# DESIGNING PATHOGEN-INDUCIBLE SYNTHETIC PROMOTERS AND FUNCTIONAL VALIDATION OF A NEW EUKARYOTIC PROMOTER-PROBE VECTOR

Thesis submitted to the
*University of Agricultural Sciences, Dharwad*
*In partial fulfillment of the requirements for the*
*Degree of*

Master of Science (Agriculture)

*in*

Plant Biotechnology

*By*

RAVEENDRA G. M.

DEPARTMENT OF BIOTECHNOLOGY
COLLEGE OF AGRICULTURE, DHARWAD
UNIVERSITY OF AGRICULTURAL SCIENCES,
DHARWAD – 580005

APRIL, 2008

**ADVISORY COMMITTEE**

**DHARWAD**
**APRIL, 2008**

**(SUMANGALA BHAT)**
**MAJOR ADVISOR**

**Approved by:**

**Chairman: _____**
**(SUMANGALA BHAT)**

**Members:**

**1._____**
**(M. S. KURUVINASHETTI)**

**2._____**
**(RAMESH BHAT)**

**3._____**
**(S.K. PRASHANTI)**

# CONTENTS

# LIST OF FIGURES



# LIST OF PLATES

# 1. INTRODUCTION

Promoter is a DNA sequence normally located upstream of the transcribed region. It contains a TATA box and serves to determine the start site of transcription (Dynan and Tjian, 1985). Transcription factors together with RNA polymerase recognize a promoter by its structural features and associate with it to initiate transcription. In this process, the newly formed complex positions RNA polymerase at the transcription initiation site and activates transcription (Lewin, 2007).

Promoters typically have a modular structure, consisting of multiple short sequence (5 to 20 nucleotides), called *cis*-acting regulatory elements, most of which comprise transcription factor (TF) binding sites. These elements can be dispersed or can overlap and usually lie within the 1kb region upstream and surrounding a transcription start site (TSS). The combination of these regulatory elements is often unique for most genes or pathways. The promoter sequence is usually located upstream from the TSS, but regulatory elements can also be located downstream, for example, in the first intron of the gene itself (Zhang *et al*., 1994; Gidekel *et al*., 1996; de Boer *et al*., 1999; Dorsett, 1999).

The promoter can roughly be divided in two parts: a proximal part, referred to as the core, and a distal part. The proximal part is believed to be responsible for correctly assembling the RNA polymerase II complex at the right position and for directing a basal level of transcription (Nikolov *et al*., 1996; Nikolov and Burley, 1997; Berk, 1999). It is mediated by elements, such as TATA and initiator boxes through the binding of the TATA box-binding protein, and other general TFs (Featherstone, 2002). The distal part of the promoter is believed to contain those elements that regulate the spatio-temporal expression (Tjian and Maniatis, 1994; Fessele *et al*., 2002). How far upstream (or downstream) such a distal part is located is not defined. In addition to the proximal and distal parts, somewhat isolated, regulatory regions have also been described, mainly in animals that contain enhancer and/or repressors elements (Barton *et al*., 1997; Bagga *et al*., 2000). The latter elements can be found from a few kilobase pairs upstream of TSS, in the introns or even at the 3' side of the genes they regulate (Larkin *et al*., 1993; Wasserman *et al*., 2000).

Depending on the activity, promoters could be classified as constitutive, tissue – specific and inducible. A constitutive promoter contains elements recognized by basal and upstream activators to initiate transcription in all tissues at all times. However, inducible promoters are activated by one or more stimuli such as hormones, chemicals, environmental conditions/stresses and biotic stresses, where as tissue-specific promoters control gene expression in a tissue-dependent manner and according to the developmental stage of the plant.

Perception of a pathogen by a plant triggers rapid defence responses via a number of signal transduction pathways. A major target of signal transduction is the cell nucleus where the terminal signals lead to the transcriptional activation of numerous genes and consequently to the *de novo* synthesis of a variety of proteins and antimicrobial compounds. Transcriptional activation is brought about by binding of transcription factors to the *cis*-acting elements present in the promoter of pathogen responsive genes. These *cis*-acting regulatory elements or boxes include W, D, GCC, S and Myb sequences which are the binding sites for various transcription factors. These *cis*-acting elements can alone mediate pathogen-inducible expression *in planta*. When taken out of their native promoter contexts, they retain pathogen inducibility directing expression that is local and that correlates with the extent of growth of the pathogen.

Optimal pathogen-inducible synthetic promoters may consist of combinations of one or two copies of defined *cis*-acting elements. Attempts have been made to check the pathogen inducibility of synthetic promoters that contain various combinations of *cis*-acting regulatory elements fused to a minimal promoter. Effects of varying the number, order, and spacing of such elements have been shown using synthetic promoters. The activity of a promoter can be characterized at the mRNA and/or protein level of the gene it controls. The most common approach to study activity of a plant promoter, however, is to employ a promoter probe vector wherein the promoter to be tested is fused to a reporter gene, and monitor the expression of the reporter gene product in stably or transiently transformed

tissues. By this approach, the expression pattern of a promoter can be analyzed qualitatively and/or quantitatively in plant tissues, and its expression pattern in response to environmental conditions can be characterized by exposing the transgenic plants to those conditions.

These promoters are valuable additions to the study of signalling and transcriptional activation during plant–pathogen interactions. The availability of a range of defined synthetic plant promoters that direct controlled local gene expression in response to pathogens would be a major advance. The utility of the synthetic promoters can be enhanced by varying several parameters. Most important is the number of copies of an individual element in a promoter. Both the strength and the inducibility of a promoter can be modulated by varying the number of copies of an element. Importantly, this also can have the effect of reducing/eliminating some background expression because pathogen inducibility appears stronger than basal or wound-induced expression. Spacing between individual *cis*-acting elements and/or between these elements and the preinitiation complex can also have a profound effect, but spacing is difficult to predict (Wray, 1998), and the optimal spacing, like the optimal number of elements, needs to be determined experimentally. Promoters with combinations of different elements may be among the best pathogen-inducible promoters, because they often combine good inducibility with low background. These promoters could be used to help define signalling pathways to isolate novel mutants using “targeted genetics” (Hooley, 1998) and to engineer plants with increased disease resistance. In the light of the above background, this research programme focused mainly on following objectives

1. To construct and validate novel promoter-probe binary vector with *SgfpS65T* (green fluorescent protein) reporter gene
2. To construct recombinant promoter-probe vectors with synthetic pathogen-inducible promoters

# 2. REVIEW OF LITERATURE

Gene expression and regulation are mediated by DNA sequences, in most instances, directly upstream to the coding sequences by recruiting transcription factors, regulators and a RNA polymerase in a spatially defined fashion. The transcription of eukaryotic gene is controlled by an intricate choreography of proteins binding to promoter, enhancer and repressor sites on the DNA sequence (Ptashne, 1988; Ptashne and Gann, 1997).

A promoter is usually defined as a nucleotide sequence which contains a TATA box and serves to determine the start site of transcription (Dynan and Tjian, 1985). A promoter is the DNA sequence, generally upstream to the coding sequence, required for basal or regulated transcription of a gene. However, only a few nucleotides within a promoter are absolutely necessary for its function (Baliga, 2001). In genetics, a promoter is a DNA sequence that enables a gene to be transcribed. The promoter is recognized by RNA polymerase, which then initiates transcription. In RNA synthesis, promoters are a means to demarcate which gene should be used for messenger RNA production.

The regulatory regions for eukaryotic genes (called promoters) typically span a few hundred to several thousand bases of DNA. Scattered through these promoters can be dozens of regulatory elements of various kinds that act as binding sites for distinct transcription factors (Arnone and Davidson, 1997). In some promoters, regulatory elements are grouped into "modules," each of which drives a discrete portion of the overall expression profile of the gene or prevents transcription at inappropriate times and places.

## 2.1 Prokaryotic promoters

In prokaryotes, the promoter consists of two short sequences at -10 and -35 position upstream of the gene. The sequence (TATAAT) at -10 is called the Pribnow box, which is absolutely essential to start transcription. The other sequence (TTGACA) at -35 allows a very high transcription rate. Promoters of prokaryotic organisms, have similar properties as the eukaryotic although there are a few basic differences. In prokaryotes, promoter serves to initiate the transcription of multiple structural genes that are immediately adjacent to it. This arrangement is called an operon. A single transcribed mRNA is translated into several proteins whose functions are interrelated. In operons, promoters have adjacent, interspersed or juxtaposed regulatory sites to which regulatory proteins bind.

## 2.2 Eukaryotic promoters

Eukaryotic promoters are extremely diverse and are difficult to characterize. They typically lie upstream of the gene and can have regulatory elements several kilo bases away from the transcriptional start site. In eukaryotes, the transcriptional complex can cause the DNA to bend back on itself, which allows for placement of regulatory sequences far from the actual site of transcription. Many eukaryotic promoters, contain a TATA box (sequence TATAAA), which in turn binds a TATA binding protein, which assist in the formation of the RNA polymerase transcription complex at start site (often within 50bp). In eukaryotic organisms promoters comprise of multiple elements, some of which are found in nearly all promoters. These include,

### 2.2.1 CAAT box

It is a consensus sequence close to -80bp from the start point (+1), playing an important role in promoter efficiency, by increasing its strength, and function in either orientation. The consensus CAAT sequence found in conserved eukaryotic promoter is GGCCAATCT. This box is replaced in plants by a consensus sequence called the AGGA box (Roa-Rodriguez and Cambia, 2003).

### 2.2.2 TATA box

A sequence usually located around 25bp upstream of the start point. The TATA box tends to be surrounded by GC rich sequences. The TATA box binds to RNA polymerase II and a series of transcription factors to an initiation complex (Smale and Kadonaga, 2003).

### 2.2.3 GC box

A sequence rich in Guanidine (G) and Cytidine (C) is usually found in multiple copies in the promoter region, normally surrounding the TATA box. The consensus GC box sequence found in conserved eukaryotic promoter is GGGCGG (Roa-Rodriguez and Cambia, 2003).

### 2.2.4 CAP site

A transcription initiation sequence or start point defined as +1, at which the transcription process actually starts. The consensus CAP site sequence found in conserved eukaryotic promoter is TAC. RNA polymerase II, the enzyme that transcribes a gene into mRNA, works in conjunction with other transcription factors that recognize signals embodied in the promoter region. It starts its journey at the TATA region where it binds and travels along the DNA until it reaches the CAP site where the actual synthesis of RNA starts. The transcription process only takes place in the downstream direction, from 5' (left) to 3' (right) (Smale and Kadonaga, 2003).

