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# Characterization of Arid/Bright Transcription Factor From Chickpea Against Fusarium Wilt

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**ABSTRACT**

The present study was undertaken with an aim to unravel the molecular basis of wilt susceptibility and immunity in Chickpea plants using two different cultivars showing differential reaction when infected with Fusarium. In order to enrich differentially expressed transcripts, SSH based libraries were constructed and were used to monitor transcript levels upon *Fusarium* infection. The ARID consensus sequence spans about 100 amino acid residues, and structural studies identify the major groove contact site as a modified helix-turn-helix motif. In Chickpea, ARID gene was isolated, cloned and found to be single copy gene and ARID protein is nuclear localized in Onion peel experiment. It is relatively a new class of transcription factor family identified in plants, and shown to express in eighteen different structures in *Arabidopsis thaliana* and also gets induced upon Rhizobium treatment in Lotus. The data has shown for the first time has shown the involvement of *CaAB* in plant immunity. The up regulation of ARID gene upon pathogen challenge shows the involvement of this gene in defense against *Fusarium*,

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

The ARID (AT-rich interaction domain) is a billion year old DNA-binding domain that has been identified in all sequenced higher eukaryotic genomes (Wilsker et. al., 2002). 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 (Iwahara and Clubb 1999). The ARID consensus was first identified in the mouse B-cell-specific transcription factor bright and is the product of the dead ringer (dri, also known as retained, retn) gene of Drosophila melanogaster. DRI and Bright were each isolated in searches designed specifically to identify proteins binding to AT-rich sequences, but neither turned out to contain a previously known DNA-binding domain. Identification of DNA binding sequences conserved between Bright and DRI defined the parameters of a new DNA-binding domain, whose name was inspired by the interaction of these proteins with AT-rich DNA elements. Since the discovery of the ARID, many additional proteins containing this domain have been identified (Webb 2001). The cellular functions of ARID proteins include participation in the regulation of cell growth, differentiation, and development (Wilsker 2002). The ARID domain is both ancient and widespread, occurring in (some) protozoa, green algae, higher plants, fungi, and metazoans (Kortschak, 2000). 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 vitis, ricinus, glycine, maize, sorghum, populous and barley. ARID-containing proteins have also been identified in higher plants and fungi. In the sequenced Arabidopsis genome, eleven ARID-containing proteins that form five subfamilies have been identified. Keeping these things, in the present study, the ARID containing proteins were characterized in chickpea against Fusarium wilt.

## MATERIAL AND METHODS

### Genomic southern

Genomic southern was performed in order to find out the copy number of *CaAB* gene in chickpea. 10ìg of genomic DNA samples were digested with restriction enzymes BamH1, Bgl II, Xba1 and SpeI. The digested samples were separated on 0.8% agarose gel, denatured and blotted on to Gene screen plus membrane (Amersham). For hybridization, ~2.0kb region of *CaAB* gene was amplified using AB5GSP-2 and AB-FRC4 primer pair. The amplified product was

run on 1% agarose gel and purified by gel extraction and this purified product was further used for preparing the 32P-dCTP labelled probe using random labelling kit (NEB).

**Northern blotting**

Northern-blot analysis was performed to determine the expression pattern of *CaAB* gene in response to *Fusarium* wilt and its tissue specific expression. For this, 20ig of RNA samples were separated on a 1.5% formaldehyde-agarose gel and then blotted onto Gene screenplus membrane (Amersham). The full length clone of *CaAB* gene (2.25Kb) was amplified from the plasmid containing this clone using gene specific primers AB5GSP-2 and AB_3PGSP-2. The amplicon was run on 1% gel, purified by gel extraction and the purified product was used for preparing 32P-dCTP labelled probe by random labelling using random labelling kit (NEB).

**Subcellular localization**

The subcellular localization of *CaAB* gene was studied by performing transient expression assay in onion epidermal cells. For this *CaAB* gene was fused in frame with 5' terminus of GFP reporter gene in pCAMBIA-1302. The fusion construct was named as pCAMBIA- *CaAB* and was prepared by amplifying *CaAB* gene from p*CaAB* using AB-FP-Bgl-pCam and AB-RP-Bgl-pCam primer pair and cloning in *Bgl*II site of vector. The fusion construct of *CaAB* –GFP was bombarded on to the onion peels which were then incubated for 24 hours before visualizing using confocal microscope.

