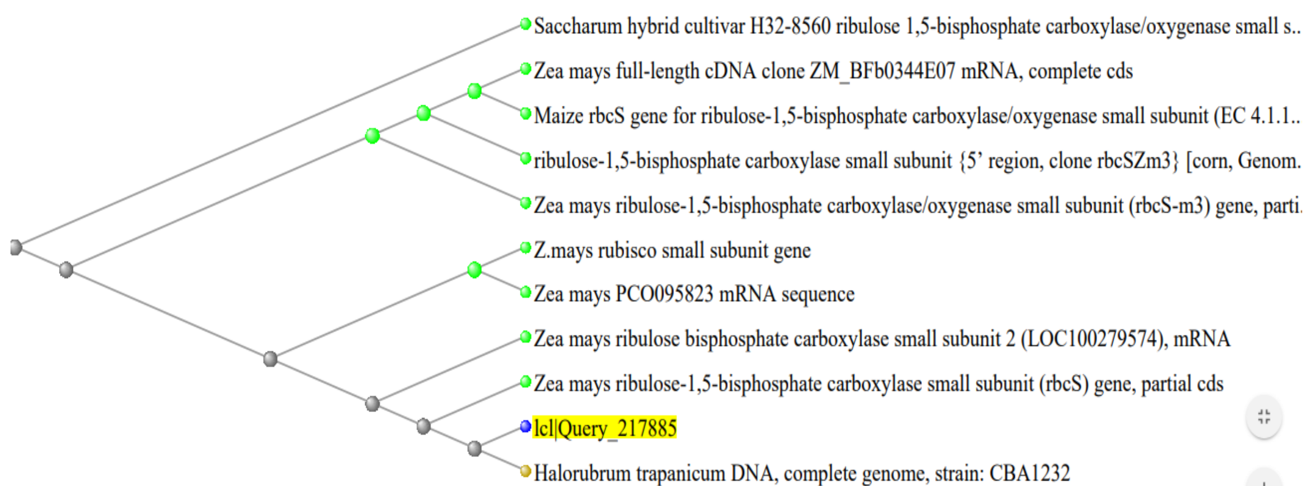

Fig. 3. Phylogenetic analysis of Rubisco gene fragment II.

Phylogenetic analysis of Rubisco promoter fragments are shown in Figs. 1, 2 and 3. RuBisCO hybrid promoter analysis by Plant CARE showed different regulatory motifs, for example G-Box, I-Box, GA-box, P-Box etc. The G-box binding factor (GBF) binds specifically, *in vitro*, to the sequence C/A-CACGTGGCA, which is highly conserved among the RbcS gene family (Shen *et al.,* 1995; Menken *et al.,* 1994). This sequence shows some synergism between certain G-boxes and other *cis*-acting elements. G box has been reported to be important for expression of the parsley chalcone synthase promoter (12). I-Box core element has been identified in the upstream region of several light regulated genes, including the rbcS of several dicot species such as Arabidopsis and tomato (Terzaghi *et al.,* 1995). The GT-1 consensus sequence, which exist in several light induced genes, is proposed to play an important role in steading the TATA box complex (Shahmaradov *et al.,* 2005). Our analysis also showed the presence of repetitive GATA sequence in promoter fragment II which contributes to constitutive expression. Furthermore, the RuBisCO hybrid promoter have fragments of CAAT box that is one of the cis sequence shown to be necessary for gene transcription, for example in pea legumin gene (Evans *et al.,* 1985; Smale *et al.,* 2001). Transient expression of the PGR1 hybrid RuBisCO construct in wheat leaves showed good expression in light resulted in stronger blue staining as compared to 35S promoter (Fig. 10). No expression was detected in root in roots of wheats as rubisco hybrid contain light responsive GT-1 elements indicating the functionality of the Rub-H promoter in directing gene expression in this tissue and also its regulation by light. The detection of the leaf specific Rub-H promoter activity in the immature embryos might be due to the fact that these tissues are totipotent at that stage (Zhang *et al.,* 2011; Craft anf Salvucci *et al.,* 2000). TFDB result of Second fragment (500 bp) matches with the ERF Family I. The AP2/ERF domain, which consists of about 60 to 70 amino acids is involved in DNA binding. It has been demonstrated that the AP2/ERF proteins have important functions in the transcriptional regulation of several biological processes related to growth and development, as well as various responses to environmental stimuli and stresses.

