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Cloning and Functional Characterization of Promoter Elements of the D Hordein Gene from the Barley (*Hordeum vulgare* L.) by Bioinformatic Tools

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***Abstract*—**The low level of foreign genes expression in transgenic plants is a key factor that limits plant genetic engineering. Because of the critical regulatory activity of the promoters on gene transcription, they are studied extensively to improve the efficiency of the plant transgenic system. The strong constitutive promoters, such as CaMV 35S promoter and *Ubiqutin* 1 maize are usually used in plant biotechnology research. However the expression level of the foreign genes in all tissues is often undesirable. But using a strong seed-specific promoter to limit gene expression in the seed solves such problems. The purpose of this study is to isolate one of the seed specific promoters of *Hordeum vulgare.* So one of the common varieties of *Hordeum vulgare* in Iran was selected and their genomes extracted then the D-Hordein promoter amplified using the specific designed primers. Then the amplified fragment of the insert cloned in an appropriate vector and then transformed to *E. coli*. At last for the final admission of accuracy the cloned fragments sent for sequencing. Sequencing analysis showed that the cloned fragment DHP-contained motifs; like TATA box, CAAT-box, CCGTCC-box, AMYBOX1 and E-box etc., which constituted the seed-specific promoter activity. The results were compared with sequences existing in data banks. D-Hordein promoters of Alger has 99% similarity at 100 % coverage. The results also showed that D-Hordein promoter of barley and *HMW* promoter of wheat are too similar

***Keywords*—**Barley, Seed specific promoter, Hordein.

I. Introduction

IN cereal grains, nitrogen and sulfur are stored mainly in the form of a complex group of proteins, the prolamins, characterized by their alcohol-soluble properties [1]. It is now known that the combined proportions of these amino acids actually vary from about 30–70% of the total among different cereals and protein groups. immunocytochemical and pearling studies of barley show that they contain mainly S-rich and S-poor prolamins (principally B and C hordeins), with the HMW prolamin (D hordein) only occurring in significant amounts below the sub-aleurone [2], [3]. Hordeins, the major storage proteins of barley seeds, are prolamins specifically synthesized in the starchy endosperm and are classified according to their mobility in SDS-electrophoretic gels into four major classes: B, C, D and g, with the B hordeins account for 70–90% of the total hordein fraction; the C hordeins form 10–30% of the hordein fraction; the g hordeins amount to 1–2% of the hordein fraction and the D hordeins occupy about 2–4% of the hordein fraction [4]. The four groups are encoded by the genes: Hor2 (B-fraction), Hor1 (C-fraction), Hor3 (D-fraction) and Hor5 (g-fraction), located on barley chromosome 5 (1H) [5].

Synthesis of the prolamin polypeptides occurs only in the endosperm where it is under tissue-specific and temporal transcriptional control [1]. The coordinate expression of all hordein genes suggests common regulatory mechanisms of transcription that should involve both *cis*-acting motifs in their promoters and *trans*-acting transcription factors [6].

Promoters used in biotechnology are of different types according to the considered type of gene expression controlling. Tissue-specific or development-stage-specific promoters could regulate the expression of a gene in specific tissue(s) or at certain stages of development. Thus, seed specific promoters provide an excellent system for studying the expression control of plant genes and are used in developing transgenic for improving the quality of seed. In this study, we report the isolation and characterization of a Hordein specific promoter in *Hordeum vulgare*.

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II. Materials and Methods

A. Materials

Hordeum vulgare L. cv. Alger seeds were provided. *Escherichia coli* strain *DH5α* was used for cloning. The sequencing vector pGEMT-Easy and Taq DNA polymerase along with restriction enzymes were purchased promega and Takara corporations, respectively. DNA gel extraction kit and plasmid extraction kit were purchased from Bioneer Corporation. Sequencing was conducted by MILLEGENE France.

B. Methods

1. Extraction of Barley Total DNA

Seeds were cultivated under standard conditions in a green house. After growing, DNA was isolated from leaf tissue by using a CTAB extraction method as described by Saghai-Maroof [7]. Its quality and quantity were determined by 0.8% agarose gel electrophoresis and its concentration was adjusted on 50ng/µl.