## 2.3 Types of promoters

Promoters are characterized according to the type or degree of control of gene expression. Control in virtually all tissues or control depending on the tissues and the developmental stage of the plant. Additionally, promoters may operate in response to external and in some cases, controllable stimuli.

### 2.3.1 Constitutive promoters

A constitutive promoter, by its simplest definition, contains elements recognized by basal and upstream activators to initiate transcription in all tissues and at all times. An example of such a promoter may be that of a housekeeping gene (homologous) or a gene derived from a plant pathogen with the ability to infect a wide range of host cells (heterologous) (Lewin, 1994). These promoters allow expression of the downstream coding region in all tissues irrespective of environmental and developmental factors (Benfey and Chua, 1990).

#### 2.3.1.1 CaMV 35S promoter

It is one of the most widely used general purpose constitutive promoter. In theory, expression from a constitutive promoter could be regulated by the interaction of *cis*-elements with factors that are present in all cell types. Alternatively, a constitutive promoter could contain multiple *cis*-elements which interact with different factors in different cell types. Benfey *et al*., (1989) isolated the promoter responsible for the transcription of the whole genome of a Cauliflower Mosaic Virus (CaMV) infecting turnips. The promoter was named CaMV 35S promoter because the coefficient of sedimentation of the whole transcript was 35S. It is a very strong promoter, causing high levels of gene expression in dicot plants. This is divided into the promoter itself, the enhancer and the minimal promoter. Deletion analysis suggested that the 35S promoter may contain at least two domains, one (domain A, -90 to +8) that confers expression principally in roots, the other (domain B, -343 to -90) that confers expression in other tissues.

Part of the domain A of the CaMV 35S promoter, which contains the TATA box and extends from -46 position to the transcription start site +8 (Benfey and Chua, 1990), is used as a "35S minimal promoter" (Odell *et al*., 1985), which does not drive the expression of a gene by itself; additional sequences, such as enhancers are required. Apart from the TATA box, which is the binding site for the RNA polymerase II, the region contains at least three CAAT - like boxes. These sequences potentiate the activity of upstream sequences and influence the efficiency of the promoter activity. These CAAT – like boxes alone or attached to heterologous promoter region drive the expression of transgene.

#### 2.3.1.2 Plant ubiquitin promoter (Ubi)

Christensen *et al.* (1992) identified two out of ten possible loci encoding ubiquitin in maize. Ubiquitin is found in eukaryotic cells and its sequence is highly conserved among

organisms as diverse as human to the fruit fly. The promoter of the *Ubi-1* gene of maize is located upstream of the structural genes and extends from -899bp of 5' of the transcription start site to about 1093bp 3' of the transcription start site. This sequence comprises; a TATA box sequence located at -30, two overlapping sequences that are similar to the consensus heat shock element found in heat inducible genes located at -214 and -204 position from the transcription start site, an 83bp leader sequence adjacent to the transcription start site (+1), an intron of around 1kb, which extends from 84 to 1093 position. The heat shock elements of the promoter region enhance the expression of ubiquitin in response to temperature stress.

2.3.1.3 Rice actin promoter (Act)

Mc Elroy *et al.* (1990) reported that the 5' region of rice *actin–1* (*Act-1*) gene successfully directed the expression of the reporter gene in transformed rice protoplasts. Since then, rice Act-1 promoter has been used as a strong constitutive promoter driving the expression of genes of interest in monocots. All the necessary *cis*-acting regulatory elements for the activity of the Act-1 promoter are in the upstream region of the transcriptional start site. Apart from the TATA box, there are two regions that play a role in the regulation of its activity. A poly (dA-dT) element of 38bp (-245 and -152) is the binding sequence for a trans-acting protein that works as a positive regulator. A region between -300 and -260 contains CCCAA pentamer repeats that appear to be involved in the negative regulation of the promoter activity.

## 2.3.2 Tissue specific promoters

Spatial and developmentally controlled gene expression can be achieved using different 'tissue-specific' promoters. A wide range of promoters has been identified in promoter trap programs and following the characterization of gene expression patterns. They allow specific gene expression in virtually any desired cell type, tissue or organs. However, iterative modes of development in plants, these promoters do not have a temporally restricted expression pattern, *sensu stricto*. For instance, the *LEAFY* (*LFY*) promoter is activated during flower initiation and is therefore to some extent temporally regulated, but it will be active in a similar way in every flower (Deveaux *et al.*, 2003), whenever initiated.

Distinct *cis*-acting DNA sequences confer spatially-regulated activation of gene expression within different regions (i.e. cotyledons, shoot apex and hypocotyl) and in different cell types of the same region (i.e. parenchyma and procambium) of the embryos. For example, the promoter of β-phaseolin gene encoding the major storage protein of bean (*Phaseolus vulgaris*) contains two upstream activating sequences (domains), UAS1 (-295 to -109) and UAS2 (-468 to -391). The activity of UAS1 was localized in the cotyledons and the shoot apex but expression in the hypocotyl required the presence UAS2. Additionally, both UAS1 and the 35S enhancer fragment directed GUS expression in the cotyledons and shoot apex, UAS1 yielded much higher activity in storage parenchyma cells and the 35S enhancer was mostly active in the procambial strands (Bustos *et al.*, 1991).

## 2.3.3 Inducible promoters

These promoters are activated by one or more stimuli such as hormones (for *e.g.* gibberellin, abscisic acid, jasmonic acid, salicylic acid, auxin), chemicals, environmental conditions (water, salt, wounding) and biotic stress (microbes, insects, nematodes). Inducible promoters are a very powerful tool in genetic engineering because the expression of genes operably linked to them can be regulated to function at certain stages of development of an organism or a particular tissue under defined conditions. There are virtually hundreds of inducible promoters that vary according to the organism source and cells or tissues where they regulate gene transcription (Singh *et al.*, 2002; Gurr and Rushton, 2005; Nakashima and Yamaguchi-Shinozaki, 2006)

2.3.3.1 Chemically regulated promoters

Systems that allow the regulation of gene expression by chemical stimuli offer great advantages both for studying gene function *in vivo* and for biotechnological applications (Gatz, 1996). Chemicals such as tetracycline, dexamethasone, copper, nitric oxide (Palmieri *et al.*, 2008) and salicylic acid have been explored as effector molecules to regulate the expression of transgenes in higher plants (Gatz, 1996; Gatz and Lenk, 1998). The

tetracycline-inducible promoter is, at present, the most advanced system and has already been used to study the function of a family of transcription factors *in vivo.* Dexamethasone inducible gene expression has been employed for cell-fate mapping in *Arabidopsis thaliana* (Schena *et al.*, 1991). In addition, salicylic acid, which induces genes involved in systemic acquired resistance against pathogens, has been used to elicit protection against insect feeding in transgenic plants.

The properties of such systems are tailored to meet the requirements of specific applications. For research experiments in the laboratory, tight control and high specificity of the inducing agent are important. For field experiments, the chemical should be cheap, easy to apply and environmentally compatible. Depending on the gene product, compromises can be made in terms of specificity and 'leakiness' of expression (Gatz, 1996).

#### 2.3.3.2 Stress regulated promoters

Stress inducible promoters regulate the temporal and spatial expression patterns of specific stress responsive genes as an important part of the plant stress response (Rushton and Somssich, 1998). Plants devote a large portion of their genome capacity to transcription, with the *Arabidopsis* genome coding in excess of 1500 transcription factors (Riechmann *et al.*, 2000). The stress responses of plants are regulated by multiple signalling pathways (Glazebrook, 2001; Knight and Knight, 2001), and there is significant overlap between the patterns of gene expression that are induced in plants in response to different stresses (Durrant *et al.*, 2000; Schenk *et al.*, 2000; Seki *et al.*, 2001; Chen *et al.*, 2002). For example, the gene expression profiles observed during an incompatible plant fungal interaction overlap with those derived from wounding (Durrant *et al.*, 2000) or by cold or drought stress (Seki *et al.*, 2001) and during senescence (Chen *et al.*, 2002). What had appeared to be several linear pathways are now being revealed as a complex regulatory network of signals that allow plants to respond optimally to their changing environment.

## 2.4 Pathogen inducible promoters

Perception of a pathogen by a plant triggers rapid defence responses via a number of signal transduction pathways. The major target of signal transduction is the cell nucleus where the terminal signals lead to the transcriptional activation of numerous genes and consequently to the *de novo* synthesis of a variety of proteins and antimicrobial compounds (Hammond-Kosack and Jones, 1996; Somssich and Hahlbrock, 1998). Transcription activation of responsive genes is mediated by pathogen-inducible promoters. An ideal pathogen-inducible promoter would be activated rapidly to a wide range of pathogens and therefore be effective against a range of pathogens. The promoter must be inactive under disease-free conditions to prevent spurious defense responses triggered by leaky expression of the transgene (McDowell and Woffenden, 2003). Further, the promoters should not be activated by transgene product itself to prevent uncontrolled spread of gene expression; so called ‘run away cell death’. Induction of the pathogen-responsive genes is regulated by one or the other type(s) of transcription factors that bind to *cis*-regulatory elements present in the promoter.

### 2.4.1 *cis*-acting elements of pathogen-inducible promoters

There are a large number of pathogen-inducible genes, and their promoters known in plants. These pathogen-inducible promoters carry *cis*-acting elements, which are the binding sites for transcriptional factors.

#### 2.4.1.1 W- Box

W box [(T) TGAC (C/T)] is the binding site for members of the WRKY family of transcription factors (Rushton *et al.*, 1996). There is increasing evidence that W boxes are a major class of *cis*-acting elements responsible for pathogen inducibility of many plant genes (Raventos *et al.*, 1995; Rushton *et al.*, 1996; Wang *et al.*, 1998). The importance of W boxes was illustrated recently by studies of the *A. thaliana* transcriptome during systemic acquired resistance (Maleck *et al.*, 2000; Petersen *et al.*, 2000). In some cases, clustering of W boxes may be associated with inducibility by different pathogens. In parsley, three WRKY proteins, WRKYl, -2 and -3, bind specifically to functional W Boxes in the PRI-1 and PRI-2 promoters

(Rushton *et al*., 1996). The optimal binding site for *A. thaliana* WRKY protein ZAP1 also has two W Boxes (de Pater *et al*., 1996; Rushton and Somssich, 1998).