Therefore, further optimization of these conditions might improve the efficiency of the Rub-H promoter fragment in directing the expression of the GUS. Since the Rub-H promoter identified in this study represents only partial fragments, we cannot exclude the possibility that other regulatory elements required for a strong expression are missing. In short, this study showed that the Rub-H promoter is functional in regulating gene expression in different parts of wheat. However; further study is required to elucidate the role of each regulatory element of the promoter in directing gene expression. Characterization and functional analysis of *cis*-regulatory elements in a promoter sequence through bioinformatics approaches provides an innovative approach in future applications of Plant biotechnology.

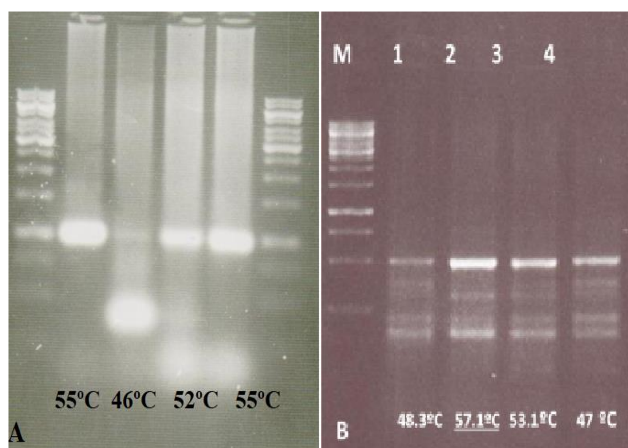

Fig. 4. (A) Gradient PCR of *Rub-I* and *Rub-II* promoter fragments: A); PCR amplifications at different annealing temperatures. M; 1Kb ladder, Lanes 1-3; Represent PCR of *Rub-I* fragment at 55ºC, 46ºC and 52ºC and 55ºC respectively. B); Gradient PCR of *Rub-II*. M; 1Kb ladder, Lanes 1-3; Represent PCR of *Rub-II* at 48.3ºC, 57.1ºC and 53.1ºC and 47ºC respectively.

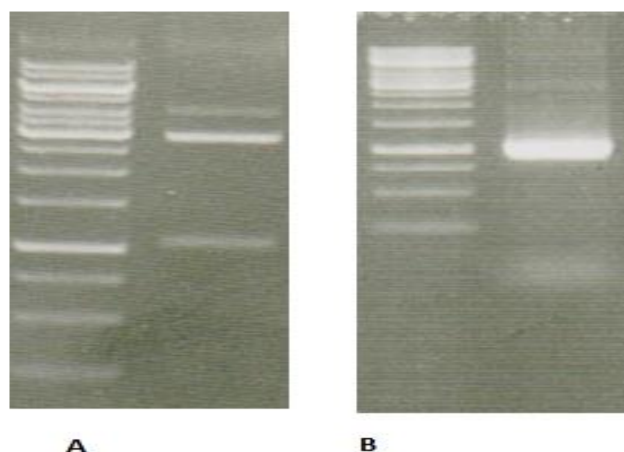

Fig. 5. Confirmation of *Rub I* promoter fragment in TA cloning vector by restriction digestion and PCR: A); Restriction digestion of Rubisco-I fragment with *Sac*I and *Apa*I. M; 1Kb ladder, Lane 1: Represent desired Rubisco-I clone of 1037bp B); PCR amplification of *Rub I*. M; 1Kb ladder, Lane1; Confirmatory PCR of *Rub I* fragment.

