J. Agric. Sci. Mansoura Univ., 33 (6): 4631 - 4647, 2008

CLONING OF NEW *AF-PRO* PROMOTER FROM KIDNEY BEAN (*Phaseolus vulgaris*, L.) AND DETERMINATION OF THE ACTIVITY IN STABLY TRANSFORMED TOBACCO (*Nicotiana tabacum var xanthi)* PLANTS

Mahmoud, Eman A.[1]; M. Solliman[1]; H. Elshemy[2]; A. Aboul-Enein[2]; M.A. Aboelsaoud[2] and S. A. Ghanem[1]

[1] **Plant Biotechnology Dept., National Research Centre, Dokki ,Cairo, 12622, Egypt.**

[2] **Biochemistry Dept., Faculty of Agriculture, Cairo University, Egypt.**

ABSTRACT

The aim of this study was to identify the highest expressing antifungal gene (*AF*) promoters (*AF-PRO*) from the tissue of germinated seedlings of kidney bean (*Phaseolus vulgaris*, L.), to drive high-level and preferably stage-specific transgenic protein expression in tobacco plants. New *AF-PRO* promoter was cloned by using several approaches, including the construction of a cDNA library and use of genome walking technique. PCR analysis of *AF-PRO* mRNA expression clearly showed that these promoters exhibited the highest activity at the germination stage of plant development. GusA expression was used as a reporter of promoter activity in tobacco (*Nicotiana tabacum var. xanthi)* plants that were transformed with the constructs using an Agrobacterium-mediated transformation strategy. Transformed tobacco seedlings expressing B-glucuronidase under control of the AF-Pro promoter region (AFb-Pro promoter from bean) showed stress specific responsive activity that depended on the type and concentration of the stress and the type of infection. These findings further the understanding of the regulation of AFb-Pro expression and provide a new stress-inducible promoter system in transgenic plant.

The mRNA level of *gusA* was quantified in transformed plants. The obtained data demonstrated that the promoter most active in seedlings under native conditions was also most active in transgenic constructs at the same stage of plant development. Plant promoter architecture is important for understanding regulation and evolution of the promoters. Several promoter elements including TATA box, and several types of transcriptional regulatory elements have been found to show local distribution within promoters, and this feature has been successfully utilized for extraction of promoter constituents from plant genome.

Keywords : promoter isolation- cloning - kidney beans- defensin gene promoter.

INTRODUCTION

Genetic transformation is a powerful tool for production of crop plants with increased resistance to phytopathogens. A number of transgenic cultivars with elevated tolerance to economically important pests and disease agents are in commercial production. However, in most of them the transgene is driven by a powerful constitutive promoter, such as the cauliflower mosaic virus *35S* (*CaMV 35S*) and its derivatives, and is expressed at high levels even in the absence of pathogen invasion. Continuous synthesis and high accumulation of transgene products, especially toxins, could interfere with plant metabolic pathways and the overall expression of other valuable traits. In contrast, the use of promoters of plant defensive genes has distinct advantages because most of them are

activated only when the plant is attacked by pests or pathogens. The use of native plant promoters can also help to avoid transgene silencing often associated with the presence of promoters of non-plant origin in the plant genome (Yevtushenko *et al.,* 2004; Vieweg *et al.*, 2004; Nishiuchi *et al.,* 2004 ; Ross *et al.*, 2004; Matarasso *et al.,* 2005 and Rubio-Somoza, *et al.*, 2006).

Plants have developed a variety of physical and biochemical defense barriers against pests and pathogens. Mechanical wounding of plant tissue (mimicking pathogen invasion or insect chewing) leads to the accumulation of mRNAs that encode proteins thought to be involved in plant defense (Yevtushenko *et al.,* 2004 and Bowles, 1990), and provides a convenient system to isolate and study defense-related genes and their upstream regulatory regions in transgenic host (Clarke *et al.*, 1994 and Hollick *and Gorden,* 1993). Potato plants can be engineered for broad-spectrum disease resistance by expression of antimicrobial peptides under control of a constitutive *CaMV 35S* promoter(Osusky *et al.*, 2000).

It has previously been shown that certain promoters are able to direct RNA synthesis at a higher rate than others. Certain promoters have also been shown to direct mRNA production at higher levels only in particular types of cells and tissues. Those promoters that direct mRNA production in many or all tissues of a plant are called constitutive promoters. Previous work had shown that the 35S promoter from the cauliflower mosaic virus (CaMV35S) is a strong constitutive promoter in plants (Odell *et al.*, 1985; Jensen *et al.*, 1986; Jefferson *et al.*, 1987 and Sanders *et al.*, 1987). This had been shown by demonstrating measurable levels of reporter gene proteins or mRNAs in extracts prepared from the leaves, stems, roots and flowers of transgenic plants. As a result, the CaMV35S promoter has been widely used by scientists in the field of plant genetic engineering.

In recent years, concerns over genetic modification issues have resulted in regulatory authorities requiring comprehensive analysis of transgene insertion events in the plants that are to be commercialized. Recent studies have suggested that applied and modified a genomic walking method that combines vectorette and suppression PCR walking. Some studies have suggested that stable expressed transgenes comprise relatively simple T-DNA arrangements flanked on at least one side by plant DNA and that unstably expressed loci tend to be composed of multiple T-DNA copies (Iglesias *et al.*, 1997). Several PCR-based walking methods are available (Ochman *et al.*, 1988; Rosenthal and Jones, 1990; Riley *et al.*, 1990; Lagerstrom *et al.*, 1991; Parker *et al.*, 1991; Trueba and Johnson 1996 and Jones and Winistorfer 1993). However, these methods generally have not been applied in determining T-DNA insertion sites because they are too inefficient and/or complicated.

Conventional PCR allows the amplification of sequences within known boundaries. Several methods have been developed for the amplification of DNA sequences that flank regions of known sequences. These include TGW-PCR (targeted gene walking PCR) (Parker *et al.*, 1991) UP-PCR (unpredictably primed PCR) (Dominguez and Lopez-Larrea 1994) and Inverse PCR (Triglia *et al.*, 1988; Ochman *et al.*, 1988; Silver and Keerikatte 1989). Inverse PCR allows the amplification of sequences that lie outside the

boundaries of known sequences by inverting the unknown sequence. This is done by self-ligating digested DNA and opening the circular DNA molecules at a different site.

