Cloning of a Novel Antifungal Promoter from *Phaseolus vulgaris* and the Determination of its Activity in Stably Transformed *Nicotiana tabacum* plants

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Abstract

To investigate the transcriptional regulation of gene expression, chimeric fusions, between the β-glucuronidase reporter gene (GUS) and the isolated promoter regions of the *pvPDF* gene (*pvPDF-PRO*: *GUS)*, were constructed and introduced into *Nicotiana tabacum.* Analysis of transgenic *pvPDF-PRO*:*GUS* tobacco plants indicated that GUS activity was observed with all the promoter constructs with the strongest being in leaf followed by stem and roots. These results clearly demonstrate that *pvPDF-PRO* is a strong inducible and near-constitutive promoter and emphasize the great application potential for plant genetic engineering studies. Interestingly, a search for putative *cis*-acting elements in the *pvPDF* promoter architecture revealed the presence of some important transcription regulatory elements including: CAAT Box, TATA Box, CATA Box, and light regulatory elements (CCA1, GATA, GT-1). Taken together, these results further our understanding of the regulation of the *pvPDF* promoter activity.

Keywords: promoter isolation, cloning, *Phaseolus vulgaris*, defensins.

Introduction

Genetic transformation is a powerful tool for production of crop plants with increased resistance to phytopathogens. A number of transgenic cultivars with heightened tolerance to economically important pests and disease agents are in commercial production. However, in most cases the transgene is driven by a powerful constitutive promoter, such as the cauliflower mosaic virus *35S* (*CaMV35S*) 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 defense genes has distinct advantages as the majority is only activated following plant attack 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; 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; Bowles *et al*., 1990), and provides a convenient system to isolate and study defense-related genes and their upstream regulatory regions in transgenic hosts (Clarke *et al.*, 1994; Hollick *et al.*, 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).

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 stably 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 have been described (Ochman *et al*., 1988; Rosenthal and Jones, 1990; Riley *et al*., 1990; Lagerstrom *et al*., 1991; Parker *et al*., 1991; Trueba and Johnson, 1996; 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.

The genes which encode the various components of the pre-mRNA splicing or rRNA processing machinery provide a rich source of promoters for transgene

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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 proves valuable for promoter isolation and exploitation (Sunter and Bisaro, 2003; Van *et al*., 2002).

This work describes the isolation and functional characterization of the plant-defensin 5′-untranscribed promoter from *Phaseolus vulgaris*, designated *pvPDF-PRO1*, *pvPDF-PRO2* and *pvPDF-PRO3* fragments. Their functional analyses and promoter activity in chimeric reporter constructs (*pvPDF-PRO: GUS*) in transgenic tobacco plants are discussed, in the light of the potential use of these promoters in plant biotechnology.

Materials and methods

Plant material and growth conditions

Kidney Bean, *Phaseolus vulgaris*, seeds were grown on MS media at 25 ± 2ºC for 7 days in dark. The harvested material was immediately frozen in liquid N_2 and stored at –70ºC. For transformation studies, *Nicotiana tabacum* var Xanthi plants were grown at 25 ± 2ºC in 16 h light and 8 h dark.

