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Cloning of HVA22 Homolog from *Aloe vera* and Preliminary Study of Transgenic Plant Development

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ABSTRACT

Aloe vera (L.) (Aloe barbadensis Miller) is a perennial succulent xerophyte, which develops water storage tissue in the leaves to survive in dry environmental conditions. Few Genes widely known for stress tolerance were isolated from Aloe vera genome and shown to have expressed as well like, DREB1. HVA22 is a stress inducible gene isolated from Hordeum vulgare aleurone layer. Homologs of HVA22 have been found in 354 plant species. The expression of HVA22 in vegetative tissues can be induced by ABA and environmental stresses, such as cold and drought. Aloe vera is known to survive without water for long duration of time. This study aimed at isolating stress inducible genes from Aloe vera. Aloe vera plantlets subjected to ABA were used to isolate cDNA pool. HVA22 homolog was obtained using RT- PCR which was verified by sequencing and bioinformatics. It was inserted into pCAMBIA 2300 and was cloned into Agrobacterium tumefaciens MTCC 431. Agrobacterium mediated gene transfer was performed in Ocimum basilicum, where integration of the transgene was shown upto the genomic level.

Key words: *ABA, pCAMBIA 2300, Stress- inducible gene, Ocimum basilicum*

INTRODUCTION

Aloe vera has been used for medicinal purposes in several countries: Greece, Egypt, India, Mexico, Japan and China[1]. This plant has many properties and one such property is it's ability to withstand shortage of water for a long period of time. This property of *Aloe vera* is useful to study stress induced proteins and the genes coding for them in the plant. Stress studies in plants is important in order to explore the mechanisms by which some plants can combat stress and some cannot. Stress inducible genes in *Aloe vera* has been studied before, some of them being DREB1, NADP-malic enzyme gene, Ty3 Gypsy Gag gene, hsp70, hsp100 and ubiquitin genes[2-4]. DREB or Dehydration responsive element binding (DREB) gene encoding AP2/ Ethylene response element binding protein transcription factor was isolated from *Aloe vera* L. It was hypothesized to be involved in the regulation of cold- responsive genes[4]. NADP malic enzyme cDNA fragment was isolated from leaves of *Aloe vera* L. which accmulated under salt stress conditions.

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It was concluded that the AvME protein expression increased with hours of treatment[5]. Gag region of a Ty3 gypsy retrotransposon was previously isolated from *Aloe vera* genomic DNA and it's active transcription was confirmed. Incomplete Retrotransposons sometimes helps in aiding the plant against pathogen attack[3]. In yet another study, the genes hsp70, hsp100 and ubiquitin were highly expressed in *Aloe vera* plants acclimated at 35^{o}C prior to a heat shock of 45^{o}C [2]. The above studies shows that there is a wide scope to find more stress inducible genes in *Aloe vera* genome. HVA22 is one such stress inducible gene that was first isolated from *Hordeum vulgare* aleurone layers. HVA22 is an ABA- inducible gene in barley, as ABA (Abscisic acid) induces the expression of a battery of genes in mediating plant responses to environmental stresses[6]. HVA22 is an ER and Golgi localized protein that is capable of negatively regulating GA- mediated vacuolation/ programmed cell death in barley aleurone cells [7]. The HVA22 homolog in Yeast, Yop1, regulates vesicular traffic in stressed cells either to facilitate membrane turnover or to decrease unnecessary secretion[8]. There are around 355 homologs of HVA22 as per the data on NCBI, but none are found in Prokaryotes. HVA22 gene codes for a 15-kDa protein with several interesting features, and it's sequence homologous to DP1 gene in human is involved in clorectal tumorigenesis. Upon obtaining the sequence of HVA22 gene, it was observed to have three elemnts, two located in the promoter and one in intron, which is essential for the high level of ABA induction of HVA22 expression[9]. Therefore, HVA22 and it's homolgs must be playing an important role in protecting the cells against environmental stress. This is the first study of HVA22 homolog isolation from *Aloe vera*. A major reason for shortage of food crops in the nation is Drought, as most of the food crops do not have the ability to overcome abiotic stress. The stress sensitive plants therefore succumb to shortage of rainfall in dry lands and global warming. One of the approaches to combat this problem is the production of Transgenic plants. A variety of crops have been engineered for enhanced resistance to a multitude of stresses such as herbicides, insecticides, viruses and a combination of biotic and abiotic stresses in different crops including rice, mustard, maize, potato etc., [10]. The promoter of HVA22 homolog from Rice was used to express the transgenes in Rice to observe it's effect on drought resistance under field conditions. The transgenic plants did show significantly higher yield per plant than wild type under drought conditions[11]. The HVA22 homolog isolated from *Aloe vera* will be subjected to transgenic plant production in *Ocimum basilicum*. Transgenic plants also allows us to check the expression pattern of the gene and it's potential products. *Ocimum basilicum* is a sacred plant which is worshipped in India. It is also used in food and medicines. It is a stress sensitive plant and cannot survive without water. Transferring this gene to *Ocimum basilicum* will answer two quesitons- The activity of HVA22 homolog in a different plant system, and also aiding the plant to overcome abiotic stress.