Zheng *et al.* (2007) analyzed the role of the WRKY25 transcription factor from *A. thaliana* in plant defense against the bacterial pathogen *Pseudomonas syringae*. WRKY25 protein recognizes the TTGACC W-box sequences and its translational fusion with green fluorescent protein is localized to the nucleus. *WRKY25* expression is responsive to general environmental stress. Analysis of stress-induced *WRKY25* in the defense signalling mutants *npr1*, *sid2*, *ein2* and *coi1* further indicated that this gene is positively regulated by the salicylic acid (SA) signalling pathway and negatively regulated by the jasmonic acid signalling pathway. Two independent T-DNA insertion mutants for *WRKY25* supported normal growth of a virulent strain of *P. syringae* but developed reduced disease symptoms after infection. By contrast, *Arabidopsis* constitutively overexpressing *WRKY25* supported enhanced growth of *P. syringae* and displayed increased disease symptom severity as compared to wild-type plants.

2.4.1.2 GCC-Box

Ohme-Takagi and Shinshi (1995) demonstrated that the GCC box, which is an 11bp sequence (TAAGAGCCGCC) conserved in the 5' upstream region of ethylene-inducible pathogenesis-related protein genes in *Nicotiana* spp. and in some other plants, is essential for ethylene responsiveness when incorporated into a heterologous promoter. Four different cDNAs encoding DNA binding proteins specific for the GCC box sequence were isolated, and their products were designated ethylene-responsive element binding proteins (EREBPs). The deduced amino acid sequences of EREBPs exhibited no homology with those of known DNA binding proteins or transcription factors and lacked the basic leucine zipper or zinc finger motif. A number of proteins that bind to GCC Boxes have been isolated and found to be members of the EREBP family of DNA-binding proteins. EREBPs contain a DNA-binding domain that is also present in the APETALA2 family of proteins. The homeotic gene *APETALA2* plays a central role in the regulation of flower development in *A. thaliana* (Okamuro *et al*., 1997).

Ethylene responsive factors (ERFs) are important plant-specific transcription factors, some of which have been demonstrated to interact with the ethylene-responsive GCC box and the dehydration responsive element (DRE). Zhang *et al.* (2004) used the GCC box, an essential *cis*-acting element responsive to ethylene and methyl jasmonate (MeJA), as bait in a yeast one-hybrid system to isolate transcription factors from tomato (*Lycopersicon esculentum* Mill.). One of the cDNAs, which was designated Jasmonate and Ethylene Response Factor 1 (*JERF1*), encodes an ERF protein, contains a conserved ERF DNA-binding motif, and shown to function as a transcriptional activator in yeast, and targeted to the nucleus in onion (*Allium cepa* L.) epidermal cells. Biochemical analysis revealed that JERF1 bound not only to the GCC box but also to the DRE sequence. Expression of the JERF1 gene in tomato was induced by ethylene, MeJA, abscisic acid (ABA) and salt treatment, indicating that JERF1 might act as a connector of different signal transduction pathways. Further research with transgenic JERF1 tobacco (*Nicotiana tabacum* L.) plants indicated that overexpressing JERF1 activated expression of GCC box-containing genes such as *osmotin, GLA, Prb-1b* and *CHN50* under normal growth conditions, and subsequently resulted in enhanced tolerance to salt stress, suggesting that JERF1 modulates osmotic tolerance by activation of downstream gene expression through interaction with the GCC box or DRE. The tomato transcription factor Pti4, an ethylene-responsive factor (ERF), interacts physically with the disease resistance protein Pto and binds the GCC box that is present in the promoters of many pathogenesis-related (*PR*) genes (Chakravarthy *et al*., 2003).

2.4.1.3 S Box

In parsley (*Petroselinum crispum*), members of the ELI7 gene family were rapidly transcriptionally activated following treatment with an elicitor derived from *Phytophthora sojae*. Several cDNA and genomic ELI7 clones were isolated. The deduced amino acid sequences revealed close similarity to fatty acid desaturases/hydroxylases. However, the precise functions are still unknown. Analysis of the promoters of two strongly elicitor-induced family members, ELI7.1 and ELI7.2, allowed Kirsch *et al.* (2000) to functionally pinpoint a novel, independently acting regulatory region (S box), the only major sequence similarity between

the two gene promoters. *In situ* RNA/RNA hybridization using an ELI7.1 gene-specific probe demonstrated that expression of this gene is rapidly and locally induced around infection sites *in planta*.

#### 2.4.1.4 D Box

Box D was discovered as a DNase1 footprint at around -76 to -52 in the parsley *PR2* promoter (Rushton *et al*., 2002). This region (box D short) had a high elicitor inducibility but was weak. Because the extent of the element was unclear, a longer version (box D) containing the next six bases from the *PR2* promoter at the 3' end was constructed and this box D was almost 30 times stronger than 4 X box D short, although inducibility was reduced as a result of increased background levels

#### 2.4.1.5 GST1 element

Usually, only one type of *cis*-acting element is required for pathogen inducibility, some pathogen-inducible promoters contain elements of more than one type. An example is the Gst1 box, which contains both a W box and an S box. This places the *gst1* gene under the control of both WRKY and AP2/ERF transcription factors. The potato *gst1* promoter has been shown to be activated transcriptionally in response to pathogens (Strittmatter *et al*., 1996). It may be common that signalling pathways operating via different transcription factors can target the same gene; another example is the parsley *WRKY1* gene (a W box and a GCC box) (Eulgem *et al*., 1999).

#### 2.4.1.6 Myb recognition elements

P box from the parsley phenylalanine ammonia lyase (PAL), L box from and 4-coumarate: CoA ligase (4CL) and H box from the bean chalcone synthase gene *Chs15* are known to be the recognition elements for Myb (myeloblastosis) transcription factor (Logemann *et al*., 1995; Faktor *et al*., 1997b). Boxes P and L were initially identified as the sites of fungal elicitor-inducible DNA-protein interactions (Lois *et al*., 1989) and were subsequently shown to bind a Myb-like protein, BPF-1 (da Costa e Silva *et al*., 1993; Feldbrugge *et al*., 1997). BPF-1 mRNA accumulates rapidly in elicitor-treated parsley cells suggesting that it participates in the plant defence response (da Costa e Silva *et al*., 1993).

However, in Arabidopsis, Myb mRNA was induced by dehydration and disappeared upon rehydration. It also accumulated upon salt stress and with the onset of treatment with abscisic acid. AtMyb2 bound specifically to a consensus sequence, TAACTG found in maize *bronze1* and Arabidopsis *rd22* promoter (Urao *et al*., 1993).

#### 2.4.1.7 G box

G-Boxes (CACGTG) function during the regulation of diverse genes by environmental cues, such as abscisic acid (ABA), light, UV radiation and wounding, as well as pathogen signals (Menkens *et al*., 1995). G-Boxes are members of the family of ACGT-containing *cis*-acting elements and have been implicated in the expression of a number of genes during pathogen attack (Kim *et al*., 1992). Often they function in concert with other *cis*-acting elements (Faktor *et al*., 1997b).

#### 2.4.1.8 *as-l* element

Another class of elements implicated in the plant defense response, consists of *as-l*-like elements. The *as-1* element (CTGACGTAAGGGATGACGCAC) was initially identified in the 35s promoter of cauliflower mosaic virus and the nos and ocs promoters of *Agrobacterium tumijkiens* (Lam *et al*., 1989; Ellis *et al*., 1993). The *as-2* element confers responsiveness to several signals, including salicylic acid, auxin, jasmonates and hydrogen peroxide (Yang *et al*., 1997). Although the *as-l* element is responsible for the activation of a number of pathogen genes, it is not clear whether functional as-Z-like elements are found widely in plant genes (Ellis *et al*., 1993). Both the G-Box and the *as-l* or ocs element are bound by bZIP proteins.

### 2.4.2 Synthetic promoters

Pathogen-inducible plant promoters contain multiple *cis*-acting elements, only some of which may contribute to pathogen inducibility. This realization has lead attempts on precise

promoter tuning by selectively including *cis*-elements or core elements or boxes that contribute significantly to promoter strength and activity. Randomizing these elements from various sources could be done by synthetic promoters. Also, defense signalling is largely conserved across species, as it has been shown with potato Gst1 box which was active in parsley and Arabidopsis (Eulgem *et al*., 1999).

Synthetic promoters might comprise consensus DNA sequences of common elements of natural promoter regions, or primary elements of a promoter region from diverse origins. A range of pathogen-inducible *cis*-acting elements can alone mediate pathogen-inducible expression. When taken out of their native promoter contexts and framed as components of synthetic promoters, they retain pathogen inducibility directing expression that is local and that correlates with the extent of growth of the pathogen. Though several *cis*-acting elements are known to be pathogen-inducible (Gurr and Rushton, 2005), very few of them have been tested using synthetic promoters. They include W, GCC, S, D and Gst1 boxes.

Although more than one type of *cis*-acting element is not required for pathogen inducibility, some pathogen-inducible promoters contain elements of more than one type. An example is the Gst1 box, which contains both a W box and an S box. This places the *gst1* gene under the control of both WRKY and AP2/ERF transcription factors (Strittmatter *et al*., 1996). It may be common that signalling pathways operating via different transcription factors can target the same gene.

## 2.5 Promoter-probe vector

Transcriptional fusions are important tools in understanding gene expression and gene regulation. The construction of such fusions is greatly facilitated by the use of promoter-probe vectors. These vectors have a common motif in which a promoterless reporter gene, encoding an easily assayable protein, is present downstream of one or more restriction sites. Known promoter sequences or uncharacterized segments of genomic DNA can be ligated into these restriction sites, and the expression of the reporter gene can then be quantified under various conditions. Initially the efforts were made to construct promoter-probe vectors for use in *Escherichia coli*. Such vectors, in order to be of the greatest use, should (i) function in as many taxa as possible, (ii) show a high degree of sensitivity to detect promoters that are of weak to moderate strength, and (iii) be stable enough to be used *in vivo* without antibiotic selection. Most of the early promoter-probe vectors that were constructed used the *lacZ* reporter gene and contained either the ColE1 or p15a origin of replication (Casadaban and Cohen, 1980; Silhavy and Beckwith, 1985; Simons *et al*., 1987).