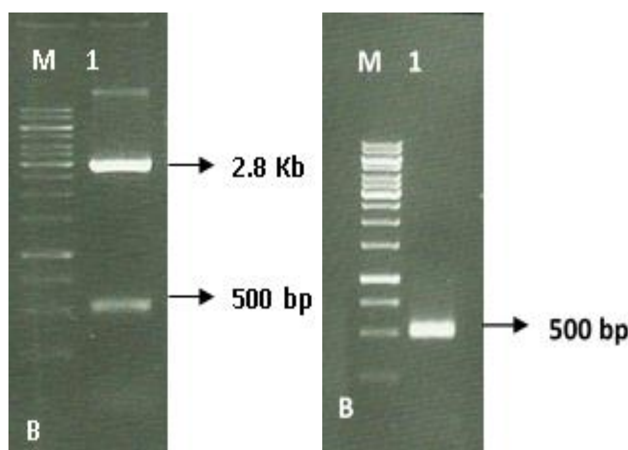

Fig. 6. Confirmation of *Rub-II* promoter fragment in TA cloning vector by restriction digestion and PCR: A); Restriction digestion of Rubisco-II fragment with *Sac*I and *Apa*I. M; 1Kb ladder, Lane 1: Represent desired Rubisco-II clone of 500bp B); PCR amplification of *Rub I*. M; 1Kb ladder, Lane1; Confirmatory PCR of *Rub II* fragment.

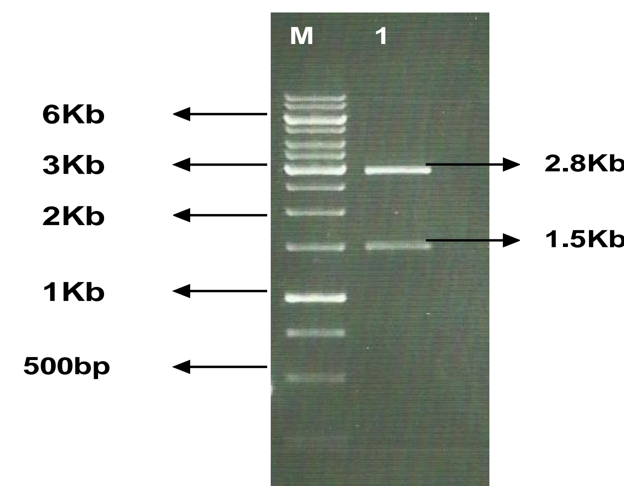

Fig. 7. Confirmation of Rubisco hybrid promoter in TA cloning vector by restriction digestion: Double digestion of *Rub* complete hybrid with *Sac*I and *Hind*III. M; 1Kb ladder, Lane 1; Clone of complete *Rubisco* promoter in TA.

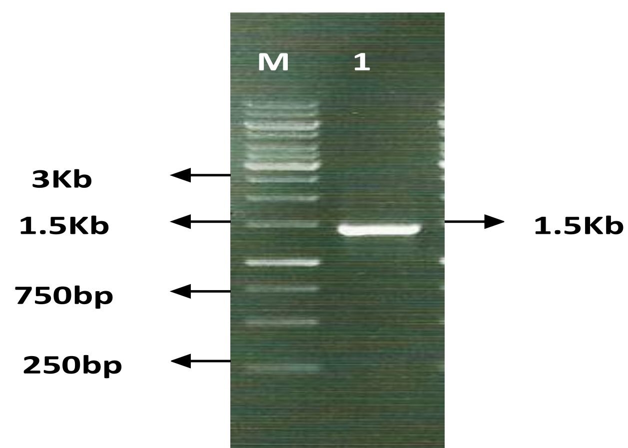

Fig. 8. Confirmation of Rubisco hybrid promoter in TA cloning vector by PCR: PCR amplification of *Rub-H*: M; 1Kb ladder, Lane1; Confirmatory PCR amplification of *Rubisco* hybrid (*Rub*-H) promoter.