MATERIALS AND METHODS

Drought stress treatment and cDNA pool synthesis:

Aloe vera plantlets were grown aseptically on MS media containing 100µg/µl ABA(S3), for 48 hours; control *Aloe vera* plantlets were grown on MS media(S2) and Pots which was watered daily(S1) for 48 hours. One gram of each tissue was used to extract RNA using Trizol. RNA was subjected to AGE to check for the integrity of the RNA bands and quantity was estimated using UV spectophotometer. Minus RT was performed with RNA to check for gDNA contamination. cDNA pool was synthesized using 1µg RNA of respective samples. PCR was then performed using the cDNA pool using Gene Specific Primers for HVA22.

RT PCR and Confirmation of HVA22 homolog by Sequencing:

Gene specific primers were designed using TB2/ DP1 domain of *Hordeum vulgare abscisic acid and stress inducible (A22) gene* sequence (GenBank ACC ID: L19119.1). Primers targeted 7bp to 26bp upstream and 353-371bp downstream of TB2_DP1 domain, which are FP: 5'AAATCATGGGCGCTCCTC AC 3' and RP: 5' CAGCCTCGGCCTTGA GTATG 3' respectively. Total length of targeted region was 364bp long. Primers were synthesized and procured from Sigma Aldrich. S1, S2 & S3 cDNA pools were subjected to RT PCT with the above primers. The amplicon obtained was sequenced and analysed using different bioinformatics softwares.

Cloning of *Aloe vera HVA22* homolog into pCAMBIA 2300 vector:

Aloe vera HVA22 homolog (~368bp) was gel purified and cloned into a cloning vector (pBlueScript II) at *EcoRI* and *HindIII* restriction sites. Probable clones were screened by colony PCR & further confirmed by restriction digestion. Positive clone was sequenced confirmed. The pBS II+368bp clone was restriction digested (EcoRI/HindIII); released fragment was then cloned into pCAMBIA 2300 (EcoRI/HindIII). Probable clones were screened by PCR & confirmed by restriction digestion.