These vectors, while suitable for use in *E. coli* either could not be maintained in many other taxa or would not be appropriate in organisms or hosts with native β-galactosidase activity. The ability to construct transcriptional fusions in organisms other than *E. coli* is increasingly important. In order for promoter-probe vectors to be more versatile, they need to contain broad-host-range origins of replication. Several such vectors have been described (Konyecsni and Deretic, 1988; Diaz and Garcia, 1990; Ronald *et al*., 1990). Additionally, many organisms are naturally resistant to varying levels of one or more antibiotics (Nikaido and Vaara, 1985). Therefore, a set of promoter-probe vectors should also contain a wide variety of antibiotic resistance genes. Some promoter-probe vectors have been described in which interference by read-through transcription is largely reduced by the addition of one or more transcriptional terminators upstream of the multiple cloning site (MCS) (Simons *et al*., 1987). Although elimination of upstream transcription increases the overall sensitivity of the vector, it is also important to consider the sensitivity of the reporter gene. The level of expression of some reporter genes that have been fused to weakly transcribed promoters may fall below the level of detectability. Some reporter genes, such as *inaZ*, which encodes a bacterial ice nucleation protein, are substantially more sensitive than *lacZ* or *gfp* (Miller *et al*., 2000). The sensitivity of the reporter gene is also an important consideration when a low-copy-number plasmid, such as a broad-host-range vector, is used.

Achieving optimal transcription and translation of foreign genes integrated into the host's chromosome is important in meeting desired product yields. Since gene expression can be influenced by promoter strength, promoter probe vectors have been used in many studies to identify promoter sequences for the heterologous expression of genes of interest

(Gat *et al*., 2003). One method of using promoter selection/probe vectors is to insert DNA fragments into a polylinker region that precedes a promoterless reporter gene. DNA fragments that turn on the expression of the reporter gene contain promoter sequences. Another method involves the use of promoter probe transposons to insert promoterless reporter genes randomly into the chromosome, which can often generate transcriptional fusions, as well as insertional mutations (Springer, 2000). Additionally, promoter probe vectors have been used to identify resident promoters that allow the expression of single-copy of recombinant genes in the chromosome (Karlyshev *et al*., 2000).

## 2.6 Expression analysis/validation with reporters

Fluorescent proteins as a tool in gene expression have revolutionized cell biology over the last decade, particularly in studies of living cells. The green fluorescent protein (GFP) is a spontaneously fluorescent polypeptide of 27kDa, derived from the jellyfish *Aequorea victoria*, which absorbs UV light and emits in the green region of the spectrum. Unlike the bacterial β-galactosidase and β-glucuronidase, which are widely used reporters in fungi, GFP does not rely on exogenous substrates or cofactors other than oxygen (Prasher *et al*., 1992). Therefore, GFP can be used as a fusion tag to localize proteins, to follow their movement, or to study the dynamics of the subcellular compartments to which these proteins are targeted (Prasher *et al*., 1992; Chalfie *et al*., 1994).

The use of GFP as a reporter gene has several advantages over commonly used reporter genes such as firefly luciferase and GUS. Assay for GFP is non-destructive, does not need any substrate or cofactors, can report in real-time and can be remotely sensed. Thus GFP has become a useful reporter marker for gene expression and regulation in plants (Stewart and Stewart, 2001). However, wild type GFP has two main disadvantages. First, folding of GFP at temperatures above $25^0$C is inefficient leading to large amounts of insoluble protein. Extensive mutagenesis of GFP in combination with DNA shuffling yielded a triple mutant GFPuv (F99S, M153T, V163A), which increased the folding efficiency at $37^0$C and concomitantly the brightness of cells expressing GFPuv increased16-fold over wild-type GFP (Crameri *et al*., 1996). In addition, the tendency of GFPuv to aggregate at high concentrations seemed to be reduced and its diffusability inside cells was increased (Yokoe and Meyer, 1996). Second, the relatively low intrinsic fluorescence of GFP was improved by mutations of the GFP chromophore (Heim *et al*., 1994; Tsien, 1998). GFPmut1, a GFP variant with the chromophore mutations F64L and S65T has a 35-fold increased fluorescence compared to wild-type (wt)GFP when excited at 488 nm (Cormack *et al*., 1996). This protein is identical to EGFP, which is commercially available and widely used and has become the de facto standard, although only about 20% of EGFP/GFPmut1 is correctly folded at $37^0$C (Tsien, 1998). Further, Scholz *et al*., (2000) constructed GFP+, which is about 130-fold brighter than wild-type GFP. Furthermore the *mgfp5-er* variant is optimized for expression in plants (Haseloff *et al*., 1997). The recent development of variants of GFP with altered codon composition has facilitated efficient expression of this reporter protein in a number of fungal species.

# 3. MATERIAL AND METHODS

The present study was undertaken to design pathogen-inducible promoters, and to construct and functionally validate a promoter-probe vector with *gfp* reporter gene. The materials used and methods employed are as follows.

## 3.1 Construction of promoter probe binary vector

### 3.1.1 PCR cloning of *gfp* reporter gene from pKU352NA into pUbi 1casER$^-$

Standard procedures for plasmid DNA isolation and manipulation were performed as described by Sambrook and Russell (2001). *SgfpS65T* (an improved *gfp*) gene was PCR amplified from the template DNA of pKU352NA (kindly donated by Dr. Narayana Upadhyaya, CSIRO PI, Canberra) using two specific primers designed to contain *Bam*HI and *Kpn*I sites.

| | |
|---|---|
| RB20_GFP_F | 5' CATGGATCCATGGTGAGCAAGGGCGAG 3' |
| RB20_GFP_R | 5' AAGGGTACCCTTGTACAGCTCGTCCATGC 3' |

PCR was carried out using a DNA Engine, Dyad PCR machine (BIORAD) with Phusion High fidelity PCR master Mix (NEB, #F-531S) containing 100ng of the plasmid DNA, 100µM of each primer. PCR condition for each cycle consisted of denaturation at 94°C for 1 min, annealing of the primers at 58°C for 1 min, and extension at 72°C for 2 min, and it was repeated for 35 cycles. PCR product of ~750bp was eluted from agarose gel, and purified using QIAGEN Gel Extraction kit (QIAGEN, #28006), and quantified by comparing with standard DNA marker. Both PCR product and pUbi1casER$^-$ vector (kindly donated by Dr. Narayana Upadhyaya, CSIRO PI, Canberra) were restricted with *Bam*HI + *Kpn*I to have compatible cohesive ends and also for directional cloning. The restricted products after purification were allowed to ligate with a molar ratio of 1:3 (ends of vector: insert) at 16°C for 16 h. The products were transferred to *E. coli* DH5α. Clones were confirmed by PCR with *SgfpS65T*-specific primers, restriction analysis with *Bam*HI + *Kpn*I to release *SgfpS65T*, and *Bam*HI + *Eco*RI to release *SgfpS65T* + *Nos*-Ter. However, open reading frame of *SgfpS65T* was checked by sequencing. Finally, the right clone carrying SgfpS65T in pUbi1casER$^-$ vector background was named as pRR19.

### 3.1.2 Sub-cloning of *SgfpS65T* + *Nos*-Ter cassette into pCAMBIA1305.1 to get promoter-probe vector

pRR19 containing SgfpS65T + *Nos*-Ter cassette was restricted with *Bam*HI+*Eco*RI. The fragment of ~1100bp was eluted and purified (as described in the section 3.1.1.) and ligated with pCAMBIA1305.1 (obtained from CAMBIA, Canberra) cut with the same enzymes (*Bam*HI + *Eco*RI) to have the directional cloning. Ligation and DH5α transformation were carried out as mentioned in the section 3.1.1. The recombinant clones were identified by blue/white colony assay. Further confirmation was done by *SgfpS65T*-specific PCR, restriction digestion with *Bam*HI + *Eco*RI, and sequencing with M13 primers. The recombinant clones were named as pRR21.

## 3.2 Construction of promoter-probe vector with CaMV 35s promoter

### 3.2.1 PCR cloning of CaMV 35S promoter from pWBVec8 into promoter-probe vector

CaMV 35S promoter was PCR amplified from pWBVec8 (kindly donated by Dr. Ming-Bo Wang, CSIRO PI, and Canberra) using two specific primers designed to contain sites for *Hind*III and *Bam*HI.

| | |
|---|---|
| RB19_35S_F | 5' ACG AAGCTT CGTCAACATGGTGGAGCA 3' |
| RB19_35S_R | 5' GAC GGATCC GTCCTCTCCAAATGAAAT 3' |

PCR product of 429bp and pRR21 were cut with *Hind*III + *Bam*HI, purified and ligated as described in 3.1.1. Ligated products were transferred to DH5α. Clones were confirmed by restriction analysis with *Bam*HI and *Hind*III to release the CaMV 35S promoter, and *Bam*HI and *Eco*RI to release the whole cassette containing CaMV 35S + *SgfpS65T* + *Nos*-Ter, and also confirmed by sequencing. The true clone was named as pRR20.

## 3.3 Designing and synthesis of pathogen inducible promoters

Novel synthetic promoters were designed by incorporating *cis*-regulatory elements such as W, GCC, S and Myb boxes which are known to be pathogen-inducible (Rushton *et al*., 2002; Gurr and Rushton, 2005; Jung *et al*., 2006) along with spacing nucleotides between the two boxes. W 2 X-BOX, GCC 2 X- BOX, GCC 3 X- BOX, S 2 X-BOX and Myb 2 X-BOX promoters were designed as per the sequence of PCU48862, AF121353, AF239835, and AY876012 (Acc. No.), respectively. Care was taken to add CaMV 35S promoter -46 region, a minimal promoter (Benfey *et al*., 1989; Sundaresan *et al*., 1995) at 3' end. All the five synthetic promoters were designed to have *Hind*III and *Pst*I sites at 5' and 3' end, respectively. These promoters were synthesized and cloned into *Kpn*I and *Sac*I sites of pGA4 by GENEART, Regenburg, Germany.

## 3.4 Cloning of synthetic promoters into promoter-probe vector

The synthetic promoters W 2 X-BOX, GCC 2 X- BOX, GCC 3 X- BOX, S 2 X-BOX and Myb 2 X-BOX cloned into pGA4 were released by restriction with *Hind*III + *Pst*I, and purified. These promoters were ligated with pRR21 cut with *Hind*III + *Pst*I to facilitate direction cloning resulting into complete expression cassette. Clones were confirmed by restriction analysis with *Hind*III + *Pst*I, and by sequencing. The clones carrying W 2 X-BOX, GCC 2 X-BOX, GCC 3 X- BOX, S 2 X-BOX and Myb 2 X-BOX promoters were named as pRR32, pRR33, pRR34, pRR35 and pRR36, respectively.

## 3.5 Mobilizing recombinant promoter-probe vectors into *Agrobacterium*

For functional validation and expression analysis, the confirmed clones of pRR20, pRR32, pRR33, pRR34, pRR35 and pRR36 were transformed to *Agrobacterium tumefaciens* strain LBA4404 by triparental mating.