2. PCR Amplification

The sequence of D-Hordein gene (Gene Bank accession number, 671536)) from *Hordeum vulgare*, which consists of promoter and coding regions of D-Hordein gene, were retrieved from Gene Bank to design primers to isolate the promoter region and signal peptide of D-Hordein gene (Fig. 1). The restriction enzyme sites *HindIII* and *NcoI* were added to 5´ end of primer PF and PR or further cloning;

PF: 5'-AT<u>AAGCTT</u>CTTCGAGTGCCCGCCGATTTG -3'
PR: 5'- AA<u>CCATGG</u>CAGCGGTGGTGAGAGCCAC-3'

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1    cttcgagtgc  ccgccgattt  gccagcaatg  gctaacagac  acatattctg  ccaaaacccc
     ---------Primer F------->
61   agaacaataa  tcacttctcg  tagatgaaga  gaacagacca  agatacaaac  gtccacgctt
121  cagcaaacag  taccccagaa  ctaggattaa  gccgattacg  cggctttagc  agaccgtcca
181  aaaaaactgt  tttgcaaagc  tccaattcct  ccttgcttat  ccaatttctt  ttgtgttggc
241  aaactgcact  tgtccaaccg  attttgttct  tcccgtgttt  cttcttaggc  taactaacac
301  agccgtgcac  atagccatgg  tccggaatct  tcacctcgtc  cttcttaggc  taactaacac
361  ctccacaatc  tcatcatcac  cgagaacacc  gagaaccaca  aaactagaga  tcaattcatt
421  gacagtccac  cgagatggct  aagcggctgg  tcctctttgt  ggcggtaatc
471  gtcgccctcg  tggctc  caccgctgaa  cgtg agatca
          <--------Primer R---------
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Fig. 1 The region of D -Hordein gene promoter and designed primers

Then the total DNA of barley Alger was amplified by PCR using RF and PR primers. The amplification profile was 94°C for 5min, followed by 35 cycles of 94°C for 50 sec, 63°C for 50sec, and 72°C for 1min, and a final extension step at 72°C for 7min. The amplified DNA products were separated by 1.0% agarose gel and were extracted by a DNA gel extraction kit. Then they were ligated to PGEMT-Easy vector at 4°C for 24 hours. The *E. coliDH5α* was transformed using ligation reaction and transformants were selected on white-blue test medium containing antibiotic ampicillin, IPTG and X-gal (Fig. 2). The plasmid DNA extractions were carried out from the positive colony PCR of white colonies using the extraction kit. The insertion of fragments in PGEMT-Easy was confirmed by reamplification with using specific primers and digestion with restriction enzymes, *HindIII* and *NcoI*. Desired fragments were sequenced by the MILLGENE company and followed by Insilco analysis any bioinformatics tools.

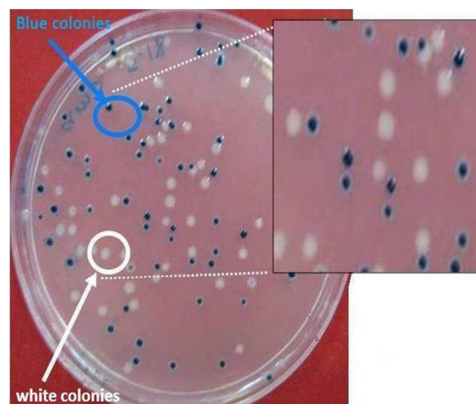

Fig. 2 Bacterial colonies transformed with pGEMT-Easy vector

III. Results and Discussion

A. Cloning of Seed-Specific Promoter Fragment Prod-Hor.A

The promoter fragment ProD-Hor.A was obtained by PCR amplification via primers PF and PR. A 506-nt-long sequence of the barley D-Hordein promoter was PCR amplified and the result is shown in Fig. 3. This fragment was ligated to PGEMT-Easy to obtain PT- ProD-Hor.A. The cloned fragments in PGEMT-Easy were confirmed by PCR and digested with restriction enzymes, HindIII, NcoI shown in Fig. 4. Individual digestion with NcoI and HindIII enzymes indicated the fragment 3015bp which included the 506bp D-hordein promoter. Double digestion with NcoI and HindIII enzymes indicated the 506bp D-hordein promoter fragment in the vector.