Transformation of *Agrobacterium tumefaciens* with pCAMBIA+ Insert

Agrobacterium tumefaciens MTCC 431 was procured from MTCC, Chandigarh. It was subjected to Heat Shock- Cold Shock treatment to transform the bacteria with pCAMBIA+ Insert. Purified (pCAMBIA2300 +HVA22) Vector at a concentration of 0.1 to 1µg/µl was prepared. A culture tube with 2ml liquid growth medium (no antibiotics) was kept ready. Frozen competent cells (prepared using $CaCl_2$) from freezer was retrieved and placed on ice. 1µg (pCAMBIA2300+HVA22) Vector DNA was added to the cells before thawing. The cell/DNA mix was frozen by lowering the tube into ICE+ Methanol for about 5 min. Immediately plunge the frozen cell/DNA mixture in 37^{o}C water bath for 5 min. The previous two steps were repeated twice. The cells are transferred to the culture tube containing the 2ml of growth medium and incubate with shaking at 25-28^{o}C for 2-4h. The cells are pelleted by spinning for 2 min at high speed microcentrifuge. The cells are resuspended in 0.1- 1ml liquid growth medium containing Kanamycin, which is to be used in selection of the plasmid. 100- 300µl cells were plated on YEB solid media+ Kanamycin, for selection of transformants. The transformed *Agrobacterium tumefaciens* will henceforth be called AtpCHVA22 for further reference. Confirmation of transformation of *Agrobacterium tumefaciens* with pCAMBIA +HVA22 insert was done in different stages: 1. Growth of transformants on YEB+ Kan (35µg/ml) Plates 2. Colony characteristics and Microscopic observations 3. Plasmid extraction and analysis by AGE 4. PCR of the plasmid extracted with GSP, M13 and 16S Primers.

Callus induction in *Ocimum basilicum*:

The stem of *Ocimum basilicum* containing nodes was selected as explants for callus induction. After surface sterilization, the explant was placed on sterile Murashige and Skoog solid media containing 3mg/lt 2,4- D in PTC tubes. Callus obtained was subcultured subsequently.

Vir gene induction in AtpCHVA22:

0.2ml of AtpCHVA22 culture was inoculated in 5ml of LB+ Kan (35µg/ml) media and incubated on shaker at RT for 24 hours. 0.5ml of AtpCHVA22 grown media was diluted in 50ml of AB media (20X AB buffer + 20X AB salts combined with sucrose water) + 35µg/ml Kanamycin and was incubated on shaker at RT overnight or till OD reaches 0.8-1.0 at 600nm. The cells are pelleted by centrifugation and resuspended in double the volume of induction medium (1X AB salts, 2mM Phosphate buffer, 50mM MES, 0.5% glucose and 100µM acetosyringone, pH 5.6) and incubated on shaker for 14- 24 hours. The cells were pelleted after incubation and re suspended in sterile distilled water depending on whether callus or leaf is used as explant.

Agrobacterium mediated transformation of *Ocimum basilicum* leaf.

The leaf explants were surface sterilized and incubated on sterile MS + BA(Butyric acid) (0.5µg/ml) plates for 24 hours. The explants were then cut at 2-3 places and exposed to vir induced AtpCHVA22 for 30 minutes in PTC bottle. Simultaneously, control explants were treated similarly but exposed to sterile D/W. The cuts were made in order to provide attachment for the bacteria. The test and control explants were rubbed dry on MS plates to get rid of excess bacterial suspension. They were then placed on MS+BA in the dark to encourage growth and infection of AtpCHVA22. The explants are transferred to selection media [MS+ Kan (35µg/ml)+ Cef (100µg/ml) + BA (0.5µg/ml)] after three days to kill AtpCHVA22 and encourage shoot regeneration.

Agrobacterium mediated transformation of *Ocimum basilicum* callus

50µl of 18 hour old culture of AtpCHVA22 was aliquoted into 25ml YEB + 200µM Acetosyringone and allowed it to grow on shaker at RT for 18 hours. The bacterial pellet was resuspended in C1 media (Callus Induction Media containing 10% Tween 20, 3mg/lt 2,4-D and 200µM Acetosyringone) along with 10-20 pieces of fresh calli for 5 minutes. The calli was blotted dry on sterile filter paper and was cultivated in C2 media (Co- cultivation solid media containing 3mg/lt 2,4-D and 200µM Acetosyringone) at RT for 3 days in the dark. Following this, the calli were subcultured on C3 (CIM containing 3mg/lt 2,4- D and 100µg/ml cefotaxime) for one week. The calli is then transferred to C4 (contains additionally 35µg/ ml Kanamycin) for selection of transformants. The transformants are finally transferred to C5 (which in addition has 0.5µg/ml Butyric acid)

RESULTS

RNA extraction and RT PCR of HVA22 primers with ABA stressed *Aloe vera* cDNA pool:

10 µl of RNA extracted from the four samples was subjected to Agarose Gel Electrophoresis. The following band pattern was observed.

