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Construction of Genome Walker Libraries and Cloning of Promoter For ARID/BRIGHT Gene in Chickpea

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ABSTRACT

The ARID (AT-rich interaction domain) is a billion year old DNA-binding domain that has been identified in all sequenced higher eukaryotic genomes The ARID consensus sequence spans about 100 amino acid residues, and structural studies identified the major groove contact site as a modified helix-turn-helix motif. In green plants 187 ARID genes are identified out of which 13 are present in Arabidopsis and 21 in Rice, one each in Lotus and Medicago etc. ARID containing genes are also present in grape, castor, soybean, maize, sorghum, populous and barley. In Chickpea, ARID gene was isolated cloned and found to be single copy gene and ARID protein is nuclear localized. Promoter cloning and characterization will greatly help in understanding gene regulation and its involvement in plant immunity against Fusarium wilt of Chickpea. So in the present study promoter region corresponding to ARID gene was cloned from Wilt resistant genotype i.e, and was found to be 2044 bp long.

Key words*: ARID/BRIGHT, Chickpea, Fusarium, Promoter.*

The promoter contains specific DNA sequences that are recognized by proteins known as transcription factors. These factors bind to the promoter sequences, recruiting RNA polymerase, the enzyme that synthesizes the RNA from the coding region of the gene. Promoters represent critical elements that can work in concert with other regulatory regions (enhancers, silencers, boundary elements/insulators) to direct the level of transcription of a given gene. Eukaryotic promoters are extremely diverse and are difficult to characterize. They typically lie upstream of the gene and can have regulatory elements several kilobases away from the transcriptional start site. Because in eukaryotes, the transcriptional complex can cause the DNA to bend back on itself, which allows for placement of regulatory sequences far from the actual site of transcription. Understanding how the regulation of gene networks is orchestrated is an important challenge for characterizing complex biological processes. Gene transcription is regulated in part by nuclear factors that recognize short DNA sequence motifs, called transcription factor binding sites, in most cases located upstream of the gene coding sequence in promoter and enhancer regions. Genes expressed in the same tissue under similar conditions often share a common organization of at least some of these regulatory binding elements. In this way the organization of promoter motifs represents a "footprint" of the transcriptional regulatory mechanisms at work in a specific biological context and thus provides information about signal and tissue specific control of expression. Therefore analysis of promoters provides a crucial link between the static nucleotide sequence of the genome and the dynamic aspects of gene regulation and expression.

MATERIAL AND METHODS

1. Isolation of Genomic DNA

Genomic DNA was isolated from two week old chickpea seedlings using DNeasy plant maxi kit (Qiagen)

1. 1g of frozen tissue sample was crushed using mortar and pestle.
2. 5ml of preheated buffer AP1 and 10ìl RNase A were added to the crushed tissue sample and incubated for 10 min and during incubation the contents were mixed by inverting the tubes.
3. 1.8ml of AP2 buffer was added and incubated for 10 min on ice.
4. The contents were centrifuged at 5000g for 5 minutes
5. The supernatant was decant into a QIAshredder Maxi spin column in a 50ml tube and centrifuged at 5000g for 5 minutes

6. The flowthrough was transferred into a new 50ml tube and 1.5 volumes of AP3/E buffer added followed by votexing.
7. The sample was then transferred into DNeasy Maxi spin column in a 50ml collection tube and centrifuged at 5000g for 5 minutes.
8. 12ml of AW buffer was added and again centrifuged for 10 min at 5000 x g and the flowthrough was discarded.
9. The spin column was transferred to a new 50ml tube and 1ml of AE buffer added to elute the DNA by first incubating for 5 min at room temperature and then centrifuging for 5 minutes at 5000 x g.

2. Polymerase chain reaction

All the PCR reactions for gene cloning from cDNA and genomic DNA were carried out using gene specific primers. The reaction mixture of 50ìl was prepared using various components as per the concentration given below following (Sambrook *et al.,* 1987, Roux, 1995) protocol.

10X PCR buffer 5.0ìl
25mM MgCl2 3.0ìl
10mM dNTP mix 1.0ìl
Gene specific forward primer (10ìM) 1.0ìl
Gene specific reverse primer (10ìM) 1.0ìl
Template (cDNA/genomic DNA) (50ng) 1.0ìl
DNA polymerase (5 units/ìl) 0.5ìl
Sterile MQ water 37.5ìl

Typically the amplification reactions were done for 30 cycles with specific alterations arrived at, empirically to optimize the yield. The PCR programme employed is given below:(Barnes 1994, and Cheng *et al.,* 1994)

1. Initial denaturation 94°C for 2 min
2. Denaturation 94°C for 30 seconds
3. Annealing of primers X°C for 30 seconds
4. Primer extension 72°C for 2 min
Step 2 to 4 were cycled 30 times
5. Final extension 72°C for 10 min
6. Indefinite hold: 4°C

"X" represents the annealing temperature which varied as per the primer combination used.

3. Restriction digestion

Enzymatic manipulation of DNA like restriction digestion was carried out essentially as described in Sambrook *et al*., 1989. All the preparative digestions for the preparation of inserts and vectors were generally set up in 50ìl volume with following components:

1ìg of DNA Xìl
buffer 5ìl
10X BSA 5ìl
Enzyme 1ìl
Water 39-Xìl

Typically the digestion reactions were carried out at 37°C overnight. Digests were resolved on 1.0% agarose gel and appropriate DNA fragments were cut out from the gel and eluted.

4. Construction of Genome Walker Libraries:

The overview of the protocol is shown as below (Figure1.1) (Ausubel *et al.,* 1994)

In brief the protocol consists of Isolation of Genomic DNA, restriction enzyme digestion and ligation to genome walker adapters. Following adapter ligation primary and secondary PCR amplification conducted and DNA bands eluted from agarose gel and cloned in pGEMT Easy vector and subsequently sequenced using adapter specific primer and also gene specific primer. The promoter sequence so obtained is compared at PLANTCARE online database for the presence of Cis Acting Regulatory elements in the promoter sequence in addition to TAATA box and CAAT box.

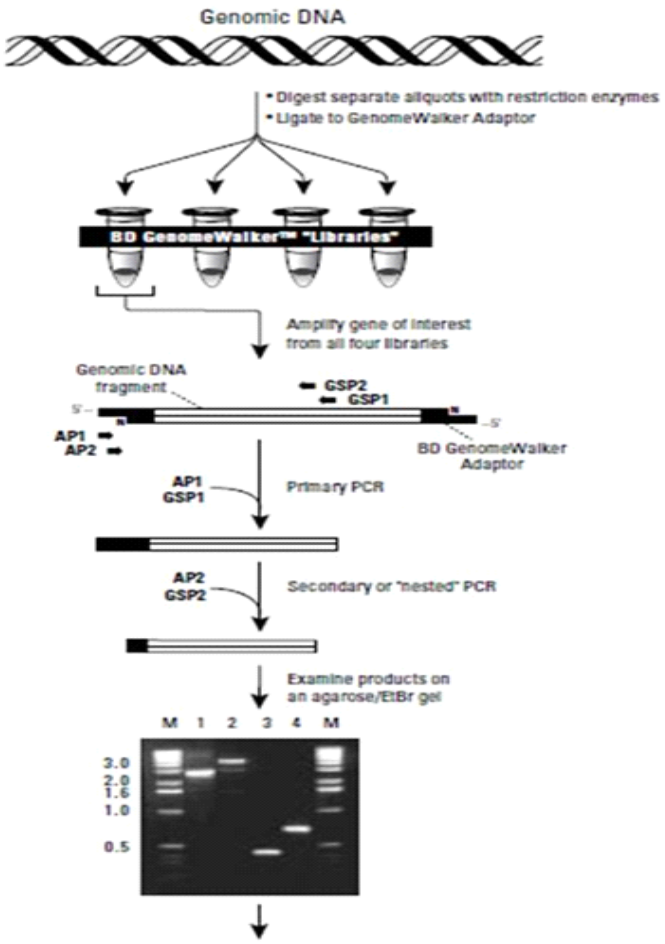
RESULTS AND DISCUSSION

Promoter isolation by Genome Walking:

Genome walking is a simple method for finding unknown genomic DNA sequences adjacent to a known sequence such as a cDNA (Siebert *et al* 1995) . The promoter of CaABgene was isolated using Universal Genome walkerTM Kit (Clontech, USA). From this kit a pool of un cloned, adaptor-ligated genomic DNA fragments were obtained which are referred to as libraries. These libraries are then used for isolation of gene specific promoter, in the following step wise manner.

1. Good quality DNA was isolated from chickpea plants using protocol of and the quality of genomic DNA was determined by resolving it on agarose/ EtBr gel. The DNA obtained was intact as no smear was observed.
2. In four different 1.5 ml sterile tubes, four digestion reactions were set up using the enzymes *Dra* I, *Eco*R V, *Pvu* II and *Stu* I, which produce blunt ends. In each reaction following components were combined.

Figure 1.1. Construction of Genome Walker Libraries.