Cloning strategy of kidney bean pvPDF *promoters*

The cloning of the *pvPDF* promoter was carried out according to Reddy *et al*. (1999 and 2002). Purified *Phaseolus vulgaris* genomic DNA was restriction-digested overnight at 37ºC, with 80 units of restriction enzymes for the concentration of 40 µg/l DNA: e.g. *Bam*HI, *Bgl*II and *Sou*3A (NEB). Following heat inactivation of enzymes at 65ºC for 10 min and centrifugation, the resulting DNA pellets were then washed with 70% EtOH, allowed to air dry and subsequently resuspended in 20 µl sterile double-distilled H_2O.The digested DNA were electrophoresed on 0.8% agarose gel and processed for cutting the region from 0.5 to 1.0 kb. Each fragment was purified using Miniprep columns (Qiagen). The digested DNA (20 µg) was partially end-filled by dGTP and dATP .The reaction mixture contained dNTPs (2mM each), 10X reaction buffer (2.5 µl), klenow enzyme (2 µl) in reaction volume of 50 µl and was incubated at 37ºC for 30 min. The DNA was purified through Miniprep purification Columns (Qiagen). The following oligonucleotide adaptors were employed: ADOP-32, 5´-AATACGACTCACTATAGGGCGGCCGCCCGGGC-3´ and ADOP-27, 5´-CACTATACCCGCCGGCGGGCCCGCT-3 (Synthesized by IDT Inc.). A volume of 20 µl of each adaptor (50 ng/l) was pipetted into a 0.5-ml Eppendorf microfuge tube and overlaid with mineral oil. The adaptors were heated at 99ºC for 4 min in a beaker of water. The heat was removed, and the solution was allowed to cool for 1 h at room temperature. The annealed adaptors were decanted from under oil and stored at –20ºC. Then, 10 µl of the genomic restriction digest was ligated to 1 µl of the annealed adaptors with 2 µl of T4 DNA ligase buffer and 2 µl of T4 DNA ligase (5 U/µl) in a 20-µl reaction. The ligation was incubated overnight at 12ºC and heat inactivated at 65ºC for 10 min. A 180-µl volume of TE (pH 8) was added to the ligation mix; this is called the adaptor library. Excess primer-adapter was removed by purification through QIAquik Columns (Qiagen).

Manipulation of plant nucleic acids

Nucleic acid manipulations were essentially carried out according to Sambrook *et al.* (1989). Genomic DNA was extracted and purified on a mini-prep scale as described by Murray and Thompson (1980). Total RNA from plant tissues was isolated as mentioned by Chomczynski and Sacchi (1987) with some modifications. Target PCR products were cleaned up by Gel Extraction Kit (Promega) and cloned into the pGEMT vector kit (Promega), then transformed into *E. coli* DH5α according to Hanahan (1985). Positive clones were confirmed by restriction analysis and sequenced using Sanger Sequencing Technology on ABI Prism 3730XL (Applied Biosystems/ Sanger) according to the dideoxy chain-termination method (Sanger *et al*., 1977).

Polymerase chain reaction

Taq-polymerase, dNTPs (deoxynucleotide triphosphate) and convergent primers achieved amplification of desired DNA fragments. The following adaptors were used to prime PCR reactions: ADOP-32, 5´-AATACGACTCACTATA GGGCGGCCGCCCGGGC-3´ and ADOP-27, 5´-CACTATACCCGCCGGCGGGCCCGCT-3. *pvPDF* promoter-based oligonucleotide primers were used: Def R1, 5′-CTCTTTATTCATCTCACTCGACT-3′; Def R2, 5′-ACAATTTTTGGTGCACCAACAACGAAATCAT-3′ and Def F1, 5′-AGTCGAGTGAGATGAATAAA GAGTTTTGAA-3. The reaction conditions for PCR involved denaturation at 94ºC for 30 seconds, annealing at 52ºC for 30 seconds and extension at 72ºC for either 30 seconds (for *pvPDF-PRO* amplification) or 2 minutes (for *pvPDF-PRO::GUS*). After 30 cycles of amplification an aliquot of this reaction mixture was loaded onto a 0.8% agarose gel and checked. For nested PCR, the following primers were employed: Pro2-F-RE, 5′-GAGTTTTGAAAGGAGAATACTTTG AAGTTT-3′; Emandef-R1-RE, 5′-GGTGCACCAACAA CGAAATCAT-3′ and ProD-R-RE, 5′-GCCAGCTAG TGATTTCTTCTCCAT-3′.

In silico *sequence analyses*

The University of Wisconsin Genetic Computer group (GCG) sequence analysis software package, PC/ GENE (Intelligenetics), and BLAST were utilized for sequence analyses (Hobohm and Sander, 1995; Stultz *et al.*, 1993; Altschul, 1997). For sequence manipulation BioEdit Sequence Alignment Editor was employed (Hall, 1999).