**Bacterial over expression of *CaAB* gene**

The full-length cDNA of *CaAB* gene was inserted at the BamHI-NotI site of pGex4T-2vector (GE), generating pGex-CaAB. For protein expression, *Escherichia coli* BL21 harboring the plasmid was induced with varying concentrations

A

| GeneFeature | BasePairs |
|---|---|
| 5Prime UTR | 263 |
| Exon1 | 946 |
| Exon2 | 41 |
| Exon3 | 51 |
| Exon4 | 59 |
| Exon5 | 80 |
| Exon6 | 108 |
| Exon7 | 51 |
| Exon8 | 79 |
| Exon9 | 64 |
| Exon10 | 53 |
| Exon11 | 93 |
| Exon12 | 100 |
| Intron1 | 356 |
| Intron2 | 105 |
| Intron3 | 829 |
| Intron4 | 816 |
| Intron5 | 551 |
| Intron6 | 383 |
| Intron7 | 102 |
| Intron8 | 99 |
| Intron9 | 174 |
| Intron10 | 81 |
| Intron11 | 364 |
| Intron12 | 118 |
| 3Prime UTR | 237 |

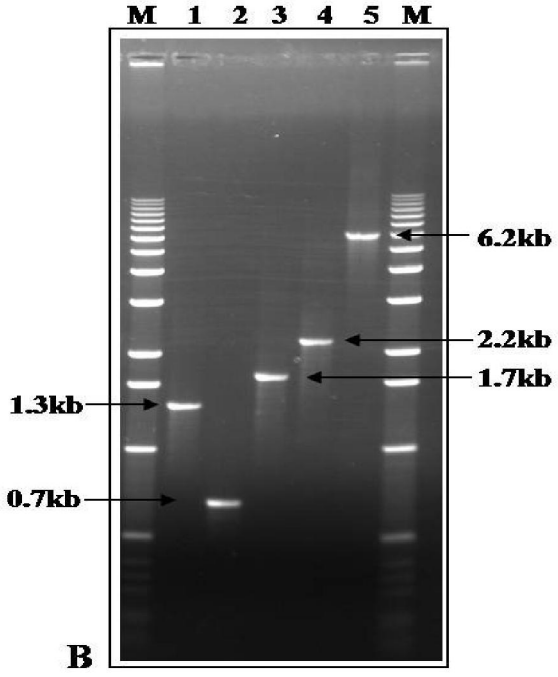



**Figure 1.1(A) Genomic organisation of CaAB gene with introns and exons (B) Gene features of CaAB gene with Lane1 showing 5pRACE product, Lane2 3pRACE product, Lane3 ORF, Lane4Full length cDNA, Lane5 Full length genomic Clone**

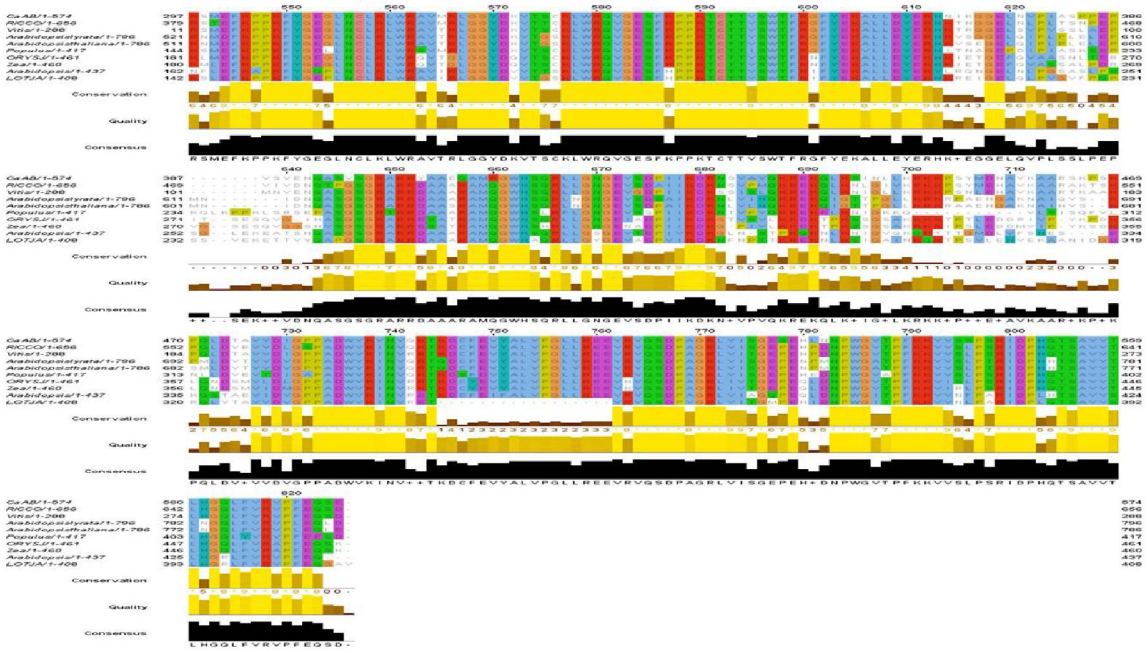


**Figure 1.2.** Sequence alignment of *CaAB* with the sequences of orthologs from other plant species. The multiple sequence alignment was done using MAFFT program LINSI algorithm