The genes which encode the various components of the pre-mRNA splicing or rRNA processing machinery provide a rich source of promoters for transgene expression in plant biotechnology. As most genes are organised in multigene families with great variability in expression levels and patterns, a novel approach has been developed to allow the identification and isolation of promoters with the required expression characteristics. This approach will prove valuable for future promoter isolation and exploitation (Sunter *et al.*, 2003 and Van *et al.*, 2002)

MATERIAL AND METHODS

Plant material and growth conditions

Seeds of kidney bean were grown at 25±2^{0}C for 7 days in dark and grown on MS medium .The harvested material were immediately frozen in liquid N_2 and stored at -70^{0}C. For plant transformation studies, tobacco was grown at 25±2^{0}C in 16 hrs light and 8 hrs. dark in the tissue culture conditions.

Isolation of plant genomic DNA

Genomic DNA was isolated on a mini-prep scale as mentioned by Murray and Thompson (1980) with some modifications.

Isolation of total RNA

Total RNA from plant tissues were isolated as mentioned by Chomczynski and Sacchi (1987) with some modifications.

The target PCR product was cleaned up by Gel extraction kit (Promega) and cloned into the pGEMT vector kit (Promega), then transformed into *E. coli* DH5α. Positive clones were confirmed by restriction analysis and sequencing.

Preparation of competent cells and transformation

DH5α Competent cells were made by the protocol of Hanahan (1985).

Spectrophotometric estimation of nucleic acids

The quantity and quality of the nucleic acid was determined by measuring the absorbance at 260 nm and 280 nm. For DNA the absorbance was calculated taking A_{260}=50 µg/ ml and for RNA its A_{260}=40 µg/ ml. The purity of the nucleic acid was determined by calculating the ratio of A_{260}/ A_{280} for each sample

Isolation of plasmid DNA

This method was adopted from Sambrook *et al.* (1989).

Purification of DNA fragment from agarose gel

After restriction enzyme digestion of plasmid, the digested product was electrophoresed on an agarose gel. By using standard molecular wt. marker the desired fragment was identified, cut and purified by one of the following methods:By Qiaquick gel extraction protocol (from Qiagen GmbH).

Polymerase chain reaction

Taq-polymerase, dNTPs (deoxynucleotide triphosphate) and convergent primers achieved amplification of the DNA fragment. The reaction conditions for PCR involved denaturation at 94^{0}C for 30 seconds, annealing at 52^{0}C for

30 seconds and extension at 72°C for either 30 seconds (for AF promoter amplification) or 2 minutes (for AF-PRO + GUS fusion). After 30 cycle of amplification an aliquot of this reaction mix. was loaded onto a 0.8 % agarose gel and checked.

Analysis of nucleotide sequence

Sequence analysis was performed using software from University of Wisconsin, Genetic computer Group (UWGCG) sequence analysis software packag, PC/GENE (Intelligenetics), and BLAST (Hobohm and Sander, 1995; and Stultz *et al.*, 1993).Constructions of plant transformation vectors.

1-pBI121-AF-promoter

Plasmid vector pBI121 was used for cloning AF-pro 1 and Ca pro 1+2 promoter in front of Gus gene Fig.1. These constructs were then utilized for tobacco transformation. To generate the pBI121- AFb-pro construct, containing the 300 and 600 pb length from ca promoter which cloned in pGEM-T first by using PCR , was cloned in plasmid pBI121.

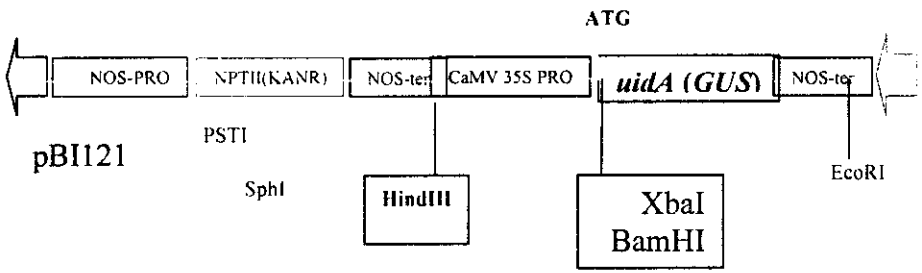

Fig. 1: pBI121 plasmid map and positions of restriction sites

Primer contain BamH1 and HindIII site for digested and clone in pBI121 was digested with BamH1 and HindIII also . T_4- DNA ligase. Ligation was done overnight 14°C and then transformed to DH5α competent cells.

2-pBI121-Gus-AF-Promoter

Transformation of Agrobacterium tumefacien

Agrobacterium tumefaceins (LBA 4404) cells were transformed with recombinant pBI 121 sense or -antisense clones by freeze thaw method. A single colony of Agrobacterium tumefacien (LBA 4404) was inoculated to YEM (0.04 % yeast extract, 10 % mannitol, 0.01 % NaCl, 0.02 % $MgSO_4.7H_2O$ and 0.05 % K_2HPO_4 at 28°C for vigorous shaking for two days. Competent cells of Agrobacterium were prepared by inoculating 1 ml of a fully-grown culture in fresh 4 ml YEM medium and grown at 28°C till an OD of 0.5-0.6 was reached. The cells were harvested by centrifugation at 3000 x g for 10 minutes at 4°C.The pellet was resuspended in 1ml of 20 mM $CaCl_2$ (chilled). Aliquot's of 0.1 ml were made and frozen at –80°C. Transformation of Agrobacterium with sense and antisense pBI 121 was done by adding 1µg of recombinant plasmid to 0.1 ml of Agrobacterium competent cells, mixed gently and immediately frozen in liquid N_2. Thereafter incubating the

Eppendorf tubes at 37°C for 5 minute to thaw the cells. Following this 1 ml of YEM was added and the Eppendorf tubes were incubated at 28° C for 6 hours with slow shaking. The revived cells were plated on YEM-agar plate containing 50 µg/ml Kanamycin and 25 µg/ml Rifampicin and incubated at 28°C. Transformed colonies appeared after 2-3 days and were further analyzed either by PCR or colony hybridization.

Agrobacterium mediated plant transformation

Tobacco transformation was done according to Horsch *et al.* (1985), with little modification. Tobacco seeds were surface sterilized by washing with 1 % bleach and 0.1 % Tween-20 for 5-10 minutes and then with 70 % ethanol for 1-2 minute. Washing with sterile water (9-10 times) followed this. Seeds were plated on MS-Basal medium [3.44 gm MS-salt (Sigma, USA), 3 % sucrose, and 1 x Gamborg B5-vitamins (Sigma, USA)]. Leaf discs of uniform size were made using a cork-borer and then immersed in a 1: 30 diluted fully grown Agrobacterium culture in MS-Basal liquid (without agar). This was incubated for 5-10 minutes and then leaf discs were blot dried and placed on MS-Basal medium. These explants were allowed to grow (co-cultivate) with Agrobacterium for 3-4 days.