Preparation of chimeric constructs

Plasmid vector pBI121 (Bevan, 1984) was used for cloning of *pvPDF* promoter upstream from GUS gene (Fig. 1). These constructs were then utilized for tobacco transformation. To generate *pvPDF::GUS* promoter construct, *pvPDF* promoter fragments, double-digested with *Bam*H1/*Hind*III, were ligated into pBI121 binary vector digested with the same restriction enzymes. Ligation was carried out overnight at 14ºC in the presence of T_4 DNA

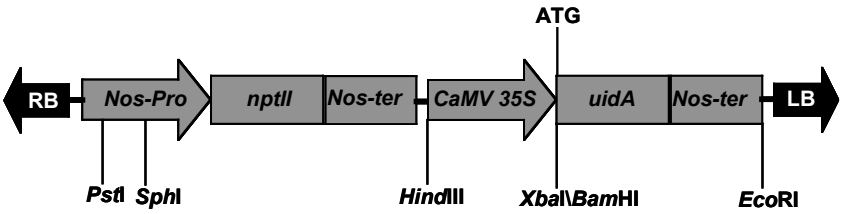


Fig. 1. The pBI121 binary vector used in chimeric construct generation including the map positions of some restriction sites.

ligase. The ligation reaction was employed to transforme DH5α competent cells.

Plant transformation

Constructs were transferred to *Agrobacterium tumefaciens* strain LBA4404 by the freeze-thaw method according to An *et al.* (1988). To test for *bona fide* transformants, 3-day-old *Agrobacterium* colonies were further analyzed by PCR for the presence of the selectable marker gene *nptII*, using the following primers combination: Neo5, 5′-GAGGCTATTCGGCTATGACTG-3′ and NeoSHR, 5′-GGCCATTTTCCACCATGATA-3′. Leaf discs of sterile-grown *Nicotiana tabacum* were transformed essentially as described by Horsch *et al*. (1985), with minor modifications. Transformed plants were selected by rooting several times on kanamycin-containing medium and transferred to compost.

GUS histochemistry and quantitative assay

This method for screening the transgenic plants was done according to (Jefferson, 1987 and Allan *et al*., 1993) and allows 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 37ºC for overnight in dark. After staining, tissues were rinsed extensively in ethanol to remove chlorophyll before examination. Preparing X-GLUC Stain by Dissolve 5 mg X-gluc in 1.0 ml dimethyl formamide and adding 50 mM $NaPO_4$ pH 7.0 to 10 ml.

4-Methylumbelliferone (4-MU) production was measured at four time points using a fluorometer fitted with a microtiter plate reader (Millipore, Cytofluor 2350). Total protein determination was carried out according to Bradford (1976) using Bradford reagent ((Bio-Rad) and BSA as standard. Measurements were performed in triplicate and GUS activity expressed as pmol 4-MU/min/mg protein.