## 3.6 Tobacco transformation

For functional validation of pRR20, A. *tumefaciens* strain LBA4404 containing pRR20 and pCAMBIA1305.1 (negative control) were used for tobacco leaf disc transformation by following the standard protocol (Hooykaas and Schilperoort, 1992) with minor modifications.

## 3.7 SgfpS65T expression analysis

Expression of SgfpS65T was observed in co-cultivated tobacco leaf discs and *de novo* callus after 15 and 30 days of co-cultivation, respectively. Co-cultivated leaf discs and calli were observed under stereo-microscope (Olympus, SZX-16) at National Centre for Biological Science (NCBS), GKVK campus, Bangalore. Images of SgfpS65T expressing tissues were captured.

# 4. EXPERIMENTAL RESULTS

In this study, efforts were made to design pathogen-inducible promoters (synthesized at GENEART, Regenburg, Germany) to construct and functionally validate a novel promoter-probe vector with *SgfpS65T* reporter gene, and to sub-clone synthetic promoters into promoter-probe vector expression analysis. The results of various experiments are presented here.

## 4.1 Designing synthetic promoters

Novel synthetic promoters (Fig. 1) were designed by incorporating *cis*-regulatory elements such as W, GCC, S and Myb boxes which are known to be pathogen-inducible along with spacing nucleotides between the two boxes. Care was taken to add a minimal promoter sequence at 3' end. All the five synthetic promoters were designed to have *Hind*III and *Pst*I sites at 5' and 3' end, respectively. These promoters were synthesized and cloned into sites *Kpn*I and *Sac*I of pGA4 by GENEART, Regenburg, Germany.

### 4.1.1 W 2 X- BOX (106bp)

W 2 X- Box promoter was designed by placing 2 copies of W Box unit (30bp) in tandem following CaMV 35S promoter -46 region (a minimal promoter). Each unit carried a W box (5'TTGACC3') along with its spacer (18bp at 5' and 6bp at 3').

### 4.1.2 GCC 2 X- BOX (104bp)

GCC 2 X- Box promoter contained 2 copies of GCC Box unit (29bp) in tandem following CaMV 35S promoter -46 region. Each unit carried a GCC box (5'AGCCGCC3') along with its spacer (15bp at 5' and 7bp at 3').

### 4.1.3 GCC 3 X -BOX (133bp)

GCC 3 X- Box promoter was similar to GCC 2 X- Box, but it contained 3 copies of GCC Box unit (29bp) in tandem following a minimal promoter.

### 4.1.4 S 2 X- BOX (102bp)

S 2 X- Box promoter was designed by placing 2 copies of S Box unit (28bp) in tandem following CaMV 35S promoter -46 region. Each unit carried a W box (5'AGCCACC3') along with its spacer (15bp at 5' and 6bp at 3').

### 4.1.5 Myb 2 X- BOX (100bp)

Myb 2 X- BOX synthetic promoter included 2 copies of Myb box unit (27bp) in tandem following CaMV 35S promoter -46 region. Each Myb-box unit carried a *cis*-regulatory element (5'TAACTG3') and spacers (15bp at 5' and 6bp at 3').

## 4.2 Construction of promoter-probe vector

### 4.2.1 PCR cloning of *SgfpS65T* from pKU352NA

Upon PCR with template plasmid DNA from pKU352NA using *SgfpS65T*-specific primers (RB20_GFP_F/R), a product of ~717bp (Plate 1) was obtained. This amplicon and the vector pUbi1casER$^-$ digested with *Bam*HI + *Kpn*I, were ligated and DH5α cells were transformed with the ligated product. The positive clones were confirmed by restriction with *Bam*HI + *Kpn*I, which release the fragment of ~717bp (Plate 2a). Also the PCR with *SgfpS65T*-specific primers yielded a product of ~717bp (Plate 2b) indicating that the clone is truly a recombinant.

From this recombinant clone (pRR19) (Fig. 2), *SgfpS65T* + *Nos*-Ter fusion fragment was released by restriction with *Bam*HI + *Eco*RI (Plate 2c) and cloned into the same sites of pCAMBIA1305.1 to get pRR21 (Fig. 3). The recombinant clones were selected based on Blue/white colony assay. pRR21 released fragments of ~717 and 988bp upon restriction with

**1] W 2 X- BOX (106)**

AAGCTTTTATTCAGCCATCAAAAG**TTGACC**AATAATTTATTCAGCCATCAA
AAG**TTGACC**AATAAT*CTCCTCTATATAAGGAAGTTCATTTCATTTGGAG*CTGCAG

**2] GCC 2 X- BOX (104 bp)**

AAGCTTAGATGGCGATGGATG**AGCCGCC**CAAGGGTAGATGGCGATGGATG**AGCCGCC**C
AAGGGT*CTCCTCTATATAAGGAAGTTCATTTCATTTGGAG*CTGCAG

**3] GCC 3 X- BOX (133 bp)**

AAGCTTAGATGGCGATGGATG**AGCCGCC**CAAGGGTAGATGGCGATGGATG**AGCCGCC**C
AAGGGTAGATGGCGATGGATG**AGCCGCC**CAAGGGT*CTCCTCTATATAAGGAAGTTCATT*
*TCATTTGGAG*CTGCAG

**4] S 2 X- BOX (102 bp)**

AAGCTTCTATGAATAAAGTAC**AGCCACC**AAAGAGCTATGAATAAAGTAC**AGCCACC**AAAG
AG*CTCCTCTATATAAGGAAGTTCATTTCATTTGGAG*CTGCAG

**5] Myb 2 X- BOX (100 bp)**

AAGCTTATGTTTTGATAAATT**TAACTG**CAATAAATGTTTTGATAAATT**TAACTG**CAATAA*CT*
*CCTCTATATAAGGAAGTTCATTTCATTTGGAG*CTGCAG

**Fig1: Nucleotide sequence of synthetic promoters**

| | |
|---|---|
| Underlined letters | : Restriction sites (*Hind*III and *Pst*I) |
| Bold letters | : *cis*-regulatory elements |
| Times New Roman letters | : Spacer sequences |
| Italics | : Minimal promoter |

*Bam*HI + *Kpn*I and *Bam*HI + *Eco*RI, respectively (Plate 3a and b). Also PCR of pRR21 with *SgfpS65T*-specific primers yielded a product of ~717bp (Plate 3c). These results indicated that pRR21 carried a promoterless *SgfpS65T* + *Nos*-Ter fusion.

### 4.2.2 Sequencing and *in silico* Analysis

Sequence of the full length *SgfpS65T* gene (about 750bp) cloned into pRR21 when sequenced using M13 primers by employing primer walking technique revealed 100% similarity with the *SgfpS65T* of pKU352NA (GenBank Acc. No. DQ225751) indicating that there is no mismatch, and the coding sequence is intact. Complete sequence and feature information of pRR21 has been deposited at GenBank of NCBI (EU760495).

## 4.3 Construction of recombinant promoter-probe vectors

Recombinant promoter-probe vectors were constructed by placing either CaMV 35S promoter or synthetic promoters upstream (5' end) of *SgfpS65T* + *Nos*-Ter fusion for functional validation of the promoter-probe vector and analysis of the pathogen-inducible promoters, respectively.

### 4.3.1 Promoter-probe vector with CaMV 35S promoter

A PCR product of 423bp was amplified from pWBVec8 (Plate 4) with specific primers (RB19_35S_F/R) carrying *Hind*III and *Bam*HI sites, and cloned into the same sites of pRR21 to get pRR20 (Fig. 4) carrying the complete expression cassette of CaMV 35S + *SgfpS65T* + *Nos*-Ter. pRR20 when subjected to PCR using CaMV 35S-specific primers yielded an amplicon of 423bp (Plate 5a). The PCR positive clones were also restricted with *Hind*III + *Bam*HI to release 423bp fragment (Plate 5b). Also when restricted with *Hind*III + *Eco*RI, entire expression cassette containing CaMV 35S + *SgfpS65T* + *Nos*-Ter was released (Plate 5c).

### 4.3.2 Promoter-probe vector with synthetic promoters

Pathogen-inducible promoters that were designed in this study were synthesized and cloned into *Kpn*I and *Sac*I sites of pGA4 by GENEART, Regenburg, Germany. Upon receipt of these recombinant vectors, they were restricted with *Hind*III + *Pst*I (Plate 6) to release the synthetic promoters. W 2 X- BOX, GCC 2 X- BOX, GCC 3 X-BOX, S 2 X- BOX, Myb 2 X-BOX promoters were cloned into pRR21 cut with *Hind*III + *Pst*I to yield pRR32, pRR33, pRR34, pRR35, and pRR36 (Fig. 5), respectively. Since the length of these promoters ranged from 106–139bp, which is too small to be observed on normal agarose gel, the recombinant promoter-probe vectors were confirmed by sequencing with M13 primers. However, the complete expression cassette containing synthetic promoter + *SgfpS65T* + *Nos*-Ter was released with *Hind*III + *Eco*RI for confirmation (Plate 7).

### 4.3.3 Transformation of *Agrobacterium tumefaciens* with recombinant promoter-probe vectors

pRR20, pRR32, pRR33, pRR34, pRR35, pRR36, and pCAMBIA1305.1 (negative control) vectors were transferred to *Agrobacterium tumefaciens* LBA4404 strain by tri-parental mating using *Escherichia coli* pRK2013 as helper strain. The recombinant *A. tumefaciens* carrying pRR20, pRR32, pRR33, pRR34, pRR35 and pRR36 were confirmed by *SgfpS65T*-specific PCR (Plate 8), where as pCAMBIA1305.1 was confirmed with *nptII*-specific PCR, which yielded 794bp expected sized product (Plate 9).

## 4.4 Functional validation of promoter-probe vector

Before analysing the pathogen-inducibility of the synthetic promoters designed in this study, it was important to make sure that the *SgfpS65T* + *Nos*-Ter fusion would function in the promoter-probe vector (pRR21). For this, *A. tumefaciens* with pRR20 carrying CaMV 35S + *SgfpS65T* + *Nos*-Ter cassette and with pCAMBIA1305.1 (as a negative control) were used for tobacco leaf disc co-cultivation. Such leaf discs and the calli originated from them were observed under fluorescent microscope for SgfpS65T florescence.