Selection, regeneration and growth of transgenic plants

After co-cultivation steps, the explants were briefly rinsed in MS-basal solution containing 500 µg/ ml Carbenicilline blot dried and plated onto MS-basal-agar plates containing 300 µg/ ml Kanamycin and 500 µg/ ml carbenicillin, and 1 mg/ liter BAP and 0.1 mg/ liter NAA. After three to four week, several shoots were separated from regenerating explants and placed in separate jar bottles for rooting in MS-basal-agar medium. Once the plants hardened, they were transferred to potted soil and then to glass house for further growth .

GUS hstochemical assay:

This method for screening the transgenic plants was done according to (Jefferson, 1987) and allowed for the verification of the expression of *uid A* gene in transgenic plants. Leaf tissue from wild type and transgenic plants was collected and rinsed in 50 mM Na-phosphate buffer (pH 7.0). Then the tissue was stained with 2 mM 5-bromo-4-chloro-3-indolyl glucuronide (X-gal from Biosynth. Inc) in 50 mM of Na-phosphate buffer (pH 7.0), followed by brief vacuum infiltration and placed at 37°C for overnight in dark. After staining, tissues were rinsed extensively in ethanol to remove chlorophyll before examination.

GUS spectrophotometric assay:

For GUS spectrophotometric analysis, frozen tissue was ground in liquid nitrogen and was suspended in 600 µl of GUS extraction buffer containing 50 mM $NaPO_4$, pH 7.0, 10 mM βME, 10 mM Na_2EDTA, 0.1 % sodium lauryl sarcosine (w/v), and 0.1 % Triton X -100 (w/v). This suspension was sonicated twice. The extract was clarified by treatment with insoluble PVP and centrifuged at 10,000 x g for 20 minutes at 4°C. GUS activity was determined by fluorimetric assay as described by Jefferson (1987), in which 4-methyl umbelliferone glucouronide (MUG) was used as a substrate. Total proteins were measured using the Bradford's solution (Bradford, 1976) and GUS specific activity was recorded as picomoles of 4-methylumbelliferone (4-

MU) formed per minute per milligram of protein from the initial velocity of the reaction (Jefferson *et al.*, 1987 and allan *et al.*, 1993).

Analysis of nucleotide sequence

Sequence analysis was performed using software from University of Wisconsin, Genetic computer Group (UWGCG) sequence analysis software package (Deverexu *et al.*, 1984), PC/GENE (Intelligenetics), and BLAST (Hobohm and Sander 1995 and Stultz *et al.*, 1993).

RESULTS AND DISCUSSION

Identification and cloning of novel plant promoter

Plant promoter architecture is important for understanding regulation and evolution of the promoters, but the current knowledge about plant promoter structure, especially with respect to the core promoter, is insufficient. Several promoter elements including TATA box, and several types of transcriptional regulatory elements have been found to show local distribution within promoters, and this feature has been successfully utilized for extraction of promoter constituents from plant genome.Promoters of various strengths and specificities are required for expression of foreign genes in plants for analysis of gene function or for biotechnological improvement of crop species. To meet such requirements and to have maximum precision in expression, a range of promoters with different expression levels and patterns is desired. Keeping this in mind, the present program was begun with isolation of plant promoters and characterize them for their suitability to express foreign genes using tobacco as a model system. One of the promoter (AFb-PRO) isolated was for a gene that code for an antifungal protein from beans.

Cloning of AF-PRO gene promoter

The isolation of unknown DNA sequences flanking known regions is critical for gene expression analysis. Several protocols have been developed to isolate an unknown DNA sequence (promoter) adjacent to DNA fragments of known sequence (cDNA) by PCR (Hui *et al.*, 1998). A number of modifications were developed to isolate the unknown 5' and 3' flanking regions of the DNA. Usually, PCR was carried out with restriction enzyme(s)-digested genomic DNA fragments after ligation (Siebert *et al.*, 1995) or cloning into a vector (Niu and Fallon, 1999) or ligated to double-stranded, partially double-stranded (Iwahana *et al.*, 1994 and Willems, 1998), or single-stranded oligonucleotide cassettes (Kilstrup and Kristiansen 2000). In these cases the amplifications were carried out with locus-specific primer(s) and a vector/oligonucleotide cassette specific-primer to amplify a fragment contiguous to the known sequence. In this method all the template molecules are likely to be amplified linearly leading to the generation of a lot of noise. In this study we have followed a protocol developed by Reddy *et al.*, (2002) that involved the use of restriction digestion of genomic DNA followed by partial fill that prevent self ligation between fragments. In addition, the present protocol also utilized the use of biotinilated primers that enrich specific template prior to nested PCR. Also, one of the adopter strands was blocked by at the 3' end by attaching an amine group. Following this method a number of unknown 3' and 5' regions of AF-PRO gene promoter were isolated Figs. 2, 3 and 4.

A 730 bp fragment that corresponds to the 5′ upstream region of AFb-PRO genes from bean, were isolated following this new method. The overlapping 60 and 70 nucleotides showed a 50 and 60 bp difference from the AFb-PRO cDNA 5′ UTR. The major transcript was found to initiate from "C", 270 bases upstream of the translation initiation site. The minor transcript was found to initiate from "c", 150 nucleotides upstream of the translation initiation site.

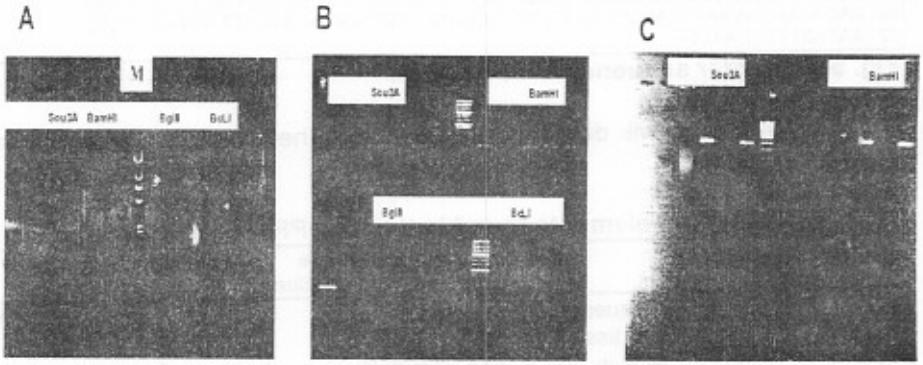

Fig.2. Isolation of AFb-PRO promoter.

(A) Genomic DNA was digested partially with Sou3A,BamHI ,BglII and BclI, end-filled and ligated with specific adapter-primer.

(B) In the Primary PCR amplification the adapter ligated genomic DNA was amplified with T7 and AFb R1 gene as reverse specific primers. The amplified products were run on 0.8% agrose gel.

(C) Secondary amplification by using various dilutions of the Primary PCR products using T7 and second gene specific AFb R2 primer.