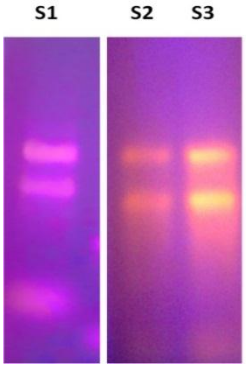

Fig. 1: RNA extracted by Trizol from S1, S2, S3 and Control as observed by AGE

RT PCR with HVA22 primers and the cDNA pools generated from the above samples gave an amplification at approximately 350bp long as seen on AGE. 'C' is the positive control of PCR reaction with HVA22 primers and Barley cDNA pool.

Fig. 2: Amplification of 350bp as observed after RT-PCR with HVA22 primers and cDNA pool of the RNA extracted from S1, S2, S3 and Control

Molecular weight Ladder from top (5Kb, 2Kb, 800bp, 400bp, 100bp). The PCR product **S3** was subjected Purification and Sequencing. From Sequencing, a 330bp long sequence information was obtained which was subjected to Bioinformatics analysis, whose results were as follows: BLASTn [13] showed 100% identity and 100% Query coverage with the original HVA22 coding gene of *Hordeum vulgare*, L19119. Other hits showed 98-95% identity with the HVA22 homologs from other monocots like *Triticum aestivum, Oryza sativa, Zea mays, Aegilops tauschii, Brachypodium distachyon* etc. BLASTx[13] also

showed results similar to that of BLASTn[13] and additionally it showed the presence of TB2/DP1 domain, which is the conserved domain of all HVA22 homologs. CDD[12] confirmed the presence of the above mentioned domain and also showed the length of the same.

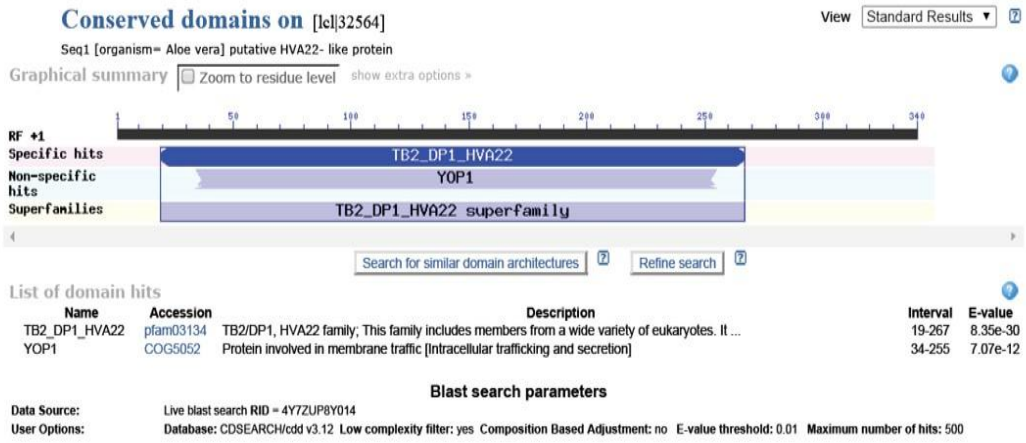

Fig. 3: Conserved domains on HVA22 homolog isolated from *Aloe vera* cDNA as observed by CDD Search Software on NCBI

This sequence was then submitted to NCBI via GenBank's Bankit Submission tool and is now available on NCBI, EMBL and DDBJ under the accession ID KP076225. The Sequence shows an ORF from +18 to +329, a reading frame of +3. The translated sequence shows complete protein product as analysed by TranSeq, BLASTp, ProteinPredict and Swiss Model. Thus, it was found to be a potential coding DNA which could be further used for cloning into Plant expression vectors.