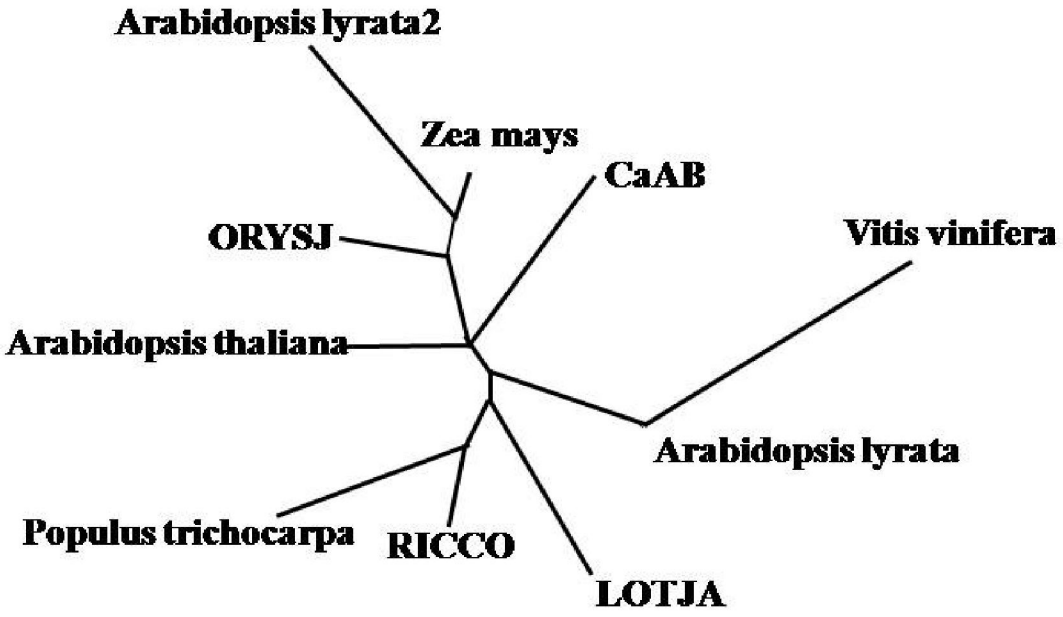



**Figure 1.3**. Phylogenetic tree showing evolutionary relationship between *CaAB* and other AB family proteins. The tree was generated using MAFFT program neighbor joining conserved method.

of isopropyl 1-thio-b-D-galactopyranoside in 2 X YTA medium, and the samples were collected at different time intervals keeping the cultures at $37^0$C and subsequently analyzed by running samples on 12% SDS-PAGE gel.

**Mass spectrometry analysis of induced CaAB protein**

The induced CaAB protein was cut from the gel and were analyzed by mass spectrometry following destaining, reduction, alkylation and trypsin digestion. In brief the excised bands were cut into small pieces and destained using 50mM Ammonium bicarbonate in 50%ACN. Following destaining, the gel pieces were reduced using 10mM DTT (prepared in 50mM Ammonium bicarbonate) and were alkylated using the buffer containing 55mM Iodo acetamide dissolved in 50mM Ammonium bicarbonate. The gel pieces were allowed to shrink using Acetonitrile and were dried subsequently, after carefully removing Acetonitrile. Dried samples were digested with Trypsin (100ng trypsin/digest) and samples were analysed using Mass spectrometry after removing digestion mixture from the samples.

**Trans-activation assay**

A yeast one-hybrid system was constructed from the DupLEX-A two-hybrid system (Origene) and applied for trans-activation assays of the CaAB protein. Recombinant plasmids used in the trans-activation experiments were constructed by inserting the PCR fragment of the full-length cDNA of *CaAB* gene amplified with primers, PCR products were cut with BamHI and XhoI and inserted in-frame to sequences encoding the LexA DNA-binding domain of pEG202.

The resulting plasmids or empty plasmid pEG202 were individually introduced into cells of yeast strain EGY48 harboring the reporter plasmid pSH18-34 (URA3, 2 mm, Apr, LexA ops-lacZ). The resulting yeast transformants were grown on