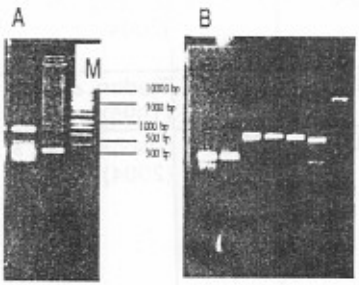

Fig.3. (A)-PCR confirmation of AF gene from beans. (B)-AFb –PRO Promoter fragments of after PCR amplification using different primers before cloned into pGEMT vector.

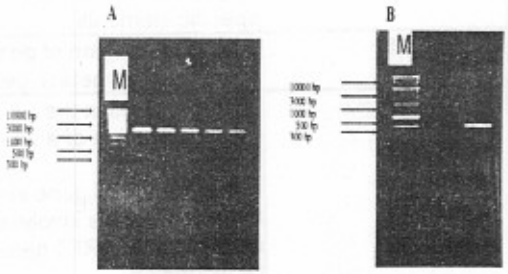

Fig.4. Photograph of a gel showing PCR products for the full-length 730 bp of AFb-PRO from beans (A), Photograph of a gel showing the purification of the PCR products from before going to pGEMT cloning and sequences.

A Similar result has been obtained by Reddy *et al.* (1999), in which they isolated genomic DNA fragments from tobacco and pea containing promoter region of DNA topoisomerase I (*top I*) using similar PCR based 5' genomic walk. In case of pea they isolated 1140 bp and in tobacco a 482 bp upstream of ATG -5' flanking region of tobacco topoisomerase I using PCR-RACE based approach. We have also shown a putative promoter region, which is present in various plant regulatory motifs Fig. 5 and Table 1.

```
1 CTTTAGAATA AATAAAAATTTCAAATCCAA ATATCTGTTATTATACTC TAAATACTAA
61 AAAATATATG TTAATTATAA ACATTTATTT ATTATTTTCT ACAAAAAAGA CTCTTTAAAA
121 AAGATTAAAA AAATTCCTCTATTTTTTCAT GTGCTCCCCT CCTCCAAACC TCTCCCCTAT
181 CCCTTCCCTA CTTTTGTTTAATTACTCTTT TATATATGAG ATGCATATTA TTGAATTTAT
241 TTTATCGATT ACTTTTGGGA AAATTACATG ATTAATTATC ACAAACAAAT CGAATGGAGA
301 ATTCAAATTG AATTGTTGCA TACTATATTT GTCTGTCTCC ATATATAGCA TTAATTATAT
361 GTTGATAAGC TCCACTCTAG CTTCCACGCC CCACACTATT GACTTTTCAT CATGAAAATA
421 CAAACTTGGG TAATAAAATA TGCTGCCACA TCAATCCGTT TCTTGTGGTT TCTTCTAGCT
481 TCTCAAACTT CATTTTCAGA TTAATAATAA TATTTGTTCA TTTCTCTTAC TAGTCAAGCC
541 CCGGCCAAAA AAGGGAAGGG GAAGAAATCG CTTTTGGCCC TTTTTTTGGG GGGGGGGGAC
601 CCAAAAAAAA AAAATGGCAG CCAAGAGTTT TTTTTCCCTT CCCCTTTTTG TGCCGCCAAA
661 AACACAACAA AATTAATGAT ATGTTTATAT AGTCTCTTTA ATAGATCATT GTTACTGCTG
721 AATACTTTAT AATT
```

Fig. 5. Promoter sequence of AFb –PRO.

The sequence will deposited in Genbank .The cis-acting elements are listed in next table 1.

Table 1: Cis-acting elements found in the AFb-PRO promoter.

Motif	Found in	Site Position	Sequence	Reference
CAATBOX1	Sequences responsible for the tissue specific promoter activity of a pea legumin gene.	452	CAAT	Shirsat, *et al.*, (1989)
GATABOX	GATA motif in CaMV 35S promoter	678	GATA	Rubio-Somoza, *et al.*, (2006)
LECPLEACS2	chlorophyll a/b binding protein the promoter of Petunia	434	TAAAATAT	Matarasso *et al.*, (2005)
OSE2ROOTNODULE	One of the consensus sequence motifs of organ-specific elements	524	CTCTT	Vieweg, *et al.*, (2004)
TATABOX5	5upstream region of pea glutamine synthetase gene	685	TTATTT	Tjaden, *et al.*, (1995)
WBOXNTERF3	"W box" found in the promoter region of a transcriptional repressor ERF3 gene in tobacco; May be involved in activation of ERF3 gene by wounding	400	TGACY	Nishiuchi *et al.*, (2004)
TATABOX2	5upstream region of pea legA gene	237	TATAAAT	Tjaden, *et al.*, (1995)
ARR1AT	ARR1; Response regulator	498	NGATT	Ross et al., (2004)
ROOTMOTIFTAPOX1	Motif found both in promoters of rolD	225	ATATT	Elmayan and Tepfer, (1995)
GT1CONSENSUS	Consensus GT-1 binding site in many light-regulated genes,	251	GRWAAW	Zhou, (1999)
GT1CONSENSUS	GT-1 binding site in many light-regulated genes	429	GRWAAW	Le Gourrierec *et al.*, (1999)

The putative cis-acting elements found in the AF-PRO promoter along with their positions are listed. These elements were identified using the Signal Scan Program at PLACE (http://www.dna.affrc.go.jp)

Database assisted promoter sequence analysis

The sequence homology of the untranslated region (UTR) of the AFb-PRO gene isolated in the present study (Fig. 5 and 6) revealed the existence of differences with the reported sequence.

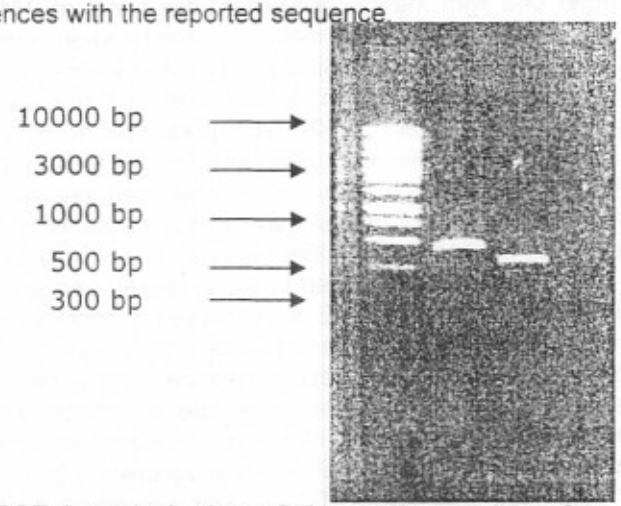

Fig. 6: PCR based cloning of the coding region of an isoform of the defensin gene from beans (lane 1) and chickpea (lane 2) using the full-length primer set.