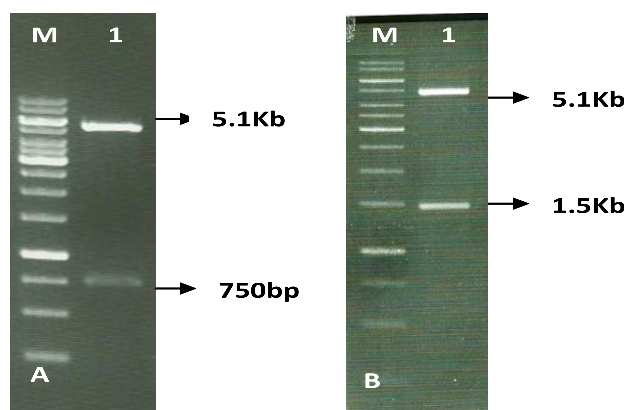

Fig. 9. Confirmation of *Rub*-H promoter in pGR1 by restriction digestion: A); Double digestion of pGR1 with *Sac*I and *Hind*III. M; IKb ladder, Lane1; Double digested pGR1 with 2X35S promoter. B); Confirmation of cloning through digestion of resultant vector with *SacI* and *Hind*III. M; IKb ladder, Lane1; Restriction of resultant vector with insert of 1.5Kb coming out.

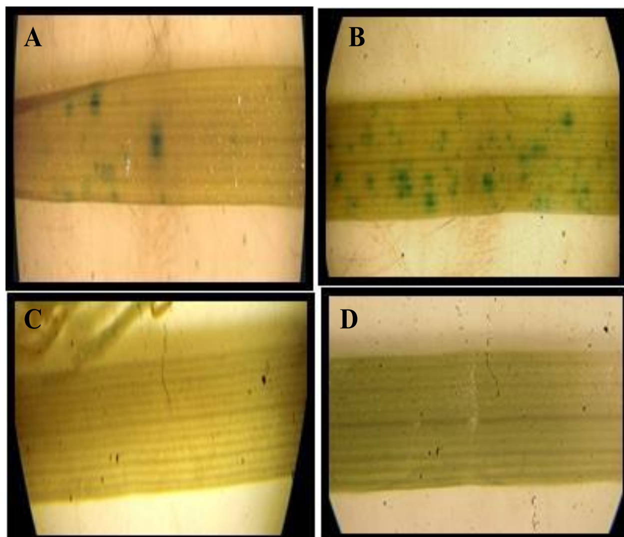

Fig. 10. Transient expression analysis of *Rub*-H promoter in wheat leaves. A): *GUS* activity under *Rub* hybrid promoter; showing the localized *GUS* activity as blue spots Positive control; B): *GUS* activity under 2X35S promoter C): Negative control; Tissues bombarded with gold particles without DNA coating, D): Negative control; Un-bombarded tissues but stained for *GUS*.

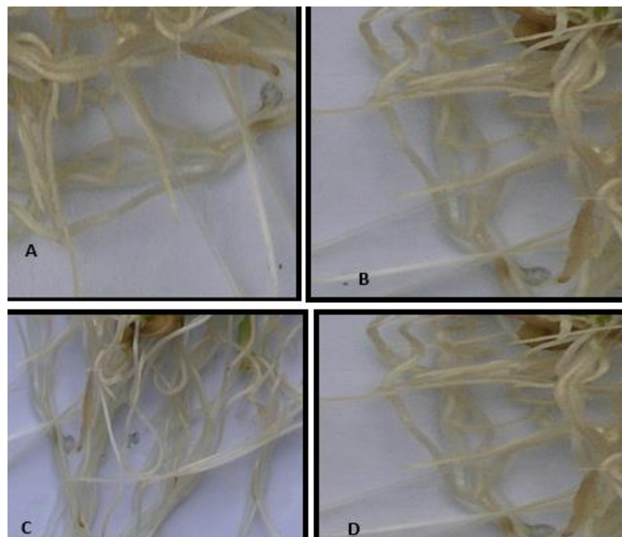

Fig. 11. Transient expression analysis of *Rub*-H promoter in wheat roots. A): *GUS* activity under *Rub* promoter, B): Positive control; *GUS* activity under 2X35S promoter, C): Negative control; Tissues bombarded with gold particles without DNA coating, D): Negative control; Un-bombarded tissues but *GUS* stained.