Cloning of *Aloe vera* HVA22 homolog into pCAMBIA 2300

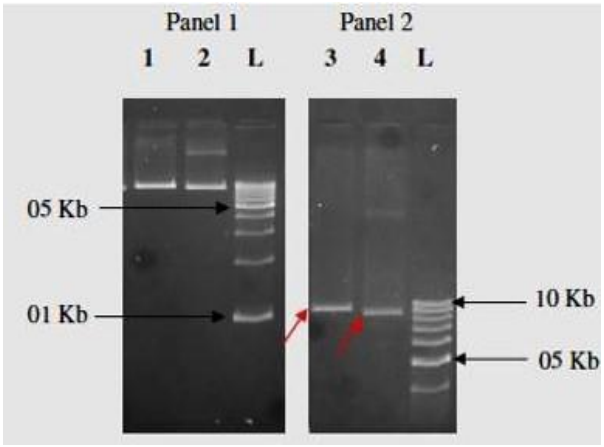

Fig. 4: Restriction digestion (BamH1) of pCAMBIA 2300+ 368bp clone

Lane Description:

1 – pCAMBIA 2300+368bp Clone digested with BamHI (Panel 1 – Short gel run)

2 – pCAMBIA 2300 plasmid digested with BamHI (Panel 1 – Short gel run)

3 – pCAMBIA 2300+368bp Clone digested with BamHI (Panel 2– after long run)

4 - pCAMBIA 2300 plasmid digested with BamHI (Panel 2– after long run)

L – 1Kb DNA Ladder

Bam HI site is present in the MCS of pCAMBIA-2300, and hence the plasmid results in an **8742bp** linear fragment on digestion with the same. Whereas, on cloning

at EcoRI & Hind III sites in pCAMBIA-2300, the Bam HI site is lost. But as Bam HI is present in 368bp fragment, the clone will be linearized to a size of **9110bp**. Hence, the difference of 368bp can be seen on gel, confirming the clone

Transformation of *Agrobacterium tumefaciens* with pCAMBIA+ Insert

Confirmation of transformation of *Agrobacterium tumefaciens* with pCAMBIA+HVA22 insert was done in different stages:

- Growth of transformants on YEB+ Kan (35µg/ml) Plates: Yellow colored, mucoid, circular and greater than 1mm colonies were observed on the above plates after 48 hrs at RT. Gram staining revealed Gram negative thin long rods.
- Plasmid extraction and analysis by AGE: The plasmid was extracted as per the protocol mentioned in the methodology section. The resuspended plasmid was subjected to AGE with 1.5% Gel. The following pattern of plasmid was observed:

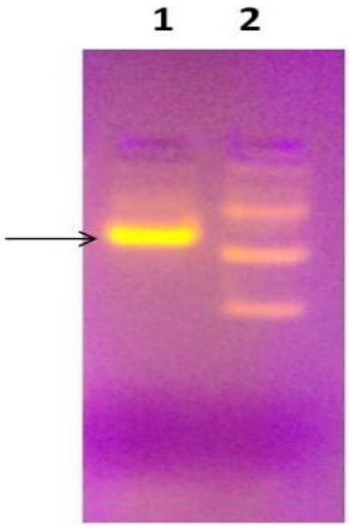

Figure 6: **PCR of AtpCHVA22: Lane 1: GSP/ HVA22 primers gave an amplification of 390bp, Lane 2: M13 gave 500bp amplification and Lane 3: 16S gave 1800bbp amplification, Lane 4: Low range DNA ladder (From well- 5Kb, 2Kb, 0.8Kb, 0.4Kb and 0.1Kb)**