Results and discussion

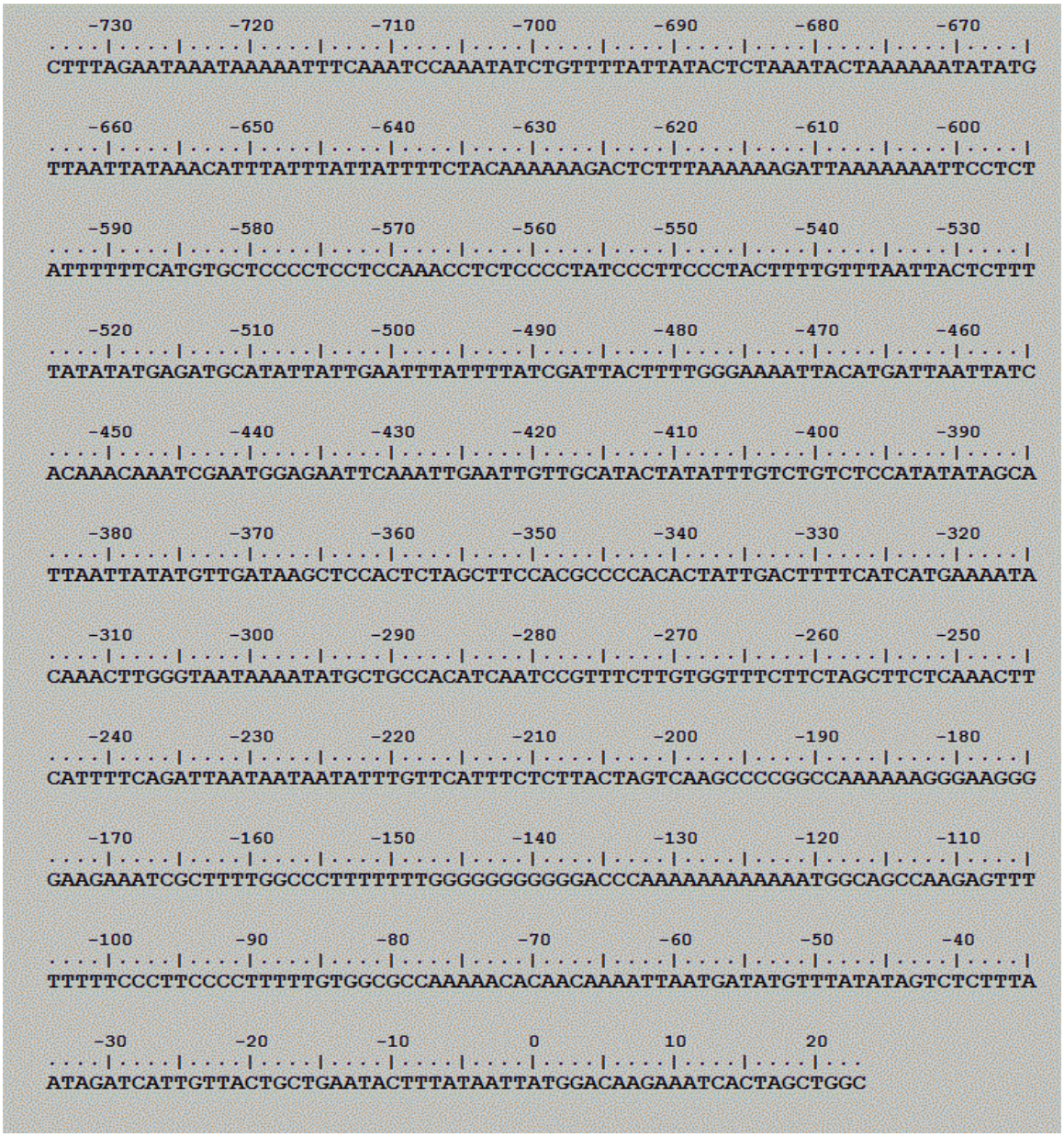
Identification and cloning of pvPDF *promoters*

Plant promoter architecture is important for understanding regulation and evolution of the promoters, but our 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, we carried out a program of isolation of plant promoters and functionally characterized them for suitability to express foreign genes using tobacco as a model system. One of the promoters, designated *pvPDF*, was identified and was shown to belong to a plant defensin gene encoding an antifungal protein from beans.

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; 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 filling whereby preventing self-ligation between fragments. In addition, the present protocol also employed biotinilated primers that enrich specific template prior to nested PCR. Also, one of the adopter strands was blocked at the 3′ end by attaching an amine group **(**Reddy *et al*., 2002). Following this method a number of unknown 3′ and 5′ regions of *pvPDF* gene promoters were isolated in this paper (data not shown). Namely, a 730 bp fragment corresponding to the 5′ UTR region of *pvPDF* gene from bean, was isolated following this new method (Fig. 2). The major transcript was found to initiate from 270 bases upstream of the translation

A



B

DEF F1 primer

ATGGACAAGAAATCACTACCTGGCTTATGCTTCCTCTTCCTTGTTCTCTT
TGTTGCTCGTAAGTCACTACTCTTATGTTTATTTTTCTGCATGTCTATAA
CTTTAATCATTCTCCTTACATCCTATAACTGTCTATTTACATATTCAGTG
TAACTTTTAGTTTTCTACAAGATTAATGTACTGTTTATACAGTCTCTTTA
GTTGTTCATTGTTACTGCTGAATACTTTATAATTCAATATCCCCGATATA
TGTTGATGAATTTATTTGAACATGCAGAAGAATGTGTGTTGCAGACAGAG
GCAAAGACTTGCGAGAACCTGGCTGATACATACAGGGGTCCATGCTTCAC
CACTGGAAGCTGCGATGATCACTGCAAGAACAAAGAACACTTGCTGAGTG
GCAGATGCAGGG**ATGATTTCGTTGTTGGTGCAC**CAAAAATTGTTAA

DEF R1 primer

Fig. 2. **A** The nucleotide sequence of the cloned *pvPDF* promoter generated by BioEdit Sequence Alignment Editor (Hall, 1999). **B** The nucleotide sequence of the *pvPDF* full length gene after isolated from Kidney Beans and sequencing.

initiation site. The minor transcript was found to initiate from 150 nucleotides upstream of the translation initiation site (data not shown). Similar result has been obtained by Reddy *et al*. (1999), in which they isolated genomic DNA fragments from tobacco- and pea-derived promoter regions for DNA topoisomerase I (*topo I*) using similar PCR based 5′ genome walking. In case of pea, they isolated 1140 bp and in tobacco a 482 bp upstream of ATG -5′ flanking region of tobacco *topo I* using 5′-RACE PCR-based approach (Reddy *et al*., 1999).

Moreover, we have managed to cloned a putative 5′-UTR promoter region for the isolated *pvPDF* gene and have found that it possess various known plant regulatory motifs (Table 1, Fig. 2 and Fig. 3A). Futhermore, the corresponding *pvPDF* gene was also successfully PCR-amplified (Fig. 3B). The *pvPDF* gene open reading frame was found to be composed of 195 bp and the exact sequence will be submitted to GenBank (Mahmoud *et al*., unpublished data).

Database-assisted pvPDF *promoter sequence analyses*

The sequence homology of the 5′-UTR region of the *pvPDF* gene isolated in the present study (Fig. 2) revealed the existence of some differences within the reported *pvPDF* gene sequence (data not shown). This could be due to the fact that the *pvPDF* exists 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 ICGEB-India, personal communication). Analysis of regulatory sequences is greatly facilitated by database-assisted bioinformatic approaches. In this context, 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/) (Hobohm and Sander, 1995; Stultz *et al.*, 1993).

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 isolated *pvPDF* promoter were identified using PLACE (Higo *et al*., 1999, http://www.dna.affrc.go.jp) (Table 1). A search for putative *cis*-acting elements in the *pvPDF* promoter revealed the presence of some important elements including: CAAT Box, TATA Box, CATA Box, and light regulatory elements or LREs (CCA1, GATA, GT-1). Interestingly, the *pvPDF* promoter has a TATA Box near the 5′ end of the *pvPDF* gene. The first TATA-like element detected is at −237 and the second TATA Box was at −685. The presence of CAAT Box1 motifs in the *pvPDF* promoter 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 *pvPDF* promoter one light regulatory element could been detected at the region between −251 to −258. Moreover, two GATA Box elements were identified, one at −678 to −682 and the other between −320 to −325. In this context, similar *cis*-acting elements have been reported in CaMV 35S promoter and in promoters from Petunia (Benfey and Chua, 1990; Gilmartin *et al*., 1990). The presence of light regulatory elements and tissue specific elements accounts for the tissue specific light regulation of *pvPDF*. 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

Table 1. Putative *cis*-acting elements identified in the cloned *pvPDF* promoter sequence. These elements were identified using the Signal Scan Program at PLACE (http://www.dna.affrc.go.jp).

Reference	Sequence	Site Position	Found in	Motif
Shirsat *et al.* (1989)	CAAT	452	Sequences responsible for the tissue specific promoter activity of a pea legumin gene	CAAT BOX1
Rubio-Somoza *et al.* (2006)	GATA	678	GATA motif in CaMV 35S promoter	GATA BOX
Matarasso *et al.* (2005)	TAAAATAT	434	chlorophyll a/b binding protein the promoter of Petunia	LECPLEACS2
Vieweg *et al.* (2004)	CTCTT	524	One of the consensus sequence motifs of organ-specific elements	OSE2 ROOT NODULE
Tjaden *et al.* (1995)	TTATTT	685	5′ UTR region of pea glutamine synthetase gene	TATA BOX5
Nishiuchi *et al.* (2004)	TGACY	400	"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	W BOX NTERF3
Tjaden *et al.* (1995)	TATAAAT	237	5′ UTR region of pea *legA* gene	TATA BOX2
Ross *et al.* (2004)	NGATT	498	ARR1; Response regulator	ARR1 AT
Elmayan and Tepfer (1995)	ATATT	225	Motif found both in promoters of rolD	ROOT MOTIF TAPOX1
Zhou (1999)	GRWAAW	251	Consensus GT-1 binding site in many light-regulated genes,	GT-1 CONSENSUS
Le Gourrierec *et al.* (1999)	GRWAAW	429	GT-1 binding site in many light-regulated genes	GT-1 CONSENSUS

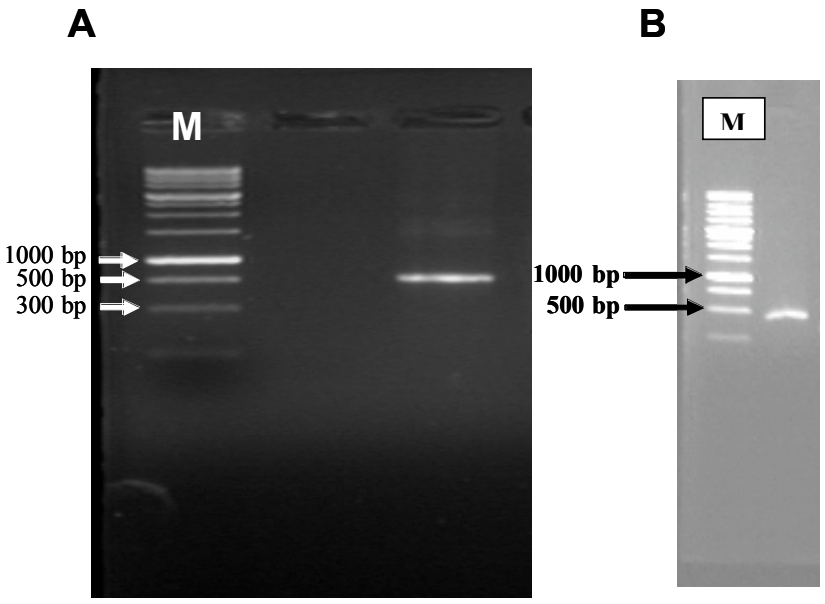


Fig. 3. **A** Photograph of a gel showing a purified PCR product corresponding to the 730 bp full-length promoter of *pvPDF* from Kidney Beans prior to pGEMT cloning and sequencing. M,marker lane. **B** PCR-based isolation of the coding region of the *pvPDF* gene from Kidney Beans using the full-length primer set (See Materials and Methods).

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 (C4-*PEPC*). 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; Yanagisawa, 2000). The *pvPDF-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 plastid *atpB* gene promoter (Kapoor and Sugiura, 1999).