ACTGAGCTCGTTTATGTGCTTGTTTATGGAAACTGGATTCTCGGTTTCTTAAAACCAAGAAGCTAGCCTCCCCTAGCTAAAA
CTAATTTATAGAAATTGAGATGGTTACAAACACGATTAACCAGTTGTTTTTATAAACCAATTTCTGGAAATTAGAGATAGAAT
CTGGCTTCTTAAAAACTAGGTTGCTTAAGGACAGGACCTTATATCAGTAGAGAAATGGTAGAATGTGTAGTCTAGAAAAATT
TATGCATATTTCTATGGTTGTTGAATTATTTTCTATAATAGATTATATTGGTAAGACAAAAATATGTGGAATATTTAGTTAATTGA
GCACGTAGGTACATAAGTAAGACACAAATTTAGCGTATTTACATTGCTTAGATACGCGTAATCTAAAAGACATATTTTTTTGAT
TATGAAAACAAAAAGTTCCCCACCTTTTGTAATTTCTTGCATACGGTTTCGCAAAACGAAAAAAAATAACAAACATCGACCAT
AAACATTCTTTGAAAAAGAACATATATCCATGAATCGAGCTCCCTTTAATCTGGCGCTAGATCTGCATCCGCGGCTTGCAAA
GATAAATGGCACATTTAGTGTGTTATTTTGCAATACCTTTCATAGTAGATATCCTTAAATGCAGTTTTAGGCATGTTTGGGTAA
TTAAATAACATTTTTAGGAGGAGTTTTAGATTTACCTTTCTTTCGTGATGACTGATGACAGACGTGGGGAATTCAAATGCAA
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GGTTGGGGAAGGCGTAGGCGCTCGTGGACGACGCCCGATAAAAGACAAGAGGCGGAATTGCCATGAATTCGAGGTAGCTA
AGTAAGGCGCATATATATGCCAAAAAATTCTACTGTCACTTTCCAATTTCAATGCGCTGCCAAACAAGCCATCCTGGAAACT
GACTTGAATTCAGCCCAATTCTGTAGATCCAAACAGGGCCCGCGCCAGGGCCCTCAGCAGCAGAGGCTATGAGCAAACAG
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CTGAACTGCTTGTGTTCGAGAGAGACAGCGGATTGAGATTGCAGGATGCCAGGAAGGGGACGACCGACTCGACCGTATAG
TCCATCCGTGGCGTGGCTCCATGTGGCGCGGACGGACGATAAGGCGACAAGTGGTGGCGGACGCGCGCGTGCGATCAGGA
TAGGCCAGGCTGGCCGGGTGCGGCCACGGGAGAACGGTGGCCACTCGTCCCACATCCGCTTCGTCACGCCCCAACGAGAG
GCGGACGCGGATCCAGCGACATGGACATGGCTCTATATATGCCGTCGGTGGGGGAGCCCCTACAGGACGACCCAAGCAAG
CAAGCTCGATCTACTACTACTACTAGCTGGTACACATACTAGCCAGCCTGCCAGCAAGCTTGCC

Fig. 12. Full nucleotide sequence of Rubisco hybrid promoter.

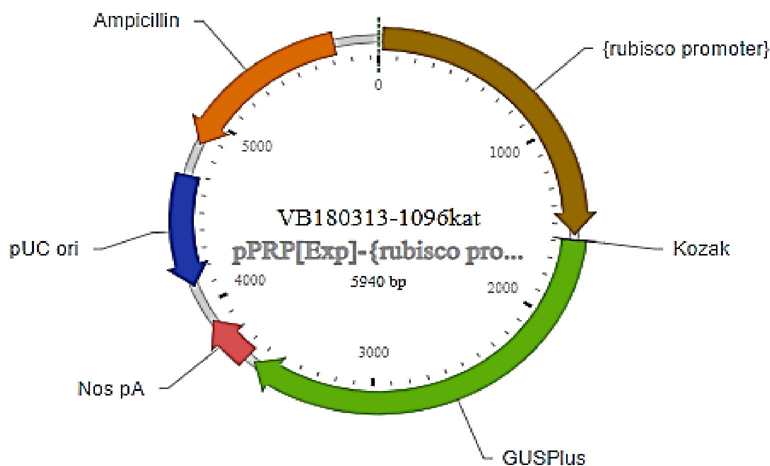

Fig. 13. Physical map of rubisco hybrid.