***Agrobacterium* mediated transformation of *Ocimum basilicum* leaf and callus**

Agrobacterium mediated gene transfer in leaf was performed 4 times. Each time 10 explants were used for transformation. Browning, shoot regeneration and callus induction in the dark was observed only in control leaves (treated with sterile water) but not with the test (treated with AtpCHVA22). Although no shoot or callus formation was observed in Test, the explants remained fresh for a longer time on the selection media as compared to the control. To confirm transformation of explants/ transgenic explants, genomic DNA was extracted from T4, C4 and an *Ocimum basilicum* callus which was later subjected to PCR with M13 primers. A single high molecular weight band was observed very close to the well. The three samples were subjected to PCR with M13 primers at 60^{o}C Annealing temperature. M13 primers gives 500bp amplification with the pCAMBIA+HVA22 insert. A single band at 500 bp was observed with T4 explant gDNA PCR and no amplification was observed with C4 or callus gDNA PCR. Amplification indicates that the vector may have been integrated into the genome of the T4 leaf explant. However, no growth was further observed with the explant.

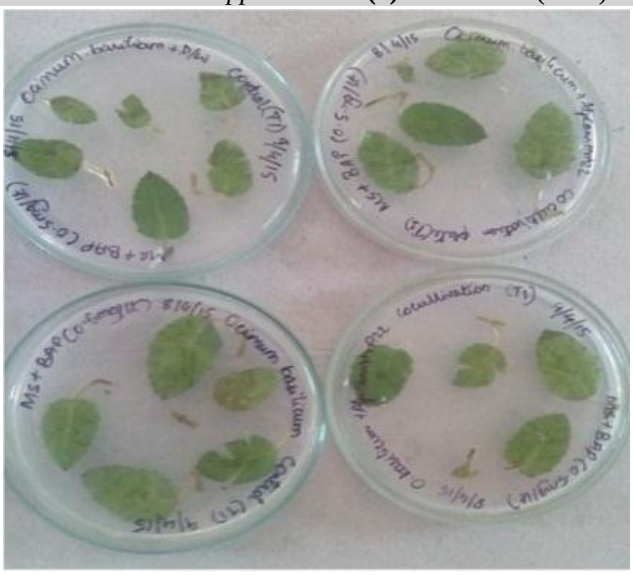

Fig. 7: Leaf explants inculatied on MS + BA media for cocoultivation wit AtpCHVA22

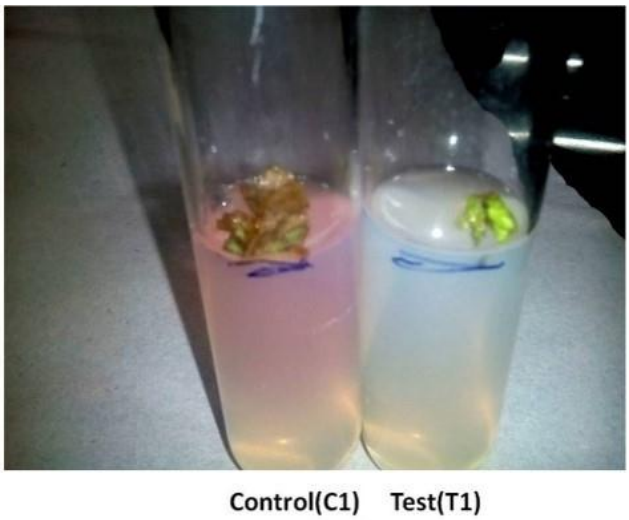

Fig. 8: Another control (C1) and Test (T1) explant transfeered on the selection media.

Browning was observed after 5 days of incubation in C1 whereas the T1 was still fresh.