B. Sequencing Analysis of the Seed-Specific Promoter Fragment Prod-Hor.A.

To find regulatory elements in promoter sequences, the PLANTCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html) software was applied. Analysis of the sequences of the seed-specific promoter fragment ProD-Hor.A revealed several kinds of seed specific promoter motifs in 506 base pairs of these cloned promoter fragments as shown in Fig. 5. For exploring DNA-binding motif similarities, the stamp (http://www.benoslab.pitt.edu/stamp/index.php) software was applied. Sequence logos of the significant motifs shown in Fig. 6 and description of each one shown in Table I.

A-box motif is present in *Petroselinum crispum* and inclusive Place ID: PALBOXAPC in PLACE database software and are involved in the light responsiveness [8]. This motif in Arabidopsis thaliana known CCGTCC-box and cis-acting regulatory element related to meristem specific activation. Another well conserved element, the CAAT-box motif with ID: CAATBOX1common cis-acting element in promoter and enhancer regions, is often present at -80 to-150 bp upstream of TIS[1] , and may operate cooperatively with other putative conserved motifs [9]. However, no unifying expression pattern for plant genes containing putative CCAAT elements

[1] Transcription initiation site

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has been observed [10], [11]. Moreover, multiple copies of the genes coding for the subunits of the CCAAT-binding protein exist in *Arabidopsis*, *rapa Arabidopsis thaliana Glycine max Petunia hybrid* and *Hordeum vulgare* suggesting the potential for multiple alternative forms of these complexes in plants [12], [13].

Prolamin-box motif with place ID: PROLAMINBOXOSGLUB1 is very similar to different types of prolamin box in rice and wheat glutenin promoter [14]. The prolamin box (P-box) is a highly conserved 7-bp sequence element (5'-TGTAAAG-3') found in the promoters of many cereal seed storage protein genes.

Regulatory elements can often be positioned quite far from the transcription initiation site (TIS) in mammalian genomes, whereas in yeast and plants they are located within a few thousands base pairs of the TIS [15].The first element described as regulating this process was a classical TATA box, TATA(A/T)A, located —25 to —30 base positions upstream of TIS [16]. However, subsequent studies suggested that AT-rich sequences completely unrelated to the TATA-box stimulate transcription with equal or increased efficiency [17]. Furthermore, although the first step of transcription initiation is highly specific, TFIID also binds with high affinity to several TATA elements that do not match the consensus sequence and is active in promoting transcription in vitro from these elements [18]. Another weakly conserved "initiator" element was described in the direct vicinity of the TIS [19].GC-boxes have also been localized in the upstream promoter regions of many plant genes tend to be surrounded by GC rich sequences. GC box, a sequence rich in guanidine (G) and cytidine (C) nucleotides, is usually found in multiple copies in the promoter region and normally surrounds the TATA box and CAP site [20].

TCCACCT-motif and CTCC sequence is similar this motif in *Petroselinum hortense*. CGTGG sequences are similar this motif in *Zea mays* and Circadian motif with place ID: CIACADIANLELHC too similar this motif in the *Lycopersicon esculentum*.

Amylase box (AMYBOX1) Conserved sequence found in 5'-upstream region of alpha-amylase gene of rice, wheat, barley [21]. CANBNNAPA is a Core of (CA)n element in storage protein genes in *Brassica napus* and involved in expression of napA gene [22].