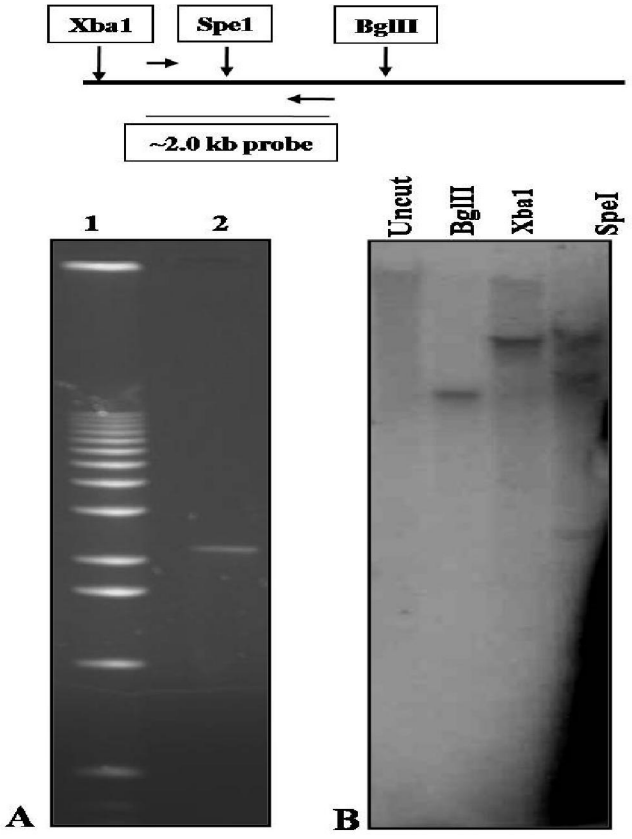
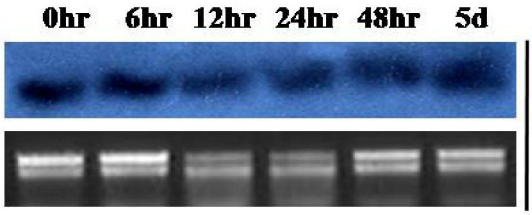



**Figure 1.4. Copy number identification using genomic southern. (A) PCR amplified product for probe using genomic clone as template. Lane 1 and 2 indicate 1Kb ladder and PCR product, respectively; (B) Southern blot hybridization analysis for predicting the copy number.**

**Figure1.5 Northern analysis of CaAB gene**



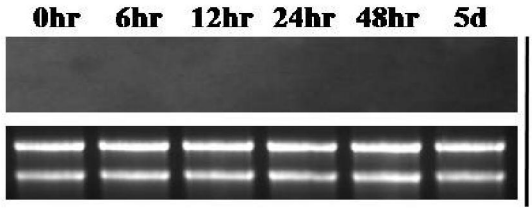
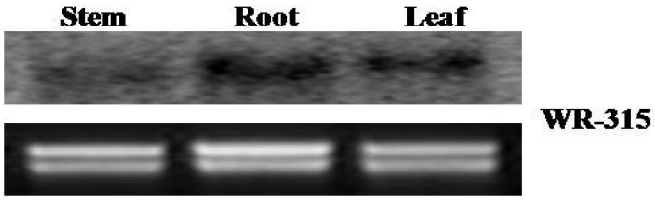




Figure1.6 Differential expression of CaAB gene




SD medium in the presence of 5-bromo-4-chloro-3-indolyl-beta-Dgalactopyranoside(X-gal), but in the absence of histidine and uracil, for 2–3 d at 30°C.

## RESULTS AND DISCUSSION

### Genomic cloning of *CaAB* gene

Genomic cloning of CaAB gene using end to end primers (AB_3PGSP-2, AB5GSP-2) on WR-315 genomic DNA generated a single product with an approximate size of 6.2kb (Figure 1.1). Further cloning, sequencing, and alignment with full length cDNA showed that it had 12 Exons with 11 Introns. There is one more intron in 5' UTR region. Since the amplified product was 6.2kb, primer walking strategy was used to get the full length sequence of genomic clone and three representative clones after confirming with digestion were sequenced on both the strands and a consensus sequence was obtained using BioEdit program. Alignment of