This could be due to the fact that the AFb-PRO existed in multiple copies and the difference could be due to isolation of promoter for the gene other than the one that was previously reported by (Siva Reddy from ICGEB personal communication).

The analysis of regulatory sequences is greatly facilitated by database assisted bioinformatic approaches. The TRANSFAC database contains information on transcription factors, their origin, functional properties and their sequence-specific binding activities (http://www.sphinx.rug.ac.be: 8080/PLANTCARE/). By employing software tools it is possible to screen the database with a given DNA sequence for interacting transcription factors. If a regulatory function is already attributed to this sequence the database assisted identification of binding sites for proteins or protein classes and subsequent experimental verification may establish functionally relevant sites within this sequence. The binding transcription factors as well as interacting factors may already be present in the database. The putative cis-acting elements present in the AFb-PRO promoter were identified using PLACE (Higo *et al.*, 1999; http://www.dna.affrc.go.jp; Fig. 5 and Table 1).

A search for putative cis -acting elements in the promoter for AFb-PRO revealed the presence of the CAAT Box, TATA Box and CATA Box, light regulatory elements or LREs (CCA1, GATA, GT-1). The cis acting elements are listed in Table 1. Interestingly, the AFb-PRO promoter has a TATA Box

near the 5′ end of the AFb-PRO gene. The first TATA-like elemen detected is at 237 and the second TATA Box was at 685. The presence of CAAT Box1 motifs in the promoter of AFb-PRO correlates with higher expression in leaves as compared to roots. Similar motifs have been reported in the promoter of the *legA* gene of pea (Shirsat *et al.*, 1989).

The presence of light regulatory elements has the consensus GT1 motif binding site in many light-regulated genes (Le Gourrierec *et al.*, 1999). In the AFb-PRO promoter one light regulatory element are seen, at 251 to 258. In the AFb-PRO promoter two GATA Box elements were seen, one at 678 to 682 and the other at –320 to -325. Similar cis-acting elements have been reported in CaMV 35S promoter and in the promoter of Petunia (Benfey and Chua 1990 and Gilmartin *et al.*, 1990). The presence of light regulatory elements and tissue specific elements accounts for the tissue specific light regulation of AFb-PRO. Three putative Dof binding sites are present in the region between 703 to –706. Dof proteins are unique to plants and contain a highly conserved DNA binding domain that binds to a core AAAG sequence. In maize, Dof1 is constitutively expressed in roots, leaves and stem and acts as a transcriptional activator while Dof2 is expressed in roots and stem and acts as a transcriptional repressor (Yanagisawa and Sheen 1998) for light mediated expression of C4 photosynthetic phosphoenolpyruvate carboxylase (C4PEPC). Thus Dof proteins may have tissue specific effects. Dof proteins have also been implicated in regulation of hormonal responses and pathogen attack (Yanagisawa and Schmidt, 1999 and Yanagisawa, 2000). The AFb-PRO promoter sequence also has a "Box II" box sequence at 231 to 234 sites. Similar cis-acting elements have been reported to be present in the tobacco (N.t.) plastid *atpB* gene promoter (Kapoor and Sugiura 1999).

Vector design to test the AFb-PRO promoter activity

In order to test the promoter specificity and the strength, we have used genetic transformation procedures following *Agrobacterium* mediated methods in tobacco (Horsch *et al.*, 1985). The binary vector pBI121 (Clontech, USA) was used for this purpose. The pBI121 is a derivative of pBin19 vector developed to express foreign genes in stably transformed plants (Bevan 1984). The pBI121 contained within the left (LB) and right (RB) border sequences, a selectable *NPTII* marker under the regulation of NOS promoter and NOS terminator. The T-DNA of the binary also had the reporter *uidA* (GUS) gene under the regulation of CaMV 35S promoter and NOS terminator. In the pBI121 vector, multiple cloning sites were present to replace 35S promoter conveniently with any other plant promoter to test. Therefore, we have utilized HindIII and BamHI sites present in the vector to clone our AFb-PRO promoter. Three different constructs were created that differed in their length. The AFb-PRO1 is the smallest promoter that contained only 350 base pairs, the AFb-PRO2 contained 500 base pairs and the longest promoter, AFb-PRO 3 contained 720 bp. All the promoters were cloned into the same HindIII and BamHI sites for a better comparison. As a control, pBI121 having 35S promoter was used Figs. 7 and 8.

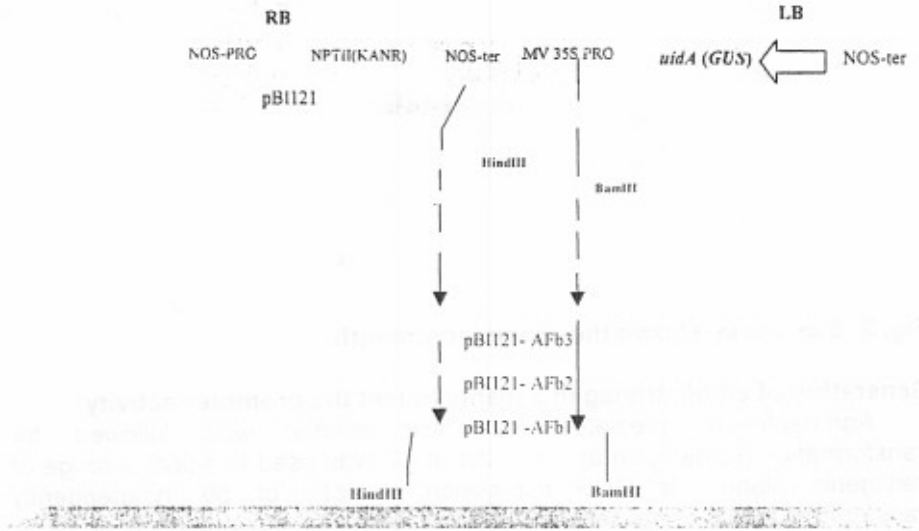

Fig. 7: Strategy used to prepared the pBI121-AF clone. In pBI121, the 35S promoter is replaced by a different fragment of AFb promoter(AF1,AF 1+2,AF 1+3).Restriction endonuclease cleavge site are indicated.

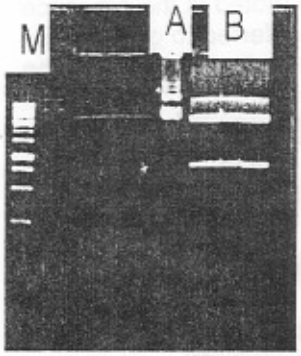

Fig. 8 . Photograph of a gel showing restriction digests of pBI121 plasmid containing the 35S promoter to replace by AFb-PRO using HindIII and Xbal Enzyme (lane A undigested and lane B digested plasmid).