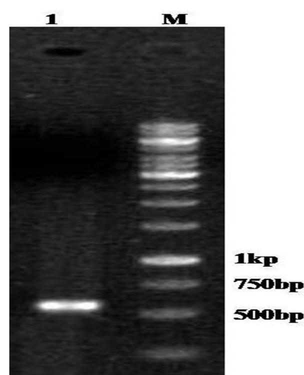
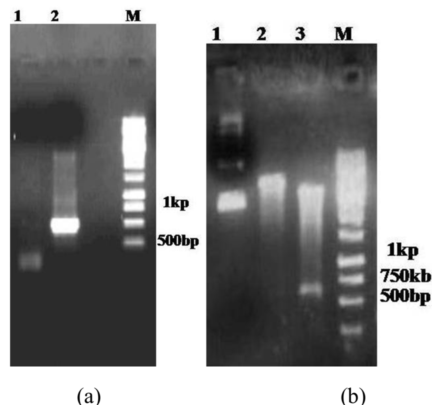


Fig. 3 PCR product 1: Alger genotype, M: DNA Marker



(a) (b)

Fig. 4 Construction and identification of ProD-Hor.A fragment
(a) 1: PCR amplification of pT- ProD-Hor.A nonrecombinant, 2: PCR amplification of pT- ProD-Hor.A recombinant
(b) M: The DNA Marker; 1: Uncut of pT- ProD-Hor.A; 2: digestion product pT- ProD-Hor.A With *NcoI*; 3: Double digestion product pT- ProD-Hor.A With *Hin*dIII/*Nco*I

C. Structural Features of the Promoter Regions of D-Hordein in Hordeum vulgare and Hordeum chilense and High Molecular Weight Glutenin Subunit 1dx2 Gene in Triticum aestivum

Although comparative analysis of promoter could not directly decide difference in function, it would useful in identification of regulatory elements variations which are relevant to gene function and evolution. All characterized promoter regions of D-Hordein were aligned to the homologous regions of D-Hordein from *Hordeum chilense* and high molecular weight glutenin subunit 1Dx2 gene in *Triticum aestivum*. The 5' flanking promoter regions of both were compared**.** A few base substitutions and insertions or deletions were found even though the alignment showed high similarity (Fig. 7). TATA box were well conserved in all compared alleles**.**

TABLE I
REGULATORY ELEMENTS IN PROMOTER SEQUENCE

Motif name	position	sequence	strand	Stamp alignment	E value	Function
A-box	173	CCGTCC	+	CCTGCC CCTGCC	3.8873e-11	light responsiveness
CAAT-box	25,64, 356,365 417	CAAT CAAT	+ -	CMAWT CCATT	4.3473e-05	common cis-acting element in promoter and enhancer regions
CCGTCC-box	173	CCGTCC	+	CCTGCC CCTGCC	3.8873e-11	cis-acting regulatory element related to meristem specific activation
Prolamin-box	187	tgtttTGCAAAGctcca	+	TGNAAAK TGAAAAT	5.3359e-08	cis-acting regulatory element involved in activation of zein gene expression during endosperm development; binding site of prolamin-box binding factor
TATA-box	340 342	ccTATAAAaa TATAAA	+ +	-CTATAAATAC CCTATAAAAA-	3.7856e-09	core promoter element around -30 of transcription start
TCCACCT-motif	329	TCCACCT	+	-CCACCTGG TCCACCT--	1.4526e-07	–
PH-Unnamed	199,208,25 1,328,360	CTCC	+	CTCCCTC CTCC---	1.0042e-03	–
Zm- Unnamed	112 478	CGTGG CGTGG	- +	ACGTGGC -CGTGG-	7.0204e-07	–
circadian	365	CAANNNNATC	+	CAANNNNATC CAANNNNATC	2.0663e-04	involved in the circadian mRNA accumulation
AMYBOX1	33	TAACARA	+	TAACARATAACARA	1.0500e-12	Conserved sequence found in 5'-upstream region of alpha-amylase gene of rice, wheat, barley
CANBNNAPA	294	CNAACAC	+	CNAACACCNAACAC	2.4440e-08	Core of "(CA)n element" in storage protein genes in Brasicanapus