**Plate 1. PCR Amplification of *SgfpS65T* from pKU352NA**

M : λ DNA /*Eco*RI/*Hind*III double digest

1-3 : Amplification of *SgfpS65T* (717 bp) from pKU352NA

**Plate 2. Restriction and PCR confirmation of pRR19**

**Plate 2a: PCR confirmation of *SgfpS65T* in pRR19**

M : 100 bp DNA ladder

1-5: Amplification of *SgfpS65T* (717 bp) from pRR19

**Plate 2b: Restriction confirmation of *SgfpS65T* in pRR19**

M : 500bp DNA ladder

1-3: pRR19 digested with *Bam*HI + *Kpn*I

**Plate 2c: Restriction confirmation of *SgfpS65T* + *Nos*-Ter in pRR19**

M : 500 bp DNA ladder

1-4: pRR19 digested with *Bam*HI + *Eco*RI

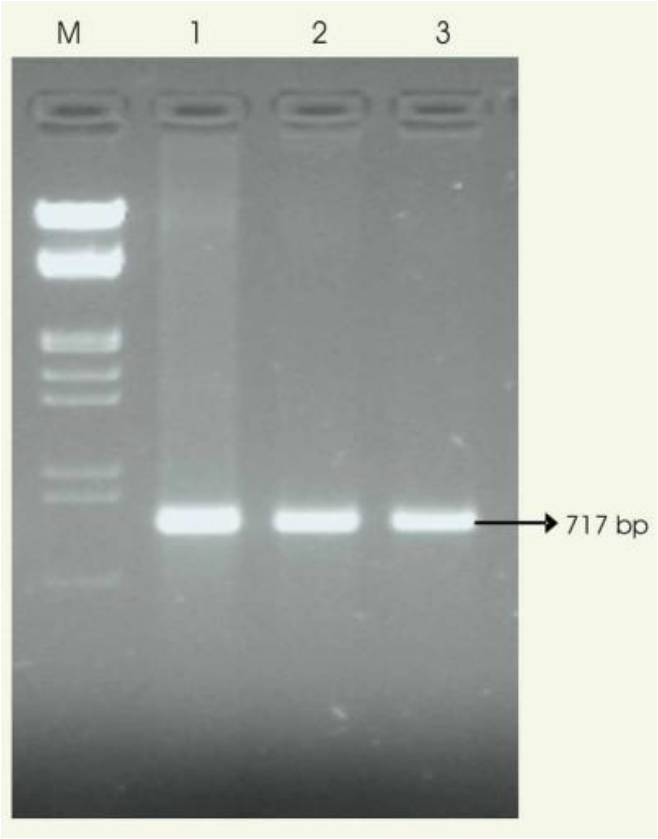


**Plate 1.PCR amplification of *SgfpS65T* from pKU352NA in pRR19**

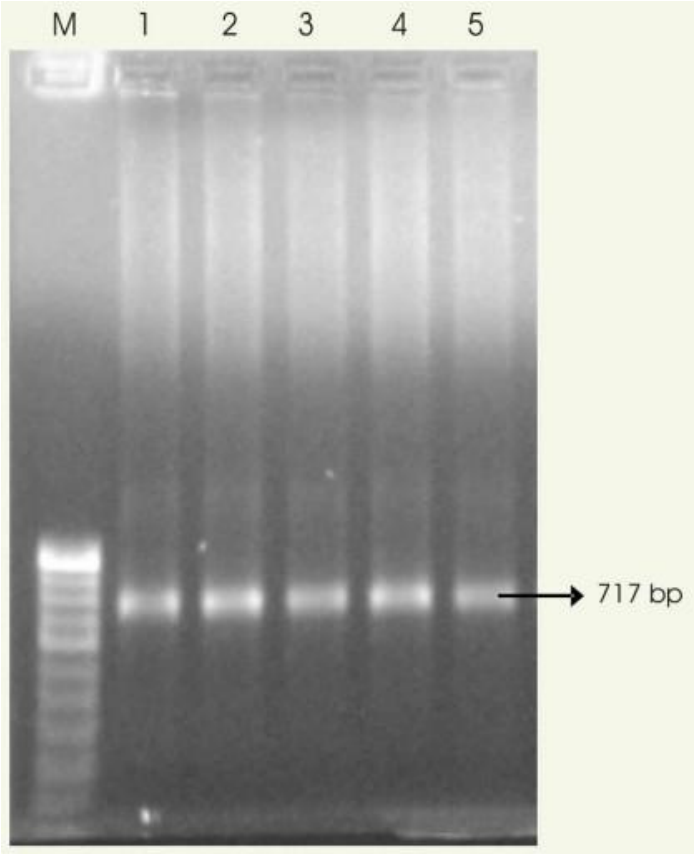


**Plate 2a. Restriction and PCR confirmation of SgfpS 65T**

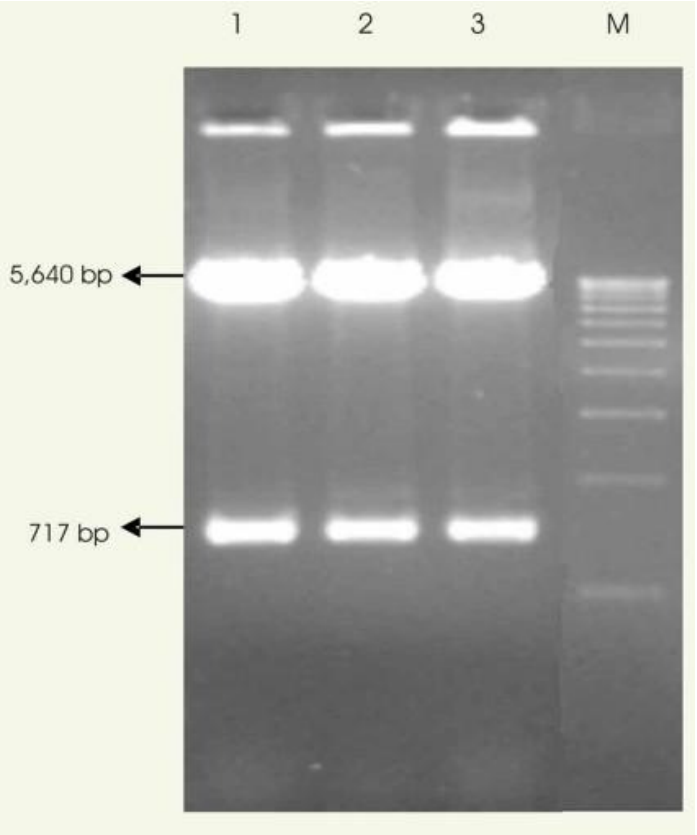


**Plate 2b. Restriction (BamHI + KpnI) confirmation of SgfpS65T in pRR19**

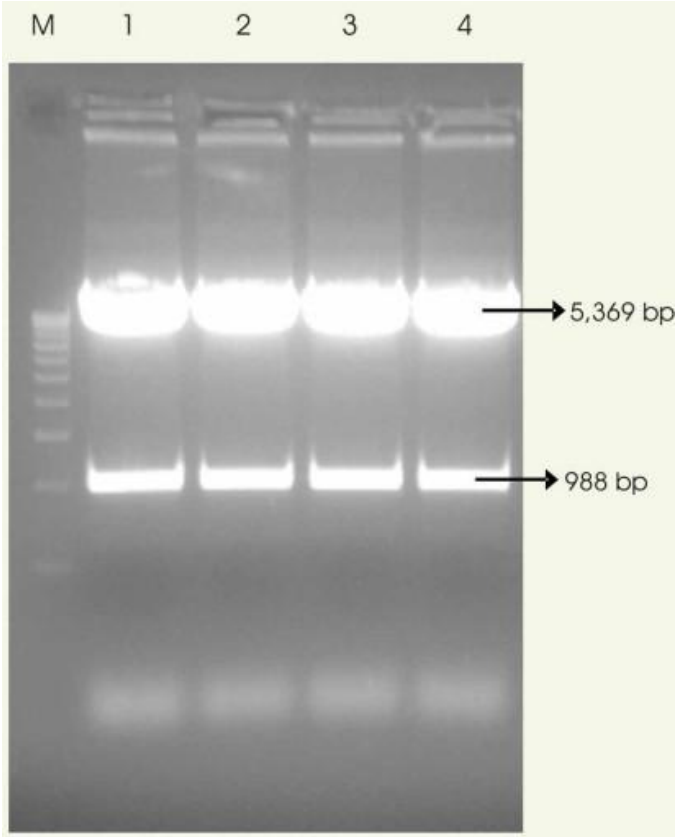


**Plate 2c. Restriction (BamHI + EcoRI) confirmation of SgfpS65T + Nos – Ter in pRR19**

**Plate 2. Restriction and PCR confirmation of pRR19**

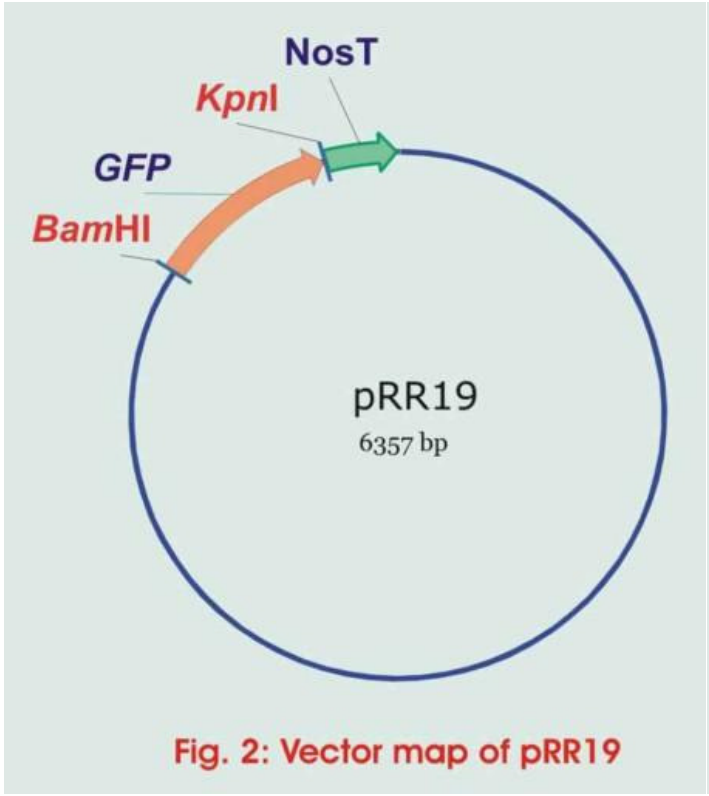


Fig 2. Vector map of pRR19

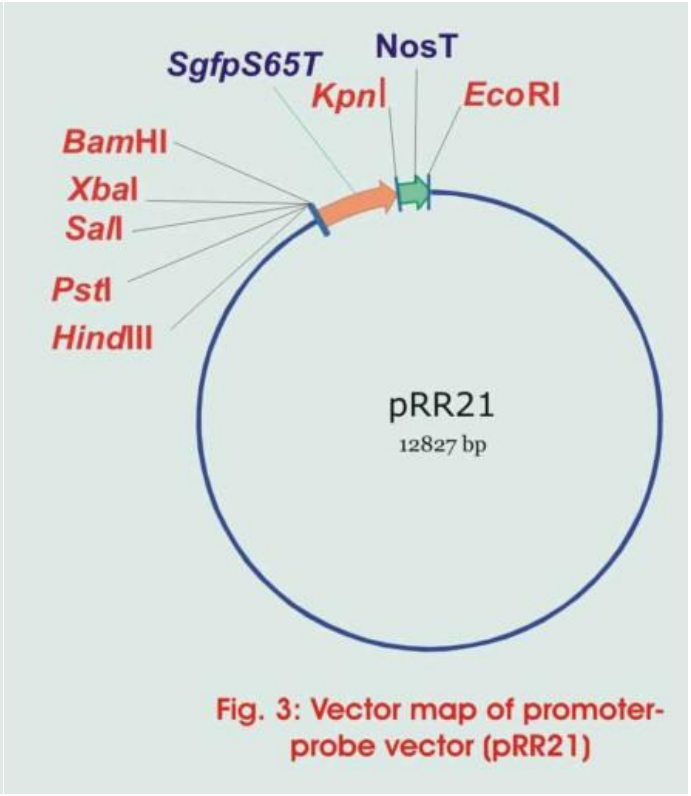


Fig 3. Vector map of promoter-probe vector (pRR21)

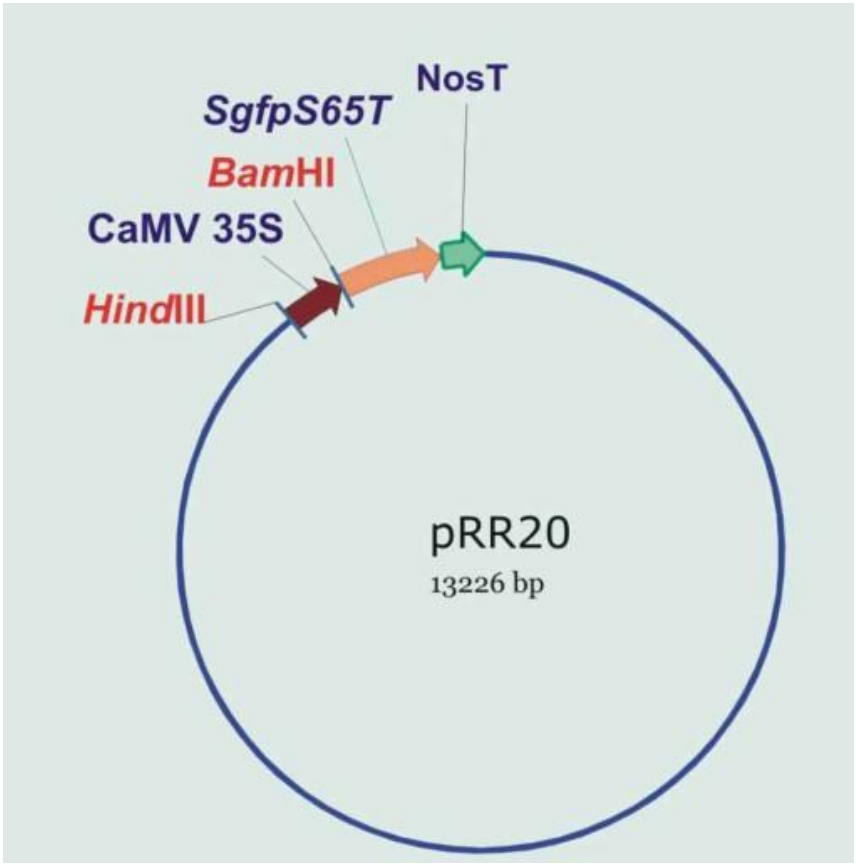


Fig 4. Vector map of recombinant promoter-probe vector (pRR20)