Responsiveness of *Promoter Transgenic* pvPDF: GUS *Tobacco Plants*

Agrobacterium mediated leaf disk method was followed for transformation, *pvPDF* promoter sequences and the GUS expression. A frequency of 5–20 plantlets were obtained per explants, which was comparable to control pBI121 binary vector transformation frequency, indicating that the cloning of *pvPDF* promoter had no adverse effects on the overall transformation frequencies. Transformed plants rooted normally and no abonormalities associated with the transformation was observed (Fig. 4).

Histochemical GUS staining of transgenic plants indicated that pvPDF: GUS promoter was active in almost all reproductive organs and the highest level was in the roots (Fig. 5).

We have utilized *Hind*III and *Bam*HI sites present in the pBI121 vector to clone *pvPDF* promoters. Three different constructs were created that differed in their length. The *pvPDF-PRO1* is the smallest promoter which was only 350 bp long, the *pvPDF-PRO2* was 500 bp and the longest promoter, *pvPDF-PRO3*, contained 730 bp. All the promoters were cloned into the same *Hind*III and *Bam*HI sites (Fig. 1) for a better comparison of promoter strength. As a control, pBI121 having 35S promoter was used. The CaMV 35S is a strong and constitutive promoter and used most extensively to express foreign genes in plants. Under 35S promoter, GUS expression was detected in all tissue types and at all developmental stages

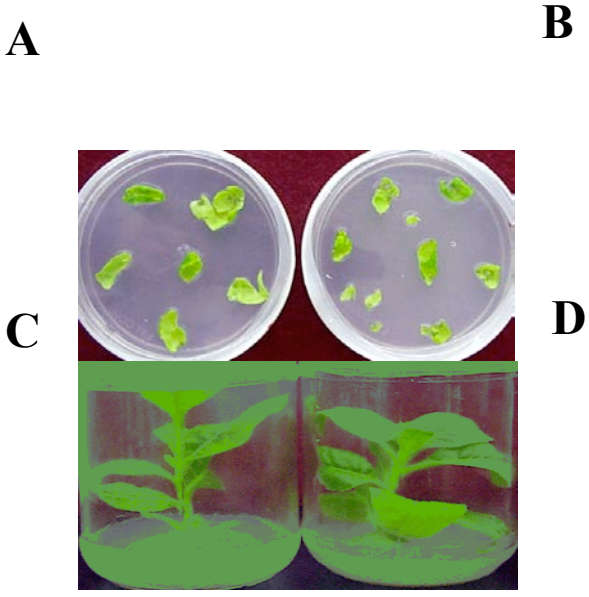


Fig. 4. Steps followed in the generation of transgenic *pvPDF::GUS* plants. **A** Tobacco explants after infecting the wounded plant tissue with *Agrobacterium* carrying the binary vector pBI121. **B** Transformed cells divide and grow. **C** Shooting and elongation medium. **D** Shoots are placed on another medium to promote root development, so that complete plantlets are produced.

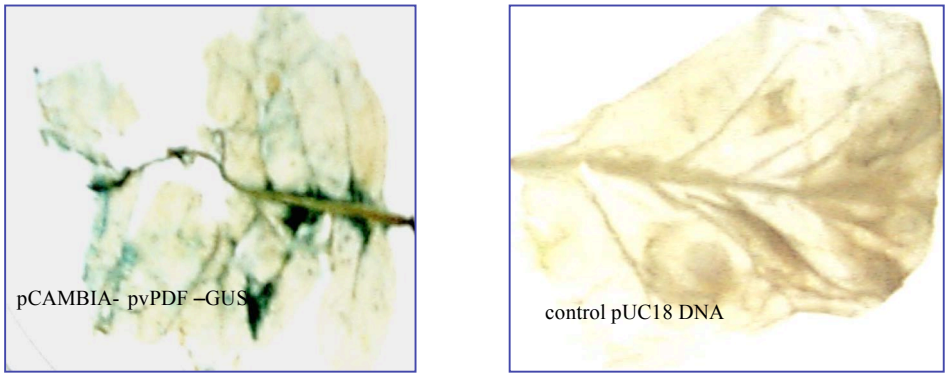


Fig. 5. Tobacco leaf was *bombarded* with plasmid DNA for transient assay of GUS. No blue spots could be seen on control leaf bombarded with pUC18 DNA. **A** pBI121 – pvPDF – GUS. **B** pUC18 DNA .