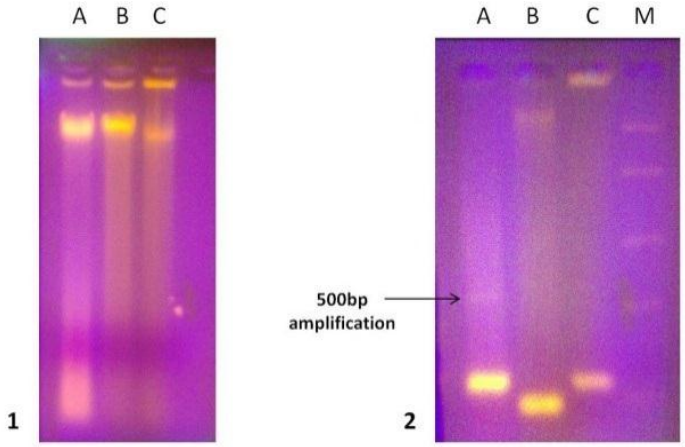

Figure 9: **1. Lane A: Genomic DNA extracted from T4(Transformed *Ocimum basilicum* leaves); Lane B: Genomic DNA extracted from C4 (*Ocimum basilicum* Callus) and Lane C: Genomic DNA extracted from untransformed *Ocimum* callus; 2. PCR performed with genomic DNA samples and M13 primers. Lane A: T4 showing amplication at 500bp position; Lane B: C4; Lane C: Callus genomic DNA showing no amplification; Lane M: Low molecular weight DNA ladder from well – 5Kb, 2Kb, 800bp, 400bp and 100bp**

Agrobacterium mediated gene transfer in callus of *Ocimum basilicum* was also performed 3 times, with 7-8 callus explants in each set. There was no growth observed in Test and Control at any stage of transgenic protocol. Browning was observed in both the cases. To confirm any positive transgenics, genomic DNA of the callus was extracted by Doyle and Doyle modified protocol. The extract showed degraded DNA when observed on AGE and hence could not be subjected to PCR with M13 primers.

A B

Fig. 10: A. *Ocimum basilicum* callus CT1 subjected to Agrobacterium mediated transformation using AtHVA22; B. *Ocimum basilicum* callus CC1 subjected to the same transformation protocol using sterile water

DISCUSSION

HVA22 is known to be most conserved stress inducible gene across all kingdoms except Prokaryotes [7]. Bioinformatics analysis of the amplification product obtained using GSP, from *Aloe vera* cDNA confirmed the presence of the same in *Aloe vera*. The cloned vector was transformed into *Agrobacterium tumefaciens* MTCC 431 and the presence of the vector was confirmed at various stages in the organism. *Ocimum basilicum* proved to be a good model to be used as transgenic plant system as callus could be induced faster and regeneration of plants was also quick. The Agrobacterium mediated leaf transformation showed positive transformants at the selection stage, whereas the Control showed no amplification with the same set of primers, viz, M13 (Primers flaking the HVA22 on pCHVA22). From this results it may be concluded that Vir gene in AtpCHVA22 was induced efficiently using Acetosyringone, and that it infected the T4 leaf samples. Upon infection, the T- DNA regions present on pCHVA22 (containing the HVA22 gene sequence, M13 sequence and Kan^R sequence) was excised and transferred to the plant cell, in which this T- DNA probably got integrated into the plant genome. However, no growth was further observed in Test but was observed in Control. This could further be standardized with respect to growth conditions of the leaf. The explant may be releasing harmful elicitors or phenolics which may be affecting the growth of the explant. As the control plant is not subjected to any kind of elicitation or infection, it may be the reason to have picked up growth after 2-3 subcultures.

The *Agrobacterium* mediated transformation of callus was performed till the selection stage where the control and treated callus was grown on MS+ Kan+ Cef+ 2,4-D media. Browning was observed on all stages and no growth was observed, in Treated nor in Control. The calli were tried growing on plate and in PTC tubes, but no difference in growth was observed. Transgenic *Ocimum basilicum* were used in many cases to study introduction of foreign genes into the system. After standardization of regeneration of transgenic Basil leaves for root and shoot production, the plantlets can be then used for effect of stress

studies. Simultaneously, expression and protein profile of the transgenic plants can be performed to detect the active transcription and translation of the transgene HVA22.

Future Prospects:

Other plant models may be used to generate transgenic lines with AtpCHVA22. Stress studies can be conducted with the transgenic lines in lab and greenhouse conditions.

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