**Plate 3: Restriction and PCR confirmation of promoter-probe vector (pRR21)**

**Plate 3a: Restriction (*Bam*HI + *Kpn*I) confirmation of *SgfpS65T* in pRR21**

M : λ DNA /*Eco*RI/*Hind*III double digest

1-2: pRR21 digested with *Bam*HI + *Kpn*I

**Plate 3b: Restriction (*Bam*HI + *Eco*RI) confirmation of *SgfpS65T* + *Nos*-Ter in pRR21**

M : λ DNA /*Eco*RI/*Hind*III double digest

1-2: pRR21 digested with *Bam*HI + *Eco*RI

**Plate 3c: PCR confirmation of *SgfpS65T* in pRR21**

M : 100 bp DNA ladder

1-4: Amplification of *SgfpS65T* (717 bp) from pRR21

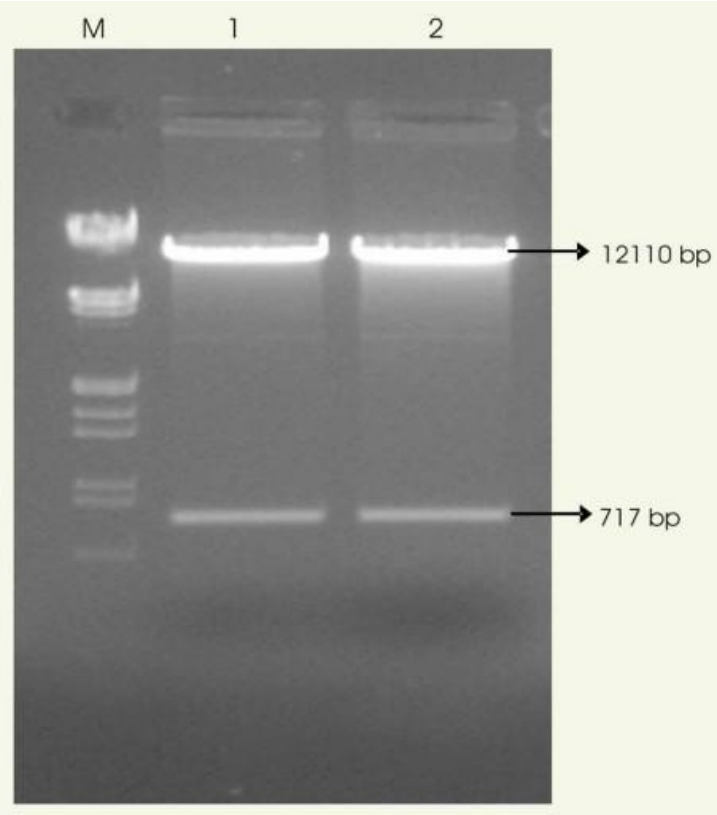


**Plate 3a. Restriction (BamHI + KpnI) confirmation of SgfpS65T in pRR21**

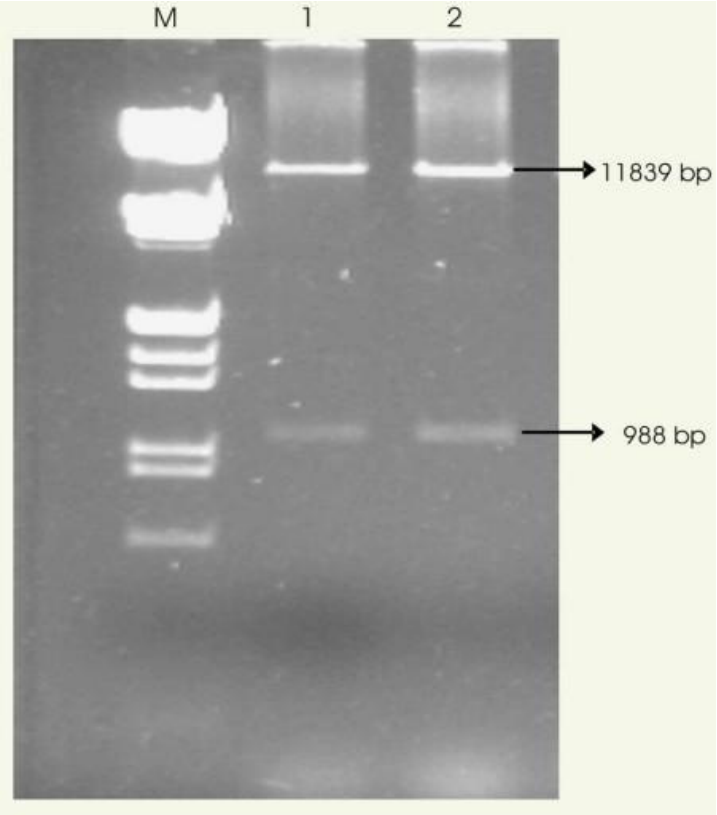


**Plate 3b. Restriction (BamHI + EcoRI) confirmation of SgfpS65T + Nos-Ter in PRR21**

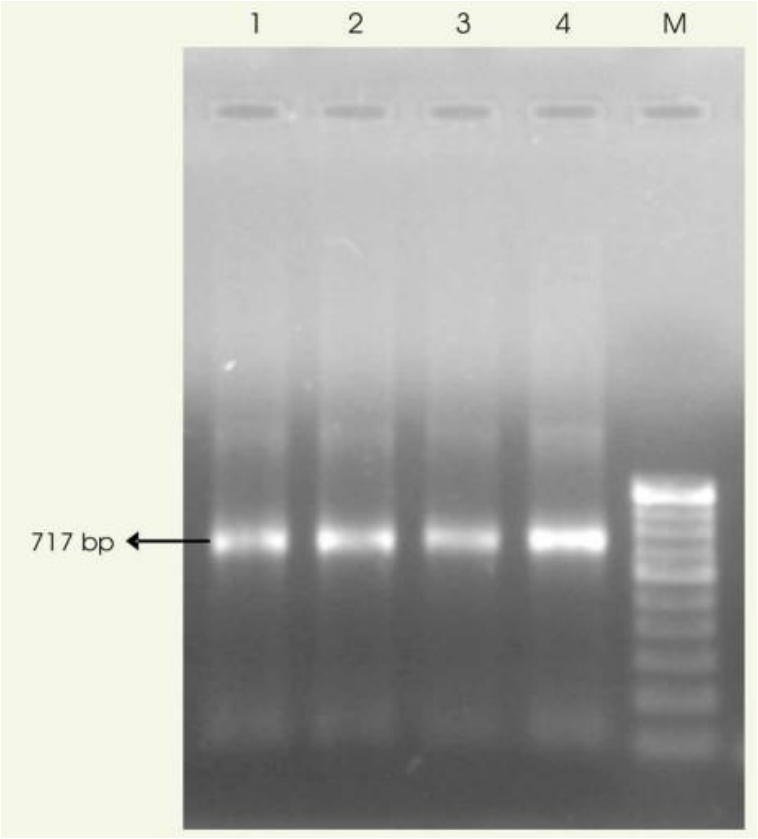


**Plate 3c. PCR confirmation of SgfpS65T in pRR21**

**Plate 3. Restriction and PCR confirmation of promoter-probe vector (pRR21)**

**Plate 4: PCR Amplification of CaMV 35S from pWBVec8**

M : 100 bp DNA ladder

1-6: Amplification of CaMV 35S (423 bp) from pWBVec8

**Plate 5: Restriction and PCR confirmation of pRR20**

**Plate 5a: PCR confirmation of CaMV 35S in pRR20**

M : 100 bp DNA ladder

1-3: Amplification of CaMV 35S (423 bp) from pRR20