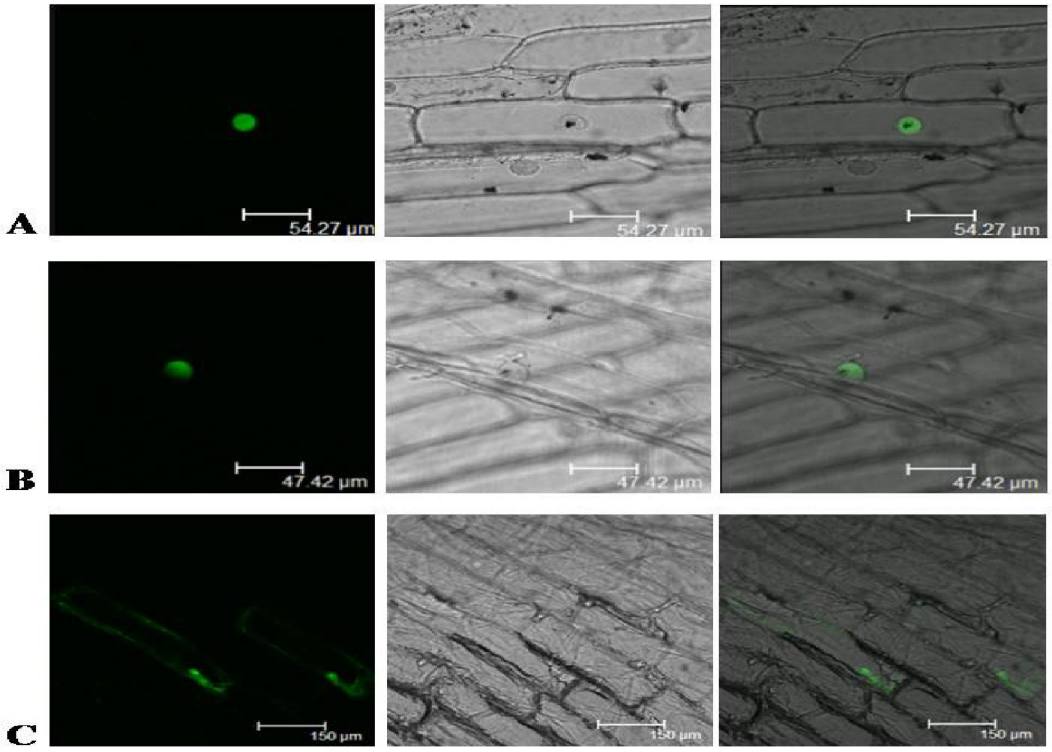



**Figure 1.7 Subcellular localization of *CaAB*. (A-B) Onion epidermal cells bombarded with vector pCaMBIA-*CaAB*-GFP. The GFP fluorescence was detected by using Confocal microscope. The middle panel shows the corresponding phase contrast image and the right panel shows the image overlay; (C) empty pCAMBIA vector as control.**

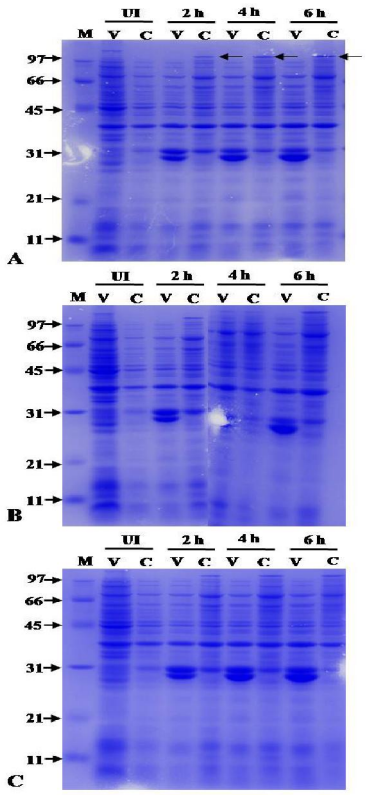



**Figure 1.8** IPTG Induction profiles of Ca*AB* protein in BL21 cells. Where V and C represent Vector and GST-CaAB Protein respectively. (A) IPTG induction 0.2 mM; (B) IPTG induction 0.4 mM; (C) IPTG induction 0.6 mM. and arrow represents induced GST-CaAB fusion protein

genomic sequence with cDNA sequence of *CaAB* gene was done using BLAST2 Program available at NCBI site. Analysis of ARID gene of model plant, *Arabidopsis thaliana* also showed multiple introns, for example the gene AT1G76510.2 had 14 exons and 13 introns with both ARID and ACD domains. The complete gene structure of *CaAB* gene was given in the Figure 3.5-C.

**Multiple sequence alignment of CaAB protein with orthologs from other species**

In order to see conservation among various ARID proteins multiple sequence alignment was done taking top hits from BLAST P search and the analysis of multiple sequence alignment using MAFFT program LINSI algorithm showed maximum conservation of amino acids in the domain regions namely ARID and ACD (Figure 1.2).
N terminal half of Chickpea ARID gene is shown to be unique as there is less conservation for the gene at this region.

**Phylogenetic analysis of CaAB protein to show the evolutionary relationship among various species**

In order to evaluate the evolutionary relationship of CaAB protein, the deduced amino acid sequence was aligned with nine orthologs of ARIDs from other plants and a phylogram was generated using MAFFT program neighbour joining conserved method. The unrooted tree (Figure 1.3) showed that CaAB protein is more close to *Arabidopsis thaliana* gene compared to Lotus even though both of them belongs to same family Leguminaceae suggesting a separate lineage of origin for this gene.

**Southern analysis to know genomic organization of *CaAB* gene**

To determine the copy number of *CaAB* gene in chickpea cultivar WR-315, genomic DNA southern blot analysis was carried out using a ~2.0kb PCR product obtained from 5' region of genomic clone as a probe. Southern transfer of chickpea genomic DNA digested separately with various restriction enzymes was also performed and hybridized with the probe under high stringent conditions and the hybridization result showed that *CaAB* gene is a single copy gene at least in WR-315 as the non cutters generated single band and single cutter in the probe region generated two bands (Figure 1.4). In contrast to this in *Arabidopsis thaliana* this gene is present as a multi copy gene.

**CaAB gene is pathogen inducible**

Earlier observation in Lotus has shown that ARID domain containing gene SIP1 was induced in response to *Rhizobacterium* infection and till date no report is available showing the involvement of ARID genes in plant pathogenesis, even though ARID containing genes have been shown to be involved in animal immunity. In *Arabidopsis thaliana* ARID containing genes are expressed in eighteen different plant structures and is mainly involved in development. Here for the first time it is shown the involvement of ARID containing gene i.e., *CaAB* gene in pathogenesis as there is up regulation of this gene upon *Fusarium* infection in resistant cultivar of chickpea WR-315, in contrast to no detectable expression in susceptible cultivar JG-62 (Figure 1.5).