Genomic DNA 25 ìl
Restriction enzyme 8 ìl
Restriction enzyme buffer 10 ìl
Deionized water 57 ìl
Total volume 100 ìl

The contents were mixed gently and incubated at 37^{0}C for 2 hr. The tubes were tapped gently and again kept for incubation for 16-18 hr. From each tube 5 ìl reaction mix was checked for digestion on 0.5% agarose/EtBr gel.

3. To each of the reaction tube, an equal amount (95 ìl) of phenol was added and slowly vortexed for 10 sec. After a brief spin aqueous layer was transferred to a new tube and again the above step was repeated to remove protein contamination.

4. After the second extraction, 2 volumes (190 ìl) of ice cold 95% ethanol, 1/10 volumes (9.5 ìl) of 3M NaOAc, and 20 ìg of glycogen was added and vortexed slowly for 10 sec.

5. To pellet the digested DNA, the tubes were centrifuged at 15,000 rpm for 10 min and the supernatant was decanted. The pellets obtained were washed with in 100ìl of ice cold 80% ethanol and centrifuged at 15,000 rpm for 5 min.

6. The supernatant was decanted, pellet was air dried and dissolved in 20 ìl of TE pH 7.5. After a slow speed vortex for 5 sec, 1 ìl of the Digested DNA quality & quantity was checked on a 0.5% agarose/ EtBr gel.

7. For ligation, 4 ìl of each digested and purified DNA was taken in 0.5 ml tubes and to each of the four tubes the following components were added 1.9 ìl Genomewalker adaptor (25ìM), 1.6 ìl 10X Ligation buffer and 0.5ìl T4 DNA ligase (6 units/ ìl). This reaction was incubated overnight at 160C.

8. Next day, to stop the reaction, the tubes were incubated at 70^{0}C for 5 min and the ligated DNA was diluted with 72 ìl of TE (pH 7.4).