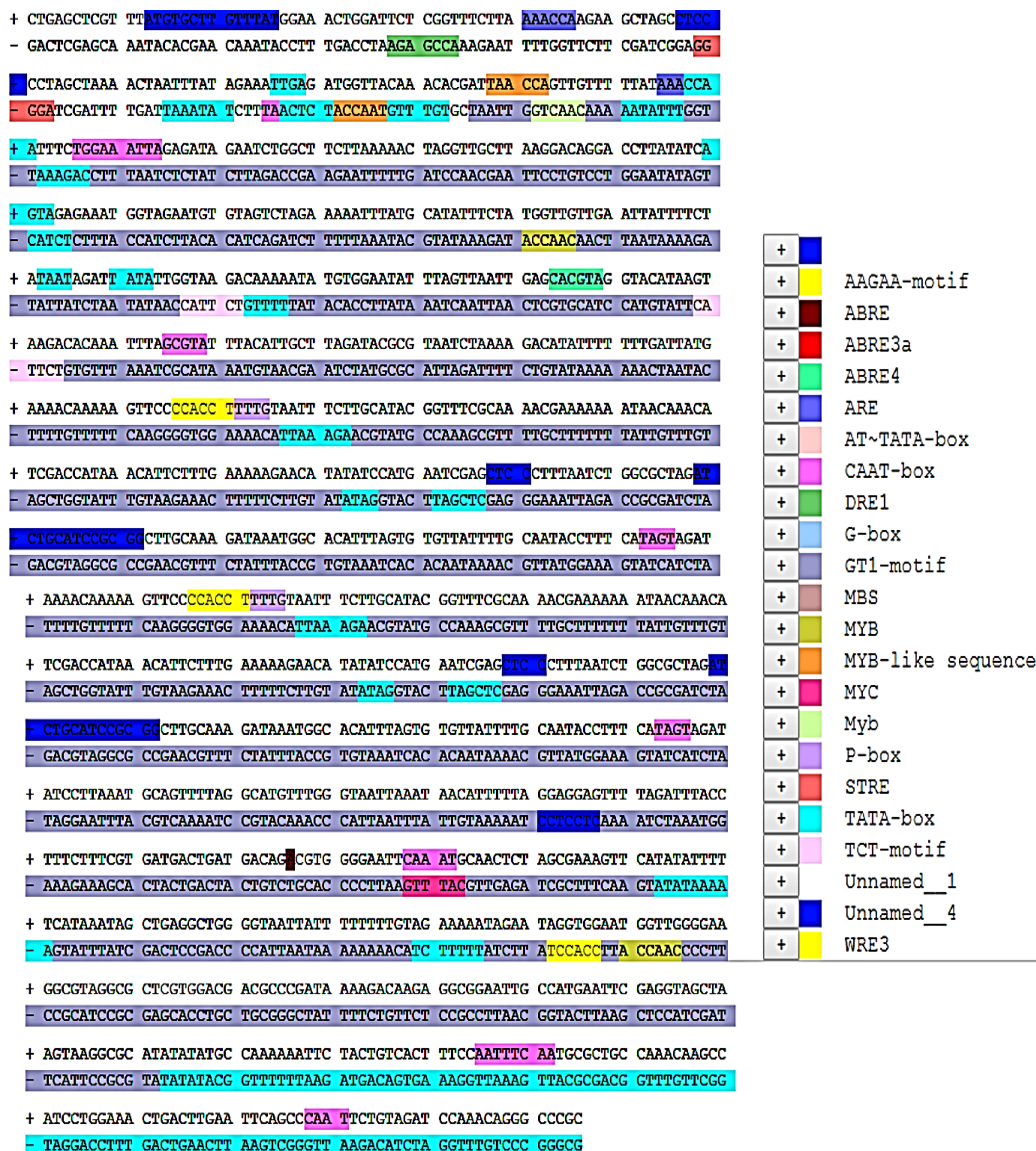

Fig. 14. Full nucleotide sequence of Rubisco hybrid promoter.

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