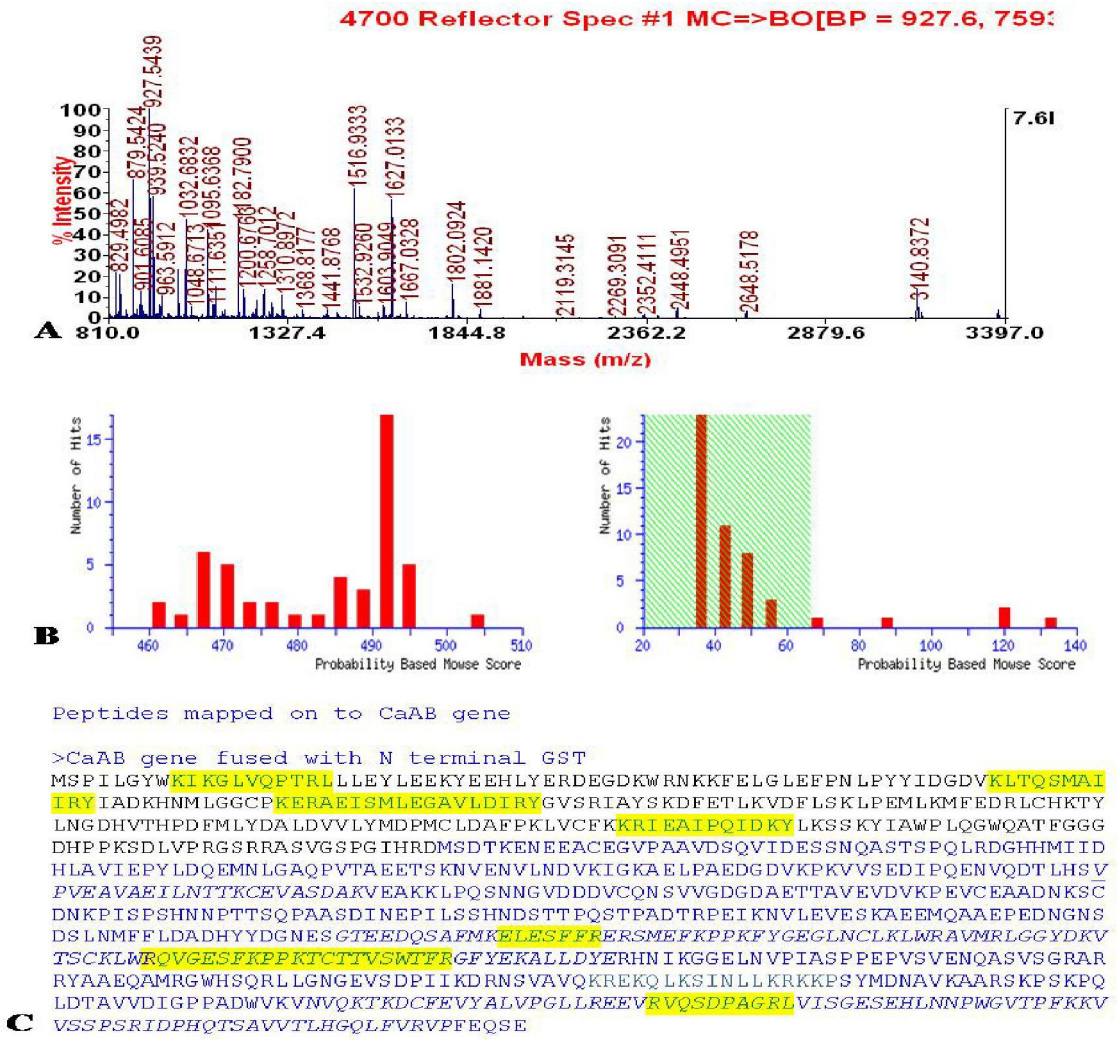



**Figure 1.9. Mass spectrometry identification of CaAB protein isolated from SDS-PAGE gel; (A) Representative Mass spectrum; (B)Score distribution for peptides; (C)Highlighted amino acids represent identified peptides.**

However microarray analysis showed up regulation of this gene in both the cultivars , showing the sensitivity of microarrays in detecting small changes in their expression pattern. But it was noteworthy to see that the induction was higher in resistant genotype as compared to the susceptible one indicating the involvement of this gene in plant immunity.