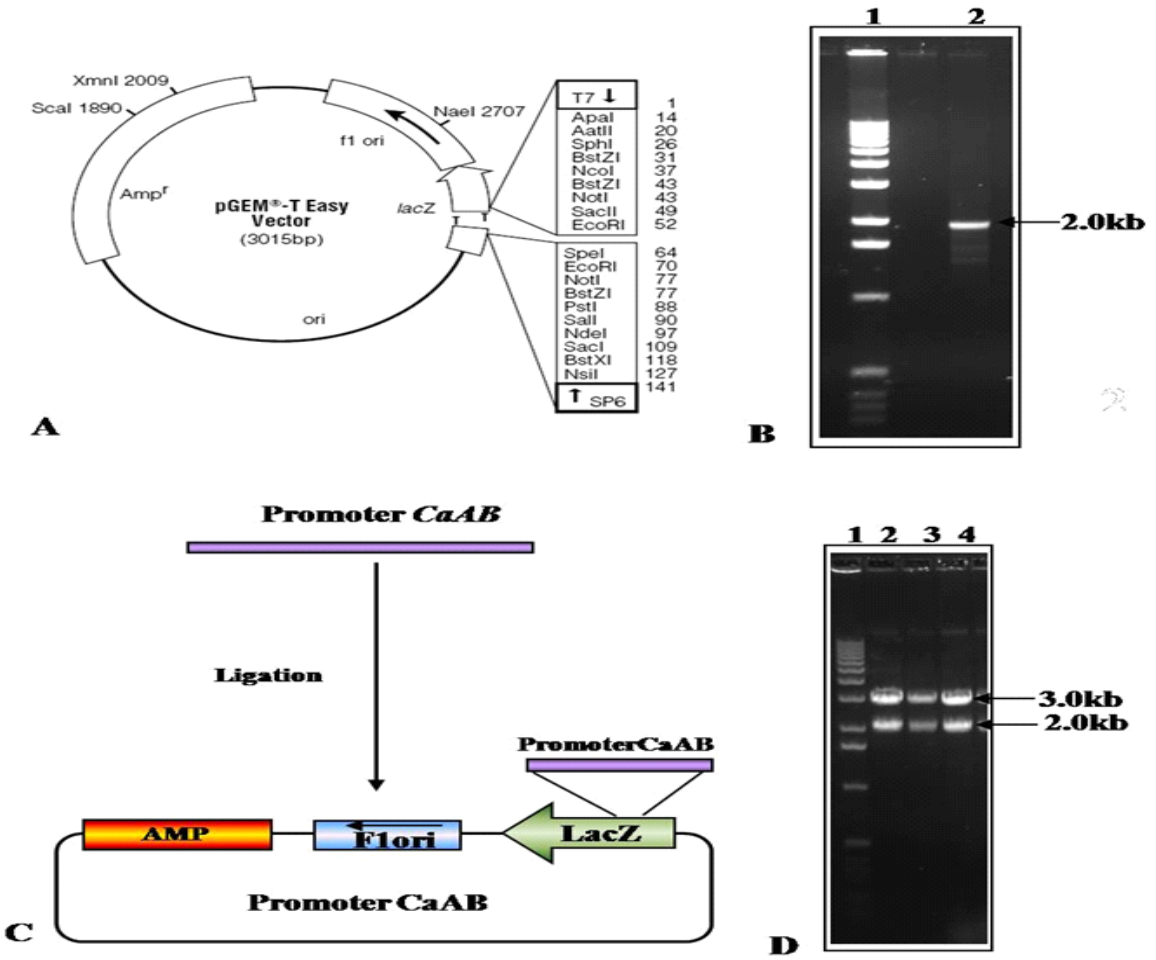


Figure 1.2-A. Promoter cloning of *Ca*AB gene. (A) Map of pGEM-T Easy vector used to clone the amplified product; (B) Amplified PCR product of CaAB promoter; (C) Diagrammatic representation of promoter of *Ca*AB cloned in pGEM-T Easy vector and the construct named as Promoter *Ca*AB; (D) 1% agarose/EtBr gel confirming Promoter of *Ca*AB by NotI digestion . Lane 1 represents 1Kb ladder and lane 2,3,4 three representative clones.

9. The promoter regions of the desired gene were amplified from the five adaptor ligated DNA library. PCR was done separately for each library with AP1 primer, and gene specific primers.

10. Some of the important points during design of gene specific primers are, they should be 26-30 mer in length and GC content should be 40-60%. The nested gene specific primer should be internal or upstream to the GSP1. There should not be more than three G's and C's in the last six positions at the 3' end of the primer.

The primary PCR reaction mix had the following components

Deionized water 40 ìl
10 X Advantage 2 PCR buffer 5ìl
dNTP mix (10ìM each) 1ìl
AP1 (10 ìM) 1ìl
DNA library 1ìl
GSP1(10 ìM) 1ìl
Advantage 2 ploymerase mix 1ìl

11. After the primary PCR, 1 ìl of it was diluted to 50 ìl to perform nested amplification using AP2 and GSP2 primer. The secondary PCR reaction mixture had the same components as that of the primary except for AP1 which was replaced by AP2 and the template was, diluted primary PCR product.

12. The amplified product was eluted after running on 1.5% agarose/EtBr gel and further cloned in to pGEM-T Easy vector, (Figure1.2).

Figure 1.3-: Cis acting regulatory elements identified in CaAB promoter. CAAT, Box; CGTCA, motif-cis-acting regulatory element involved in the MeJA-responsiveness; ATTTCAAA, ERE ethylene-responsive element; AAAAAATTTC, cis-acting element involved in heat stress responsiveness; TAACTG, MYB binding site involved in drought-inducibility; CCATCTTTTT, cis-acting element involved in salicylic acid responsiveness; TGACG, cis-acting regulatory element involved in the MeJA-responsiveness; TATA, core promoter element around -30 of transcription start.

Cloning and sequencing of the product in pGEMT Easy vector showed that it is 2044bp long. Three representative clones after confirming with digestion (Figure 1.2) were used for sequencing. All the clones showed overlap with the 5prime UTR and when analysed using promoter scan program showed the presence of TATA box, CAAT box, TF2D binding site with a promoter prediction score of 0.54, well above the cut off score of 0.53. Identification of Cis Acting Regulatory Elements using PLANT-CARE showed the presence of various Cis elements as Light responsive, heat stress responsive, Methyl Jasmonate responsive, Salicylic acid responsive, Ethylene responsive, etc. (Figure1.3).

Of these MeJ, SA and Ethylene are important as these elements have been shown to be present in the promoters of defense related genes and have implications in modulating resistance against various stresses especially stress caused by biotic factors. Since *CaAB* promoter contains these Cis elements, it would be interesting to see the role of this promoter against *Fusarium* infection in chickpea. The complete sequence of *CaAB* promoter obtained is shown in the Figure 1.4. Factors binding to Promoters will shed light on gene regulation and its involvement in immunity or resistance mechanism.

Conclusion:

In Conclusion a 2044 base pair long ARID/ BRIGHT Promoter was cloned from Wilt resistant Chickpea genotype and it was found to contain various Cis acting regulatory elements such as Light responsive, heat stress responsive, Methyl Jasmonate responsive, Salicylic acid responsive, Ethylene responsive and defense responsive elements in addition to presence of TATA box, CAAT box and TF2D binding site.