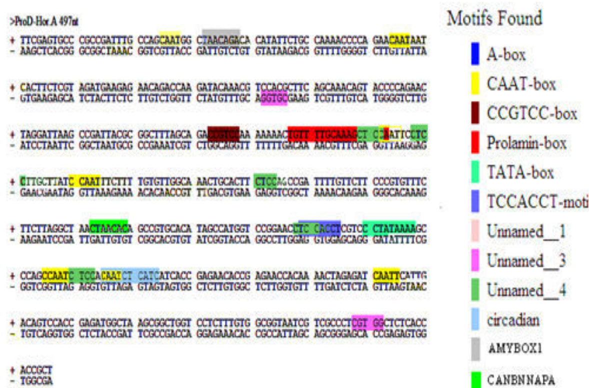


Fig. 5 Sequence analysis of D-Hordein specific promoter fragment ProD-Hor.A

D. Evolutionary Analyses of the Promoter Regions of D-Hordein

The phylogenetic analysis was conducted to investigate the evolutionary relationships among the alleles encoded by D-Hordein in *Hordeum vulgare* and *Hordeum chilense* and high-molecular-weight glutenin in *Triticum aestivum* (Fig. 8). The promoter sequences plus the sequences encoding the signal peptides were chosen to construct the phylogenetic tree under several principles for the sequence selections. Firstly, we found that the regulatory elements that control the tissue specificity and expression level of different D-Hordein and HMW genes are well conserved in D-Hordein and HMW alleles from different species. Secondly, the sequences encoding signal peptides high conservation with enough variations suggested these D-Hordein and HMW sequences are phylogenetically informative. The resulted phylogenetic tree was divided into 2 clusters, comprising the *HMW* alleles at the top and the alleles of *D*-Hordein at the bottom. In the cluster of *HMW* alleles, *HMW* genes from each cultivar were clustered together, respectively. In spite all *HMW* alleles from different wheats show a close relationship. D-Hordein in *Hordeum vulgare* shows 94% identity with D-Hordein in *Hordeum chilense* and 87% with HMW in *Triticum aestivum*.

Fig. 6 Sequence logos of motifs enriched in seed storageprotein gene promoter sequences
Sequence logos of significant DNA motifs discovered in SSP gene promoter sequence. Left, Forward motif, right, reverse complement of motif

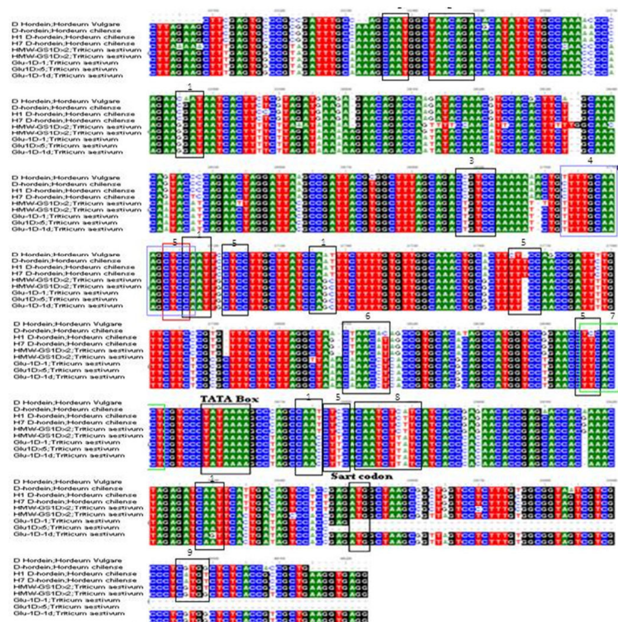


Fig. 7 Comparison of the of the promoter regions of D-Hordein in *Hordeum vulgare* and *Hordeum chilense* and high molecular weight glutenin subunit 1Dx2 gene in *Triticum aestivum*
1: CAAT-Box 2: AMYBOX1 3: A-box and CCGTCC-box4: Prolamin-box5: PH-Unnamed 6: CANBNNAPA 7: TCCACCT-motif 8: circadian9: Zm- Unnamed

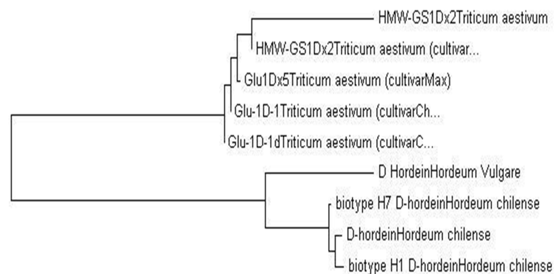
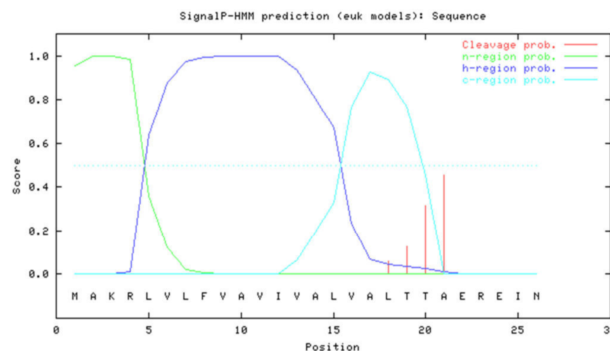


Fig.8 Phylogenetic relationships of *D-Hordein* from *Hordeum vulgare, Hordeu mchilense* and high molecular weight glutenin in *Triticum aestivum*

E. nal Peptide Detection for the Barley D-Hordein Protein

Nucleotides 435 to 506 constitute a signal peptide that begins with ATG codon, and encodes a signal peptide [23]. The presence of a signal peptide would be consistent with the evidence that the D-Hordeins are synthesized on the rough endoplasmic reticulum and deposited in protein bodies [24], [25]

Based on this seed-specific motif by the bioinformatics analysis, the isolated regions were selected as candidates to promote gene expression in seed [26]. The 5′noncoding region is highly similar to those of the genes encoding D-Hordein polypeptides Results obtained from the neural network algorithm showed three scores. The high S-score indicates a signal peptide at the beginning, i.e., 20 amino acids at the first sequence; the C-score indicates the mature protein possibility starts at (and includes) position 24, and the Y-score indicates the true cleavage site between amino acids 19 and 21 with 97% probability. The results also showed that signal peptide is comprised of three distinct regions: a polar N-terminal end (n-region) that may have a net positive charge, a central hydrophobic core (h-region) that consists of 6±15 hydrophobic amino acids, and a polar C-terminal (c-region) end that contains prolines and glycines (Fig. 9).Blast homology for the Barley D-Hordein signal peptide showed Total score of 61%, Query coverage of 87% and high identity of 91% with the *Triticumaestivum*1Dx high molecular weight glutenin signal peptide.



>Sequence
Prediction: Signal peptide
Signal peptide probability: 0.955
Signal anchor probability: 0.045
Max cleavage site probability: 0.453 between pos. 20 and 21

(a)

(b)

Fig. 9 (a) Signal peptide prediction for the Barley D-Hordein protein using neural networks (NN) by SignalP 3.0 server (b) The n-, h- and c-regions of the D-Hordein signal peptide are shown
The site of signal peptide cleavage is indicated by an arrow

IV. Conclusion

The aim of this research was to isolate one of the seed specific promoters of *Hordeum vulgare.* One barley varieties that were common in Iran were selected and their genomes were extracted using the specific designed primers for amplifying the D-Hordein promoter. The amplified fragment of the insert was cloned in an appropriate vector and then was transformed to *E. coli*. At last, for the final admission of accuracy, the cloned fragments were sent for sequencing. The results were compared with the sequences existing in data banks. The promoter region contained motifs; like TATA box, $(CA)_n$ box, A box, CAAT-box, motif AMYBOX1 etc., which constituted the seed-specific promoter activity.

The results obtained from the neural network algorithm showed three scores in signal peptide: the high S-score, C-score and the Y-score.

ABBREVIATIONS

ProD-Hor.A: Promoter D-Hordein Cultivar Alger
PT- ProD-Hor.A: pGEMT-Easy vector- promoter D-Hordein Cultivar Alger
DHP: D Hordein Promoter

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