of tobacco plant growth. The expression was strongest in leaf tissue. The expression of GUS was observed with all three promoter constructs. The expression was strongest in the leaf followed by stem and roots (data not shown). Among the three constructs, was the highest *pvPDF-PRO3* followed by *pvPDF-PRO2*, whereas GUS activity was low with *pvPDF-PRO1* promoter construct (data not shown). However, the expression pattern for various tissues was similar in all the constructs.

The activity of the *pvPDF* gene promoter, encoding a bean cysteine-rich antifungal peptide, was investigated in transgenic *pvPDF::GUS* tobacco plants. Quantitative GUS activity analysis of the transgenic plant leaves showed the average activity of the bean *pvPDF-PRO* was 2- to 3-fold higher than that of the *CaMV35S* promoter. Histochemical GUS staining of transgenic plants indicated that *pvPDF::GUS* promoter was active in almost all reproductive organs and the highest level was in the roots (data not shown).

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 *pvPDF* full-length promoter, respectively. These results demonstrate that the bean *pvPDF* 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, one reason is attributed to the fact 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; Ross *et al*., 2004).

Promoters of various strengths and specificities are required for expression of foreign genes in plants for functional analysis of gene expression and for biotechnological improvement of economically-important crop plants. To meet such requirements, a range of promoters with different expression levels and patterns is required. Keeping this in mind, we begun a program of isolation of plant promoters and characterize them for their suitability to express foreign genes using tobacco as a model system. In this work, we described the cloning and functional analysis of a *Phaseolus vulgaris* promoter (*pvPDF*), controlling the expression of a plant defensin gene encoding an antifungal protein. Based on the obtained results, we envisage that this promoter can be valuable for various plant biotechnological applications.

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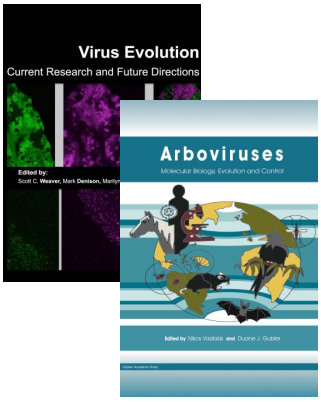

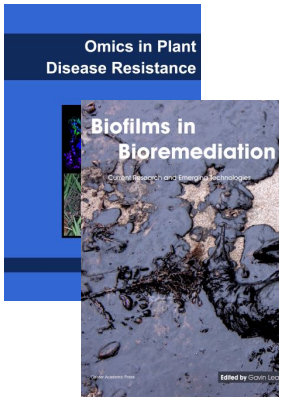

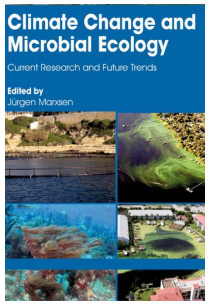

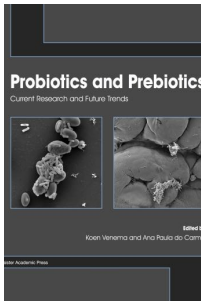

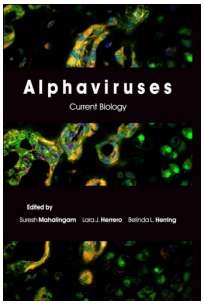

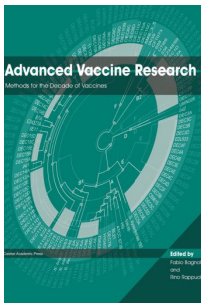

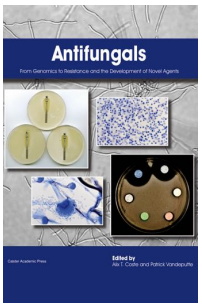

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