>CaAB-consensus promoter sequence(From+1 to 2044)
AACGTTTAAAGTTAGTTCGTACTTTTGTGTTGTTGGAGGGATGTGGTTTTAGTAATGTAATTAAGTTAAAG
TTCTAATTTTTTTACCTGGGCTCTTTCTTTTTTTTTTTGTCTTGAAGTCATAGGTGGTGGTTGATTGTGTT
TTTTTTTTTTTTTTTTTTTTAGGTATTTCTGTCTATTGAAAAAGATGGGATTTATGATTTGGGATCGGTCAT
TATTAGATATTTACTTTGTTGGAACCATATGATTTGGGGTTTTTTTATGTACGAACACTTGAAGTGTTTCG
AGTTTTGAAACTAAAAGTACCGATGAATTCAGTAATATCGCTTTAAGTTTCACTTTTTCTTTAACTTGTTA
TTGGTTTTTTATCTTCTTTGTTGCTTTACATCTGTAAATGGACACGCACAACTCTAACTTCCCACTCAGAT
GTCACTTTCGTCCCTTACGGGATTTAACTTCTTGTCCTAATTAACTTAAACCTTTTTGGGATTTAATAAAA
AGGGGAAGACTCGAAACGTCCTTAAAAAGTCTTCTTGTGTTTAAAGAACAACTTCGCCGTCTAAAACTACG
CGCAAAGTATGTGCGCGGCGCAATGTAACCTACTATTTTTGACGCTACCGTAAGCATGCTGCGGCAAAACT
AGATTTTTTTTGTTCACTTGAATCGTAATTCTCTTAATCTTCTTTTGTTCCATCAAACCCGAAAACTTAAA
TAAATAACCTATACTGAAAAACGTGGTGGTAAAAAAAATGATAACAAATATAAAACTAGGTACAACAAGAA
AAACCTTCATTCATATGTAAACCTCGATGGATACTTTTATTTGATCTGGCTCAATTTGAAACATTTCAAAC
TCTCGTCAGAAATTTTTTTAACTTTCACACATCATACAATATTCAAAAAAACAAATCCAAATTAGAAATAA
TATCAGATCATACTAAACAATTTAATAAATTTTCAAATAAAGTATAATTATAAACTTCAATCAACAGTTTG
TATAAACACTATCTGGCTGTTTAATTTACCAGTTAGTTTAGTTTATAAAAACATCTAAATTTTACTATAAA
ATTCCTGAAACTAAAATTTACTATAGTATAAAGTAAAGTTAAAACACTATTATATAAATTTATGCTTTTTT
TTTGATTATATCTTATTATACTAATTTAAGAATTTTCAAATCGTCTAAATATTAAATTATAATCATAATTT
TATTATAATAAAATCTAAATAAATTTATCTAGCCAAACCATATAGAATTTACTAAAAACATACCGTACATC
AGTCCATAAAAATCGATTTATCTGAAGATTTATTCATATTTAAAACGAATAAATTTTTTTTTTTAGAGTAG
ATTATACCTAAATAATATCTCATCTGACATCGGGGAACAGCTTACCAGACTCAGTAACTTTCACATGAAAA
ACTAACTTTACCTTCTAAATATACATAACCCAATACAAAGATTTACCTGACACTACCAACTTAGTTATACA
ATTCAGGTTACTCTCCAGAAACAGGAGTAAGTAAATTGATAAATTAACAATAGTATAATCTACTAATTAAA
TTAGAAATTTGAATTGCTATTACTAACATCAAATGGTGAGTAAAATAAGAAAGTTGTTTACGGAGAGTTTA
AGATTTTTTATACCCCAAGAGATAAAAAGCCCTATATGGGATTATGCATAGATAAACTCAAACTATAAATT
GTAAATAAAACAACTCTAAATCTTTGGAAGAACGTATACGCGTGGAGGTCTGCATGTATGATGTTATTCTC
CATTTTTAACTAACCATAATATAAACATCTTATAATTGATTGAATATTCAATGTTTATTTTTTATTATTAT
TTTTGTTTTCTTTATTTAATTTATAATATGTGATATTTTAGTTTGCGATACTTGAATTTTTATTATAATAT
TTAAGCACTCTTTTGTATTAATAGTTTGTCCTAATAACTCAATTTGCGATATTTGAAAAAAAAAAGGGAAA
GGATTGTGTGGGATTTTTCTTTTCCTATTTTGGAATATAGATTAGCCACTCTTGCT

Figure 1.4. Consensus promoter sequence of *CaAB* gene from Chickpea.

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