### *CaAB* gene is preferentially expressed in root tissue

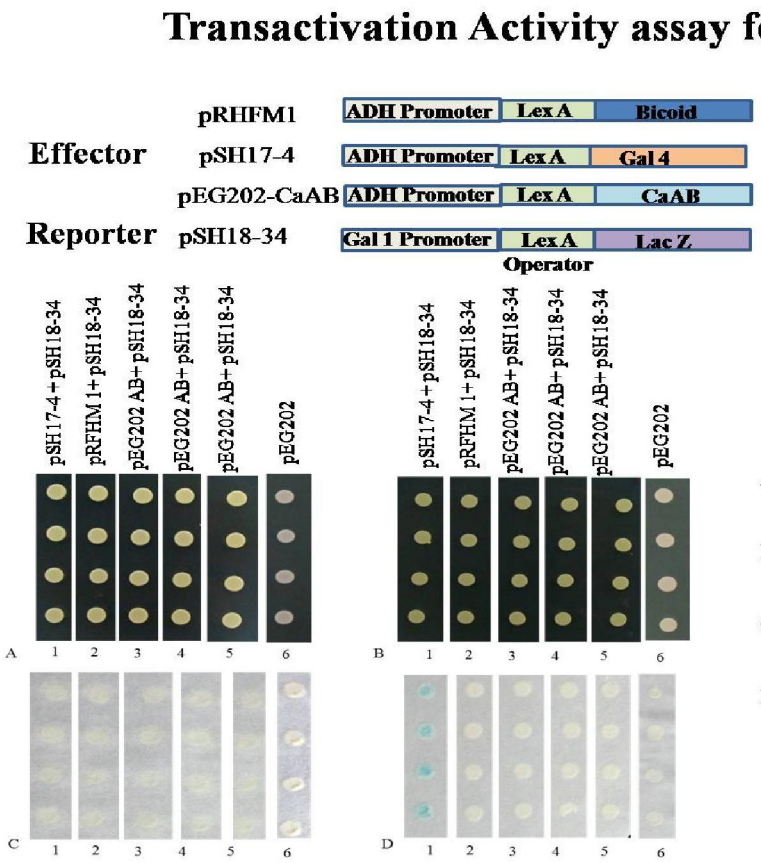
Since *CaAB* gene was found to be differentially regulated, it was of interest to know the tissue specificity of its expression. Total RNA was extracted from root, stem and leaf tissue of 21-day old chickpea seedlings infected with *Fusarium* and the northern blot was prepared and probed with the CaAB full length cDNA fragment. Varying levels of *CaAB* gene transcripts were detected in stem, leaf and root with expression in root being significantly higher than in other tissues (Figure 1.6).

This may be due to the fact that *Fusarium* infects through roots and the infection is later on spread to other parts of the plant system. Thus root being the first tissue to come in contact with the pathogen leads to increased expression of *CaAB* gene in this tissue.

### *CaAB* gene is nuclear localized

In silico analysis using WOLF P-SORT for sub cellular localization predicted CaAB protein to be nuclear with a prediction probability of greater

**Figure1.10 Transcriptional activation assay for CaAB gene**




| Constructs | Reporter | Result |
|---|---|---|
| pRHFM1 | pSH18-34 | - |
| pSH17-4 | pSH18-34 | + |
| pEG202-CaAB | pSH18-34 | - |

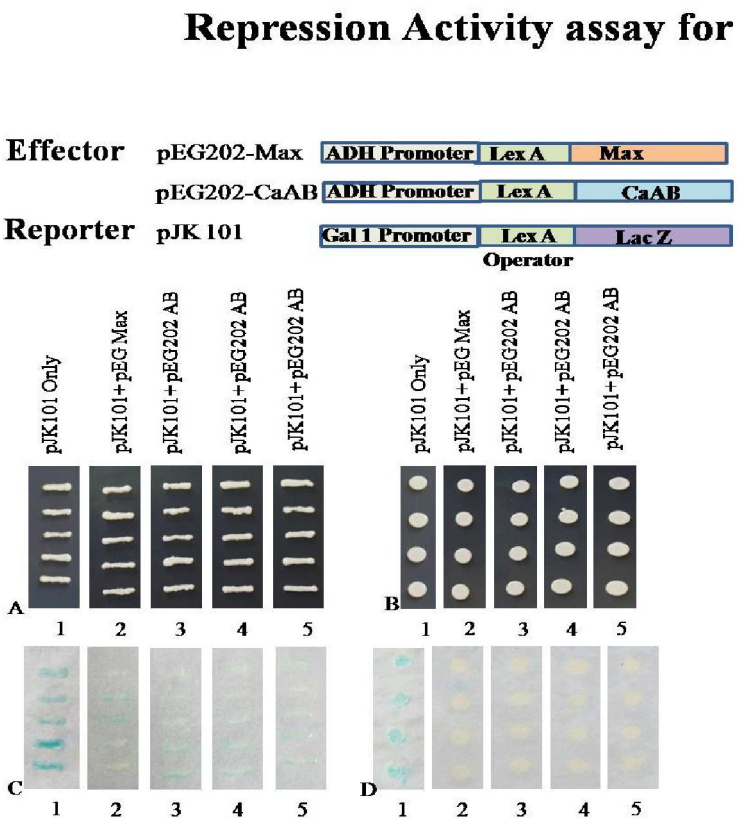
A- YNB + Glucose – his – Ura (1-5) YNB + Glucose – his (6) on Plate

B - YNB + Galactose – his – Ura (1-5) YNB + Galatose – his (6) on Plate

C - YNB + Glucose – his – Ura (1-5) YNB + Glucose – his (6) on Filter

D - YNB + Galactose – his – Ura (1-5) YNB + Galatose – his (6) on Filter

**Figure1.11 Repressor activity of CaAB Gene)**




| Constructs | Reporter | Result |
|---|---|---|
| pEG202Max | pJK101 | + (No Color) |
| pJK 101 | - | - (Blue Color) |
| pEG202-CaAB | pSH18-34 | + (No Color) |

A-B YNB + Galactose – Ura (1) YNB + Galatose – his – Ura (2-5) on Plate

C-D YNB + Galactose – Ura (1) YNB + Galatose – his – Ura (2-5) on Filter

than 0.9 and shown to contain the NLS "KREKQLKSINLLKRKKP". To test this experimentally pCAMBIA 35S- CaAB -GFP was constructed. In this construct the CaAB gene was fused in frame to the 5'terminus of GFP reporter gene. The expression of the fusion gene construct CaAB -GFP was driven by the 35S promoter of cauliflower mosaic virus (CaMV-35S). The plasmid construct was named as pCAMBIA- CaAB. The plasmids containing vector control DNA and fusion gene were introduced into onion (*Allium cepa)* epidermal cells by particle bombardment. Observations using confocal microscope, showed the nuclear localization of CaAB - GFP construct whereas the GFP protein alone was distributed all throughout the cell (Figure 1.7).

This result is consistent with its potential function in DNA binding and transcription regulation. Nuclear localization of ARID containing protein SIP1 was also shown in Lotus (Zhu et. al., 2008).

**Bacterial expression of *CaAB* gene followed by Mass spectrometry analysis of induced protein**

The full-length cDNA of *CaAB* gene was inserted at the BamHI-NotI site of pGex4T-2vector (GE), generating GST-CaAB. Recombinant clones were confirmed by digestion and sequenced to check the correctness of junction sequence. For protein expression, *Escherichia coli* BL21 harboring the plasmid were induced with varying concentrations of isopropyl 1-thio-b-D-galactopyranoside viz 0.2mM, 0.4mM and 0.6mM, in 2X YTA medium, and the samples were collected at different time intervals viz 0 hr, 2 hr, 4 hr and 6 hr keeping the cultures at $37^0$C and subsequently analyzed by running samples on 12% SDS-PAGE gel (Figure 1.8) and the results showed that CaAB gene was induced at all the concentrations of IPTG, equally at time intervals 2hr, 4,hr and 6hrs. The induced GST-CaAB protein was taken from the gel and was analyzed by mass spectrometry following distaining, reduction, alkylation and trypsin digestion. Mass spectrometry identified peptides corresponding to both GST and CaAB protein as shown in Figure 1.9 with an acceptable score upon MASSCOT search done against Viridi plantae database.

**Trans-activation assay**

To determine whether the CaAB protein has transcriptional activity, a yeast-one-hybrid system with two plasmids (effector: pEG202 and reporter: pSH18- 34) were selected from a DupLEX-AM yeast-two-hybrid. CaAB cDNA was fused in-frame with a LexA protein and the recipient yeast contains a LacZ gene, whose promoter elements can be recognized by LexA. As a result, the yeast transformants should change to blue color in SD plate supplemented with the lacZ (beta-galactosidase) substrate X-gal. In our experiments, yeast transformants with CaABLexA showed no blue color indicating no transactivation in Yeast based system and the positive control showed blue colour (Figure 1.10) as expected. Similar results were obtained when ARID containing protein SIP1, isolated from Lotus was tested for transactivation using GAL-4 system. It would be interesting to see transactivation of CaAB in plant based system or it might also function as a repressor as confirmed by repression assay (Figure1.11)

## LITERATURE CITED

**Iwahara J and Clubb R T 1999** Solution structure of the DNA binding domain from Dead ringer, a sequence specific AT-rich interaction domain (ARID).*EMBO J* 18: 6084–6094.

**Kortschak R D, Tucker P W and Saint R 2000** ARID proteins come in from the desert. Trends Biochem Sci 25*:* 294–299.

**Webb C F 2001** The transcription factor, Bright, and immune globulin heavy chain expression. *Immunol Res* 24*:* 149–161.

**Wilsker D, Patsialou A, Dallas PB and Moran E 2002** ARID proteins: a diverse family of DNA binding proteins implicated in the control of cell growth, differentiation, and development. *Cell Growth Differ* 13: 95– 106.

**Zhu H, Chen T, Zhu M, Fang Q, Kang H, Hong Z and Zhang A 2008** A novel ARID DNA-binding protein interacts with SymRK and is expressed during early nodule development in *Lotus japonicas*. *Plant Physiol* 148: 337–347.

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