AFb-PRO promoter strength

The CaMV 35S is a strong and constitutive promoter and used most extensively to express foreign genes in plants. Under 35S promoter, GUS expressed in all tissue types and at all developmental stages of tobacco plant growth. The expression was strongest in leaf tissue. The expression of GUS was observed under all three promoters (AFb-PRO). The expression was strongest in the leaf followed by stem and roots. Among the three constructs, AFb-PRO 3 were high followed by AFb-PRO 2. The expression was low under AFb-PRO 1. However, the expression pattern for various tissues was similar in all the constructs. When compared to 35S promoter, the high expressing AFb-PRO3 promoter activity was 20-30% less and may find it's use in transgene expression in crop plants as shown in fig. 9.

Fig. 9: Gus assay shown the promoter strength

Generation of stable transgenic plants to test the promoter activity

Agrobacterium mediated leaf disk method was followed for transformation. Kanamycin at 100 – 200 mg/L was used to select a range of transgenic plants for GUS expression. A total of 50 independently transformed plants were selected and screened for the presence of GUS, AFb-PRO promoter sequences and the GUS expression. A frequency of 5-20 plantlets were obtained per explants, which was comparable to control vector (pBI121) transformation frequency, indicating that the cloning of AFb-PRO promoter had no adverse effects on the overall transformation frequencies. Transformed plants rooted normally and no abonormalities associated with the transformation was observed. Transformed shoots rooted in the presence of 100 mg/L kanamycin and all the plants that were transferred to greenhouse grew normally and set seeds. However, the obtained results referred that the successful transformation in tobacco plants as shown in fig. 10.

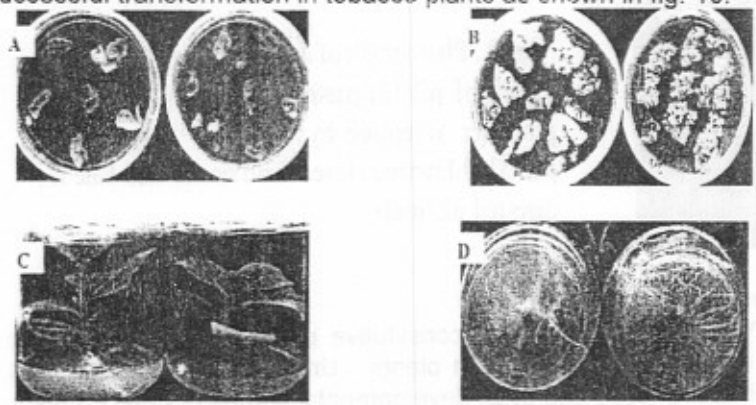

Fig..10. Establishment of the Protocol for Culture Conditions and *Agrobacterium* – Mediated Transformation for Tobacco. (A)-Tobacco explants after infecting the wounded plant tissue with *Agrobacterium* carrying the pBI121. (B)-Transformed cells divide and grow; these are transferred to medium to allow shoots to develop. (C)- Shooting and elongation medium. (D)- Shoots are placed on another medium to promote root development, so that small plantlets are produced.

The identification and isolation of novel specific plant promoters with customer-defined expression profiles is one of the most new applications technology. This is a highly efficient gene expression profile analysis technology for the detection of those genes that are exclusively expressed in tissues or conditions of interest. With our expertise in long-range linker-PCR for the cloning of upstream and downstream elements from a given expressed sequence, we able to identify any gene (known or unknown), and isolate and characterize the corresponding promoter and terminator regions, that match the predefined expression criteria.

The activity of the AFb-Pro gene promoter of bean a cysteine-rich antifungal peptide was investigated in transgenic plants with the *gus* gene as a reporter gene. Quantitative GUS activity analysis of the transgenic plant leaves showed the average activity of the bean AF-Pro gene promoter was 2- to 3-fold higher than that of the CaMV 35S promoter, with maximal expression being 3-fold higher. Beans AFb-Pro gene promoter activity was about 2-fold that of the CaMV 35S promoter. Histochemical GUS staining of the transgenic plants indicated that the bean AFb-Pro gene promoter was active in almost all reproductive organs and the highest level was in the roots.

Functional analysis of promoter 5'-deletion series indicated that promoter activity of a 355 nucleotide fragment (-355 to the transcription initiation site) and a 460 nucleotide fragment (-460 to the transcription initiation site) were 2-fold and 3-fold stronger than that of the full-length AFb-Pro promoter respectively. These results demonstrate that the bean AFb-Pro promoter is a strong inducible and near-constitutive promoter in plants and has great application potential for plant genetic engineering studies. Although the CaMV35S promoter appeared to be a strong, constitutive promoter in assays involving cell extracts, detailed histological analysis of a reporter gene product that is detectable at the cell and tissue level showed a rather high degree of variability of expression of this gene product. This histological analysis revealed an unknown and unexpected variability in the expression of a gene product driven by the CaMV35S promoter. This variable level and site of expression is believed to have two primary causes. The first is that variability is an intrinsic property of the CaMV35S promoter (Rubio-Somoza, *et al.*, 2006; Matarasso *et al*,. 2005; Vieweg, *et al.*, 2004; Nishiuchi *et al.*, 2004; Tjaden, *et al.*, 1995 and Ross *et al.*, 2004).

REFERENCES

Allan, G. C., Hall, G. E., Childs, L. C., Weissinger, A. K., Spiker, S. and Thompson, W. F. (1993). Scaffold attachment regions increase reporter gene expression in stably transformed plant cells. Plant Cell 5: 603-613.

Benfey, P.N., and Chua, N.H. (1990). The cauliflower mosaic virus 35S promoter: combinatorial regulation of transcription in plants. *Science* 250: 959-966.

Bevan, M. W. (1984). Binary Agrobacterium vectors for plant transformation. Nucleic *Acids Res* 12: 8711-8721.

Bowles, D.J. (1990). Defense related proteins in higher plants, Annu. Rev. Biochem. 59: 873–907.

Chomczynski, P. and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem., 162: 156-159

Clarke, H.R.; Davis, J.M;. Wilbert, S.M. Bradshaw H.D. and Gordon, M.P. (1994). Wound-induced and developmental activation of a poplar tree chitinase gene promoter in transgenic tobacco. Plant Mol. Biol.25, 799–815.

Devereux , J., Haeberli, P. and Smithies, O. (1984). A comprehensive set of sequence analysis programs for VAX. Nucleic Acids Res., 12: 38.

Dominguez, O. and Lopez-Larrea, C. (1994). Gene walking by unpredictably primed PCR. Nucleic Acids Res., 22: 3247-3248.

Elmayan T and Tepfer M. (1995). Evaluation in tobacco of the organ specificity and strength of the rol D promoter, domain A of the 35S promoter and the 35S^2 promoter Transgenic Res., 4:388-396.

Gilmartin, P.M., Sarokin, L., Memelink, J. and Chua, N-H. (1990). Molecular light switches for plant genes. *Plant Cell* 2: 369-378.

Higo, K., Ugawa, Y., Iwamoto, M. and Korenaga, T. (1999). Plant cis-acting regulatory DNA elements (PLACE) database: *Nucleic Acids Res* 27: 297-300.

Hanahan, D .(1985). Heritable formation of pancreatic β-cell tumours in transgenic mice expressing recombinant insulin/simian virus 40 oncogenes. Nature 315: 115-122.

Hobohm, U. and Sander, C. .(1995). A sequence property approach for sequencing protein databases. J. Mol Biol 251: 390.

Hollick, J.B. and Gordon, M.P. (1993). A poplar tree proteinase inhibitor-like gene promoter is responsive to wounding in transgenic tobacco, Plant Mol. Biol., 22.561–572.

Horsch, R.B., Fry ,J.F., Hoffman, N.L., Eichholtz D, Rogers, S.G .and Fraley ,R.T. (1985). A simple and general method for transferring genes into plants. Science 227: 226-228.

Hui, E. K., Wang, P. C., and Lo, S. J. (1998). Strategies for cloning unknown cellular flanking DNA sequences from foreign integrants. Cell Mol. Life Sci., 54: 1403-1411.

Iglesias, V.A., Moscone, E.A., Papp, I., Neuhuber, F., Michalowski, S., Phelan, T., Spiker, S., Matzke, M. and Matzke, A.J. (1997). Molecular and cytogenetic analyses of stably and unstably expressed transgene loci in tobacco. Plant Cell 9: 1251-1264.

Iwahana, H., Tsujisawa. T., Katashima, R., Yoshimoto, K., and Itakura, M. (1994). PCR with end trimming and cassette ligation: A rapid method to clone exon-intron boundaries and a 5'-up-stream sequences of genomic DNA based on a cDNA sequence. PCR Methods Appl., 4: 19-45.

Jefferson, R. A., Kavanagh, T. A. and Bevan, M. W. (1987). GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J., 6: 3901-3907.

Jensen, J. S., Marcker, K. A., Otten, L. and Schell, J. (1986). Nodule-specific expression of a chimeric soybean leghemoglobin gene in transgenic *Lotus corniculatus*. Nature 321: 669-674.

Jones, D.H. and Winistorfer, S.C. (1993). Genome walking with 2- to 4-kb steps using panhandle PCR. PCR Methods Appl., 2: 197-203.

Kapoor, S. and Sugiura, M. (1999). Identification of two essential sequence elements in the nonconsensus type II PatpB-290 plastid promoter by using plastid transcription extracts from cultured tobacco BY-2 cells. Plant Cell 11: 1799-1810.

Kilstrup,M., and Kristiansen, K.N.(2000). , Rapid genome walking: a simplified oligo-cassette mediated polymerase chain reaction using a single genome-specific primer, *Nucleic Acids Res.* 28: p. E55.

Lagerstrom, M., Parik J., Malmgren, H., Stewart J, Pettersson U. and Landegren, U. (1991). Capture PCR: Efficient amplification of DNA fragments adjacent to a known sequence in human and YAC DNA. PCR Methods Appl., 1: 111-119.

Le Gourrierec, J., Li, Y.F. and Zhou, D.X. (1999). Transcriptional activation by Arabidopsis GT-1 may be through interaction with TFIIA-TBP-TATA complex. Plant J. 18: 663-668.

Matarasso N, Schuster S, and Avni A. (2005).A novel plant cysteine protease has a dual function as a regulator of 1-aminocyclopropane-1-carboxylic Acid synthase gene expression. Plant Cell. 17: 1205-1216.

Murray, M.G. and Thompson, W.F. (1980). Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res., 8: 4321-4325.

Nishiuchi T, Shinshi H, and Suzuki K. (2004). Rapid and transient activation of transcription of the ERF3 gene by wounding in tobacco leaves: Possible involvement of NtWRKYs and autorepression. J. Biol. Chem., 279: 55355-55361.

Niu, L. L., and Fallon, A. M. (1999). Retrieval of flanking DNA using a PCR-based approach with restriction enzyme-digested genomic DNA template. BioTechniques 26: 630-634.

Ochman, H., Gerber, A.S. and Hartl, D.L. (1988). Genetic applications of an inverse polymerase chain reaction. Genetics 120: 621-623.

Odell, J. T., Nagy, F. and Chua, N. H. (1985). Identification of DNA sequences required for activity of the cauliflower mosaic virus 35S promoter. Nature 313: 810-812.

Osusky, M.; Zhou, G.; Osuska, L. Hancock, R.E.; Kay W.W. and Misra, S. (2000). Transgenic plants expressing cationic peptide chimeras exhibit broad-spectrum resistance to phytopathogens, Nat. Biotechnol. 18, 1162–1166.

Parker, J.D., Rabinovitch, P.S. and Burmer, G.C. (1991). Targeted gene walking polymerase chain reaction. Nucleic Acids Res., 19: 3055-3060.

Reddy M.K., Nair S., Tewari K.K., Mudgil Y., Yadav B.S. and Sopory S.K. (1999). Cloning and characterization of a cDNA encoding topoisomerase II in pea and analysis of its expression in relation to cell proliferation. Plant Mol Biol., 41: 125-137.

Reddy M.K., Nair S. and Sopory S.K. (2002). A new approach for efficient directional genome walking using Polymerase Chain Reaction. Anal Biochem 306: 154-158.

Riley, J., Butler, R., Ogilvie, D., Finniear, R., Jenner, D., Powell, S., Anand, R., Smith, J.C. and Markham, A.F. (1990). A novel, rapid method for the isolation of terminal sequences from yeast artificial chromosome (YAC) clones. Nucleic Acids Res., 18: 2887-2890.

Rosenthal, A. and Jones, D.S. 1990. Genomic walking and sequencing by oligo-cassette mediated polymerase chain reaction. Nucleic Acids Res., 18: 3095-3096.

Ross EJ, Stone JM, Elowsky CG, Arredondo-Peter R, Klucas RV, and Sarath G. (2004). Activation of the Oryza sativa non-symbiotic haemoglobin-2 promoter by the cytokinin-regulated transcription factor, ARR1. J Exp Bot. 55: 1721-1731.