**Plate 5b: Restriction (*Hind*III + *Bam*HI) confirmation of CaMV 35S in pRR20**

M : 500 bp DNA ladder

1-2: pRR20 digested with *Hind*III + *Bam*HI

**Plate 5c: Restriction (*Bam*HI + *Eco*RI) confirmation of CaMV35S + *SgfpS65T* + *Nos*-Ter in pRR20**

M : λ DNA /*Eco*RI/*Hind*III duble digest

1-2: pRR21 digested with *Bam*HI + *Eco*RI

3-4: pRR20 digested with *Bam*HI + *Eco*RI

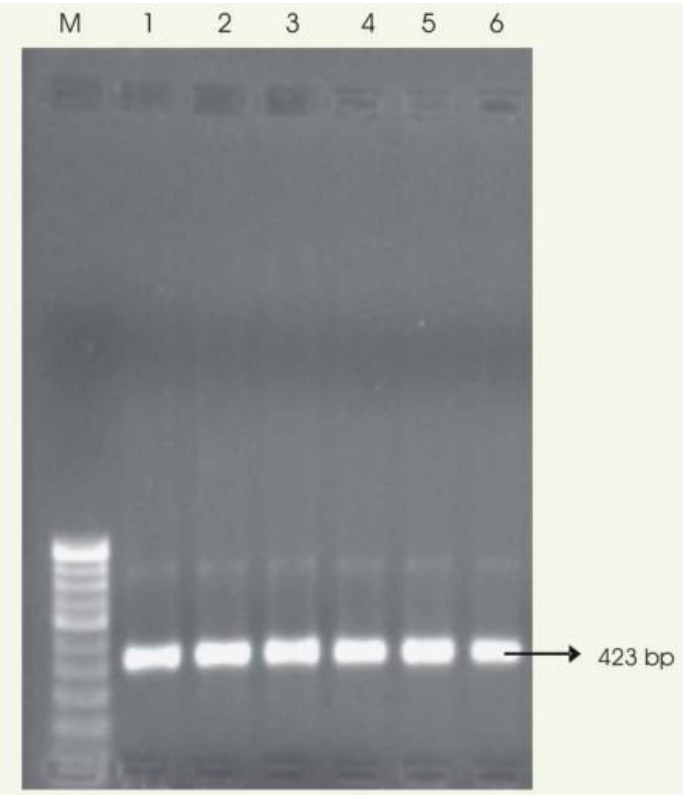


**Plate 4. PCR amplification of CaMV 35S from pWBVec8**

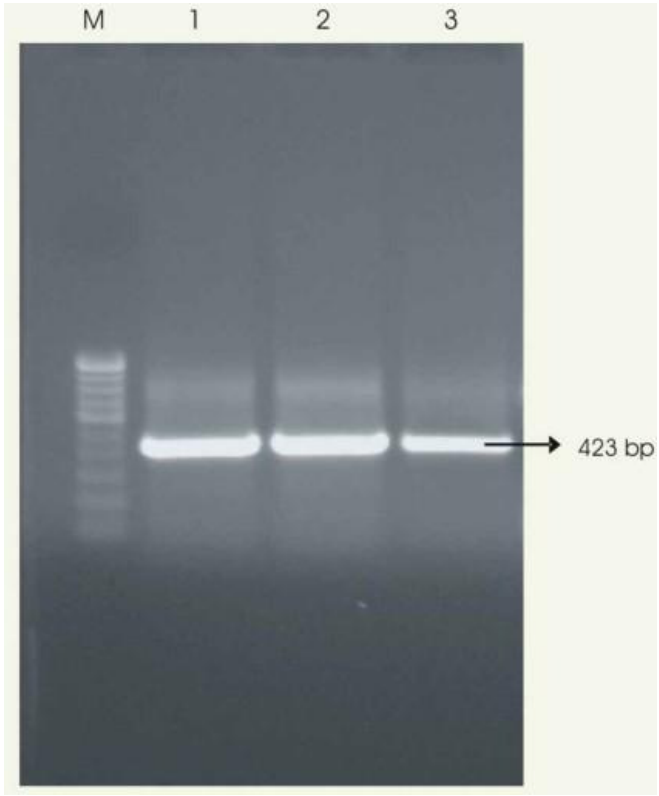


**Plate 5a. PCR confirmation of CaMV 35S in PRR20**

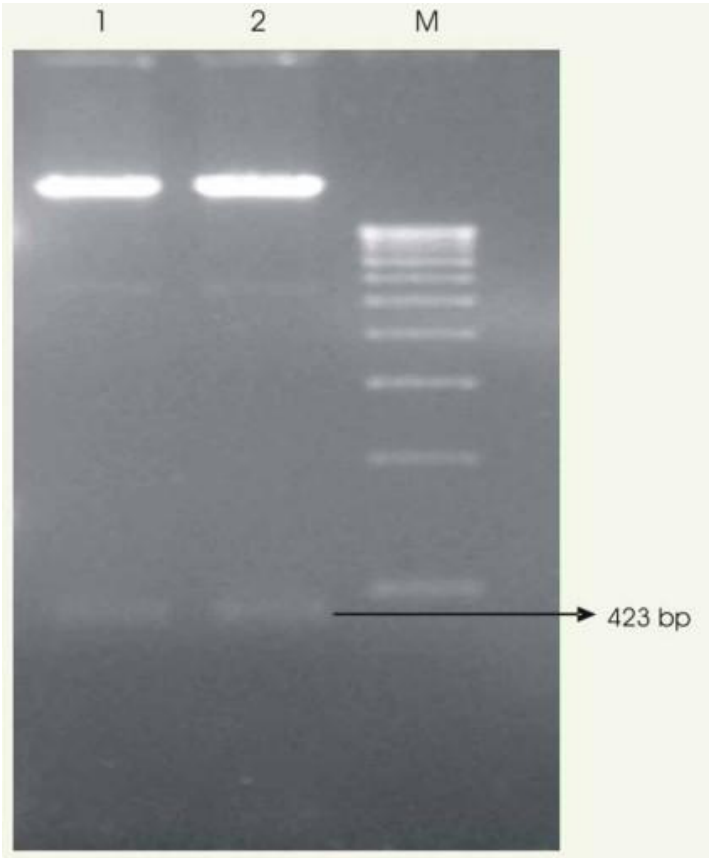


**Plate 5b. Restriction (HindIII + BamHI) confiramation of CaMV 35S in PRR20**

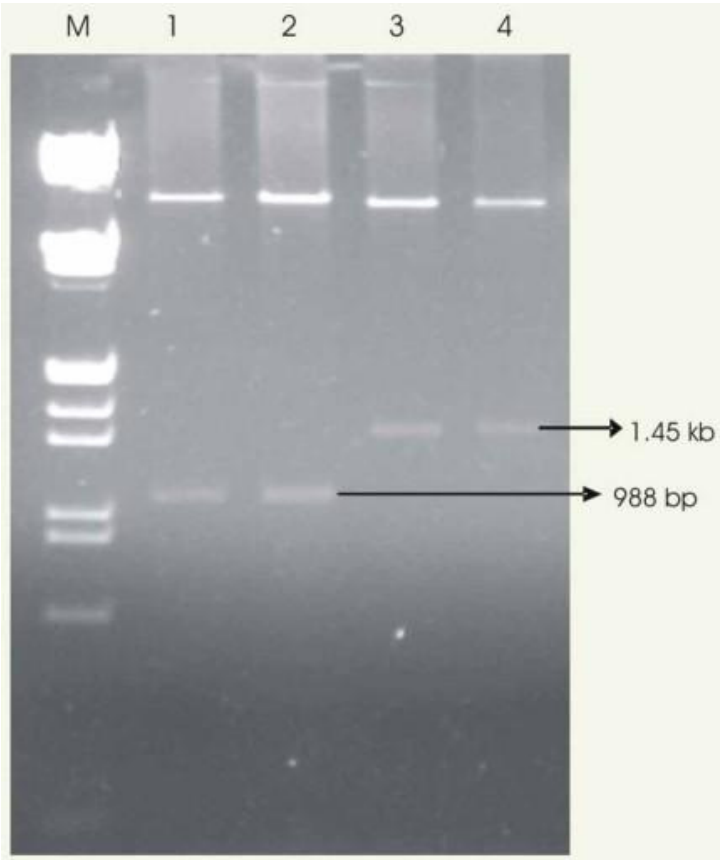


**Plate 5c. Restriction (BamHI + EcoRI) confirmation of CaMV 35S + SgfpS65T + Nos – Ter in pRR20**

**Plate 5. Restriction and PCR confirmation of pRR20**

**Plate 6: Restriction (*Hind*III + *Pst*I) confirmation of synthetic promoters in pGA4**

**Plate 6a: W 2 X- Box and GCC 3 X- Box promoters released with *Hind*III + *Pst*I**

M : 100 bp DNA ladder

1-3: GCC 3 X- Box promoter

4-7: W 2 X- Box promoter

**Plate 6b: GCC 2 X- Box and Myb 2 X- Box promoters released with *Hind*III + *Pst*I**

M : 100 bp DNA ladder

1-3: GCC 2 X- Box promoter

4-6: Myb 2 X- