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Engineering for Expression of the Cold Regulated Barley Protein HVCR21 in E. coli

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ABSTRACT

HVCR21 is a barley protein known to be cold regulated at the mRNA level. However, its function as well as the affect of low temperature on its translation are unknown. The purpose of this project was to engineer *E. coli* to express recombinant HVCR21. PCR primers were designed for the 5' and 3' ends of the HVCR21 coding region. The primers were also designed with a 5' SacI restriction site and a 3' Pst I restriction site. After ligation of the PCR product into the pCR4-TOPO vector, bacteria were transformed and plated and the successful transformant verified by PCR. The new pCR4-TOPO-HVCR21 construct was therefore available as a ready source of the SacI/Pst I fragment. The expression vector pQE100 was prepared by digestion with SacI and PstI. The SacI/Pst I fragment from pCR4-TOPO-HVCR21 was ligated to the SacI/PstI pQE100 vector. Transformants were analyzed by PCR with the HVCR21 primers. Sequence analysis of the engineered region of pQE100-HVCR21 confirms the correct orientation and frame for expression of HVCR21 in *E. coli.* Future studies will involve growth of bacteria containing this construct and SDS-polyacrylamide gel electrophoresis analysis of the expressed proteins.

INTRODUCTION

Low nonfreezing temperatures are required to acclimate plants to varying degrees of freezing tolerance (Levitt, 1980). Since the ability to cold acclimate is under genetic control, investigations have focused on the identification of cold-regulated genes (Thomashow, 1999). Several investigators have concentrated their efforts on characterizing barley and wheat cold-regulated genes. Ndong *et al* (2002) have described cold-regulated chloroplast proteins. Tsuda *et al* (2000), Vaguijfalvi *et al* (2000) and Gana *et al* (1997) are only a few of the investigators studying wheat cold-regulated genes. Brown (2001), Shen (2001) and Zhu (2000) have reported on stress-regulated barley multi-gene families.

We isolated a number of full-length barley cDNA clones for cold-regulated genes (Chang, 1993). Southern blot hybridization analyses revealed that two of these genes designated *hvcr8* and *hvcr21* belong to a small gene family (Gana *et al.*, 1997). The transcript size for *hvcr21* is 690 nt. Northern blot hybridization analyses revealed that

hvcr21 is regulated by low temperature (Chang *et al* 1993). The function of the class of proteins encoded by this multi-gene family has yet to be elucidated.

This study was undertaken to generate the necessary tools to examine the location and possible function of the encoded protein HVCR21 *in planta.*

We have placed the cDNA insert corresponding to the complete coding region under the control of the lac promoter. Successful expression of the protein in *E.coli* will allow us to test an antibody generated against the imunogenic region of the deduced amino acid sequence. The antibody will then be available for *in planta* studies.

MATERIALS & METHODS

Primer design

The sequence of the coding region for pHVCR21 (Accession No, L28092, Chang *et al*, 1996) was analyzed and primers with Pst I and Sac I restriction sites designed and synthesized (Figure 1.). Two different restriction sites were chosen, since the aim was to directionally subclone the fragment. The 5' primer was designated primer 1. The 3' primer was designated primer 2.

Isolation of HVCR21 ORF from pHVCR21

PCR amplification of HVCR21 ORF was performed with primers 1 and 2. Template pHVCR21(50 ng), 500 uM dNTP, primer 1 and primer2, 5U Taq polymerase in a final volume of 50ul. Amplification was performed in thin walled reaction tubes in an Idaho Technology Rapid Cycler using the following program; Link (H2-C5-H4)= 94 C 50 sec, 95 C 30sec, 40 C 1 min, 72 C 1min, 29 cycles , 72 C 10 min.

Subcloning into pCR4-TOPO

The resulting amplicon was added directly to the ligation reaction containing the pretailed pCR4-TOPO vector (Invitrogen life Technologies) and incubation performed at room temperature for 5 min[utes] then transferred to ice. One Shot TOPO10 cells (*E. coli* strain Topo 10 F' *{lacIq....}*) were transformed with 2 ul of the PCR reactions. The transformation mixture (50 ul) was spread on LB 50ug/ml Kanamycin and incubated overnight at 37C.

Verification that transformants contained first construct, pTOPO-HVCR21

A PCR master mix consisting of PCR buffer, dNTPs, primers 1and 2 and Taq polymerase at concentrations listed above was prepared. Ten colonies were picked and individually spotted on an LB 50ug/ml kanamycin plate before resuspending in 20 ul of the master mix. The reaction was placed in the Idaho Technology Rapid Cycler using the previously described program with an additional first 10 min[ute] 94 C incubation. The inoculated plate was incubated overnight at 37 C and served as a source for the positive clones.

Preparation of pQE100 for insertion of Sac I/Pst I pTOPO-HVCR21 insert

pQE100 (Qiagen Inc.) contains a double tag. 5' to the MCS is a 6xHis tag and 3' to the MCS is the Tag 100. pQE100 was restricted with Sac I and Pst I. The Sac I/Pst I vector fragment was purified from the MCS Sac I/Pst I region by column chromatography.

Preparation of pTOPO-HVCR21 insert

A positive colony from the pCR4-TOPO/ HVCR amplicon ligation transformation was streaked for purity on LB 50 ug/ml Kanamycin. A single colony was used to start a 5 ml culture and a miniplamid isolation procedure performed. The resulting plasmid designated pTOPO-HVCR21 was double digested with Sac I and Pst I. The resulting products were resolved on a 1.5% agarose Tris acetate gel. The 261 bp Sac I/Pst I fragment representing the pHVCR21 coding region (Figure 2) was eluted from the gel slice.

Generation of pQE100-HVCR21

The purified Sac I/ Pst I pQE100 vector was ligated to the gel eluted Sac I/ Pst I pHVCR21 fragment in the presence of T4 DNA ligase, DTT and ATP as per instructions, Novagen Inc.

E. coli strain Topo 10 F' *{lacIq....}* was transformed with the ligation reaction and 50 ul of the transformation mixture was spread on LB plates containing 100 ug/ml Ampicillin.

Verification that transformants contained new construct pQE100-HVCR21

Since the HVCR21 insert of pQE100-HVCR21 was flanked by Primer 1 and 2, PCR amplification was performed with Primers 1 and 2 with single transformants from the previous ligation. The PCR products were resolved on a 2% agarose, Tris Acetate gel (Figure 3).

A positive transformant was streaked for purity. Plasmid DNA was isolated and the engineered region sequenced (Figure 4).

RESULTS

Isolation of HVCR21 ORF from pHVCR21

The designed HVCR21 primers 1 and 2 are displayed in Figure 1 relative to the HVCR coding region. Since the pQE100 vector into which it would be ligated contained the start codon ATG 5' to the MCS, the endogenous HVCR start codon was not included in Primer1.

Primer 1
GAGCTCGCAAAGTGTCTCG

Nucleotide sequence:
GAGCTCGCAAAGTGTCTCGCCGCCGCGCTCCTGCTACTG
GTGGTGCTCGCGTACTGCGACGGTAGGGAGCTGAACCAG
AAGGACCAGGCACTAGCAACGGCACGCGGTGCCGGTGC
CGGTGCCGGTGGTGGCGTCGGCGAGGAGAAGATCTTGGG
GTTGCCAGAGTTGCCGCTTGTGGGAACCGTTACCGGTAC
CAGCACCGTTACCGGTCCGGTGGTGGTGCTTCCTGCCCTT
CCTGGGATCCCTGCTCACCCACTGCAG

Primer 2
CTGCAGTGGGTGAGCAGG

Primer 1 was designed with Pst I site
Primer 2 was designed with Sac I site
This facilitates insertion into pQE100 vector

Figure 1. HVCR21 nucleotide sequence

The result of an amplicon of approximately 261 bp in the reaction containing pHVCR21 plus primers indicated that the PCR was successful. No product was observed in the control reaction minus template (data not shown).

Preparation of pTOPO-HVCR21 insert

The amplicon ligated into pCR4-TOPO (Invitrogen, inc.) produced the plasmid designated pTOPO-HVCR21. Digestion of pTOPO-HVCR21 with SacI/ PstI (lane2, Figure2) produced two fragments. The 261 bp fragment contained the HVCR21 sequence and a 4 kb fragment corresponding to the pCR4-TOPO vector.

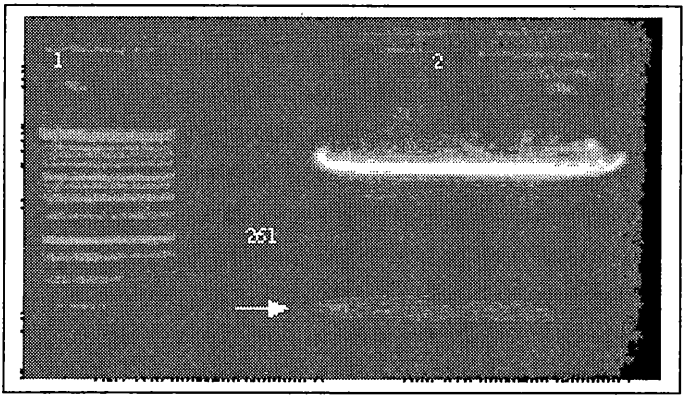

Figure 2. Pst I/Sac I double digest of pTOPO-HVCR21
Lane1. 1 kb DNA ladder. Lane2. PstI/SacI pTOPO-HVCR21

Generation of pQE100-HVCR21

The SacI/PstI digest of pQE100 (Quiagen inc.) resulted in a single fragment visible by agarose gel electrophoresis. The insert was not detectable due to its short length within the multiple cloning site (data not shown).

The product of the ligation of the SacI/PstI pQE100 and SacI/PstI 261 bp HVCR21 fragment produced the protein expression plasmid designated pQE100-HVCR21.

Result of analysis of the transformants with the ligation products is depicted in Figure 3.

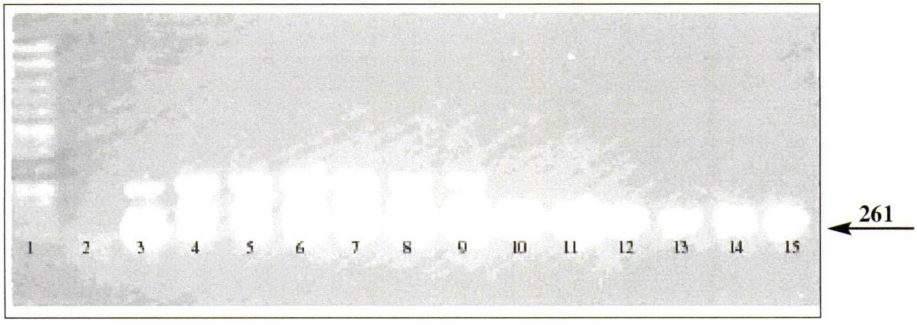

Figure 3. PCR analysis of putative pQE100-HVCR21transformants. Lane 1. 1 kb DNA ladder. Lane 2. PCR control minus template. Lane 3-15 PCR plus single transformants as template.

Transformants corresponding to lanes 10-15 (Fig 3) were considered positive for proper insertion of SacI/PstI HVCR21 insert into SacI/PstI pQE100 vector. The resulting plasmid was purified for sequencing.

The result of sequencing across the engineered region of the plasmid produced from a pQE100-HVCR21 transformant is depicted in Figure 4.

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atgagaggatcgcatcaccatcaccatcacggatccgcatgcgag
  M R G S H H H H H H G S A C E
ctcgcaaagtgtctcgccgccgcgctcctgctactggtggtgctc
 L A K C L A A A L L L L V V L
 gcgtactgcgacggtagggagctgaaccagaaggaccaggcacta
 A Y C D G R E L N Q K D Q A L
gcaacggcacgcggtgccggtgccggtgccggtggtggcgtcggc
 A T A R G A G A G A G G G V G
gaggagaagatcttggggttgccagagttgccgcttgtgggaacc
 E E K I L G L P E L P L V G T
gttaccgtaccagcaccgtaccgtnccgtgtggctctnc
 V T V P A P Y R X V W L X
```

Figure 4. Sequence of N-terminus coding region of pQE100-HVCR21. Note the 6x His tag from pQE100 (red) and SacI restriction site (blue). The immunogenic region for HVCR21 is highlighted in green.

CONCLUSION

Based on the sequence analysis of pQE100-HVCR21, we have successfully placed the cDNA for the barley cold-regulated gene *hvcr21* under the control of the *lac* promoter. It is now possible to induce expression of HVCR21 in *E. coli*. We will then analyze the protein products and verify that the anti-HVCR21 peptide antibody generated in an earlier study recognizes the recombinant protein.

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