Rubio-Somoza, I., Martinez, M., Abraham, Z., Diaz, I. and Carbonero, P. (2006).Ternary complex formation between HvMYBS3 and other factors involved in transcriptional control in barley seeds.Plant J. 47: 269-281.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989). Molecular Cloning: A laboratory manual. 2 [1,2,3]. Cold Spring Harbour, New York, Cold Spring Harbour Laboratory Press.

Sanders, P.R., Winter, J.A., Barnason, A.R., Rogers, S.G. and Fraley, R.T. (1987). Comparison of cauliflower mosaic virus 35S and nopaline synthase promoters in transgenic plants. Nucleic Acids Res., 15: 1543-1558.

Siebert, P. D., Chenchik, A., Kellogg, D. E., Lukyanov, K. A., and Lukyanov, S. A. (1995). An improved PCR method for walking in uncloned genomic DNA. Nucleic Acids Res., 23: 1087-1088.

Silver, J. and Keerikatte, V. (1989). Novel use of polymerase chain reaction to amplify cellular DNA adjacent to an integrated provirus. Virol., 63: 1924-1928.

Shirsat, A., Wilford, N., Croy, R. and Boulter, D. (1989). Sequences responsible for the tissue specific promoter activity of a pea legumin gene in tobacco. Mol. Gen. Genet 215: 326-331.

Stultz, C. M., White, J. V. and Smith, T. F. (1993). Structure analysis based on state-space modelling. Protein Sci., 2: 305.

Sunter, G .and Bisaro, D. M. (2003). Identification of a minimal sequence required for activation of the tomato golden mosaic virus coat protein promoter in protoplasts. Virology. 305: 452–462.

Tjaden, G., Edwards, J.W. and Coruzzi, G.M. (1995). Cis elements and trans-acting factors affecting regulation of a nonphotosynthetic light-regulated gene for chloroplast glutamine synthetase. Plant Physiol., 108: 1109-1117

Triglia, T., Peterson, M.G. and Kemp, D.J. (1988). A procedure for in vitro amplification of DNA segments that lie outside the boundaries of known sequences. Nucleic Acids Res. 16: 81-86.

Trueba, G.A. and Johnson, R.C. (1996). Random primed gene walking PCR: a simple procedure to retrieve nucleotide fragments adjacent to known DNA sequences. Biotechniques 21: 20.

Willems, H. (1998). Adaptor PCR for the specific amplification of unknown DNA fragments. Bio Techniques 24: 26-28.

Van, W.R., Dong, X., Liu, H., Tien, P., Stanley, J. and Hong, Y. (2002). Mutation of three cysteine residues in tomato yellow leaf curl virus-China C2 protein causes dysfunction in pathogenesis and posttranscriptional gene-silencing suppression. Mol. Plant- Microbe Interact. 15: 203–208.

Vieweg MF, Fruhling M, Quandt HJ, Heim U, Baumlein H, Puhler A, Kuster H, and Andreas M. P. (2004).The promoter of the *Vicia faba, L.* leghemoglobin gene VfLb29 is specifically activated in the infected cells of root nodules and in the arbuscule-containing cells of mycorrhizal roots from different legume and nonlegume plants. Mol Plant Microbe Interact. 17: 62-69.

Yanagisawa, S. and Sheen, J. (199). Involvement of maize Dof zinc finger protein in tissue-specific and light regulated gene expression. Plant Cell 10: 75-89.

Yanagisawa, S. and Schmidt RJ (1999). Diversity and similarity among recognition sequences of Dof transcription factors. Plant J. 17: 209-214.

Yanagisawa, S. (2000). Dof1 and Dof2 transcription factors are associated with expression of multiple genes involved in carbon metabolism in maize. Plant J., 21: 281-288.

Yevtushenko, D.P., Vladimir A. Sidorov , Rafael Romeroa, William W. Kaya and Santosh Misra (2004). Wound-inducible promoter from poplar is responsive to fungal infection in transgenic potato.Plant Science *167: 715–724.*

Zhou, D.X. (1999).Regulatory mechanism of plant gene transcription by GT-elements and GT-factors.Trends in Plant Science 4:210-214.

الحصول علي محفز جيني جديد معزول من نباتات الفاصوليا (*Phseolus vulgaris, L.*) وقياس النشاط في نباتات التبغ (*Nicotiana Tabacum var. xanthi*) المعدلة وراثيا .

ايمان عبد المطلب محمود[1]، محي الدين سليمان[1]، هاني الشيمي[2] ، أحمد ابو العينين[2] ، مراد عبد المجيد ابو السعود[2] و صبحي عبد الرازق غانم[1].

١- قسم التكنولوجيا الحيوية النباتية –المركز القومي للبحوث – الدقي – القاهرة.

٢- قسم الكيمياء الحيوية كلية الزراعة – جامعة القاهرة .

تهدف هذه الدراسة الي التعرف علي التعبير العالي للمحفز الجيني المقاوم للفطريات (AF-pro) المعزول من نبات الفاصوليا (*Phaseolus vulgaris, L.*)، و الحصول علي بروتين trans genic عالي التعبير للمحفز الجيني في نباتات التبغ. وتم عمل محفز جيني Af-pro باستخدام اكثر من طريقة يتضمن ذلك بناء cDNA مكتبة وإستعمال تقنية المورّثِ المُتنقلة. تحليلُ PCR للمحفز الجيني و تعبير mRNA يوضح النشاط الأعلى لمرحلة الانباتَ و تطور النبات. و تم استخدام تعبيرَ gusA كتقرير لنشاط المحفزالجيني في نباتات و ذلك باستخدام تقنية النقل الوراثي للاجروباكتريم . التبغ المعدل وراثيا و التعبير ل B-glucuronidase تحت السّيطرة للمحفز الجيني اي المنطقة المعزولة من جينوم الفاصوليا (AF-PRO) يوضح التعرض للاجهاد و المقاومة يعتمد علي نوعية الاجهاد و نوع الاصابة . و مستوى RNA الرسول منْ gusA حُدّد في النباتات المعدلة وراثيا . البياناتُ المتحصل عليها توضح ان المحفز الجيني اكثر نشاطا عند الانبات تحت الظروف الاصلية و كان ايضا اكثر نشاطا في التركيبة المعدلة وراثيا لنفس المرحلة لتطور النبات . بناء المحفز الجيني هام لفهم عملية التنظيم للمحفز الجيني ، لكن معرفتنا الحالية حول تركيبِ المحفز النباتي، خصوصاً فيما يتعلق بمركز المحفز الجيني ، غير كافيُة. العديد من عناصر المحفز الجيني تحتوي علىَ TATA box ، وعِدّة أنواع لعوامل التنظيم و النسخ ايضا وُجدت لتوضح التوزيع الداخلي للمحفز، وهذه الميزّة إستعملتْ بنجاح لعزل المحفز الجيني من جينوم النبات.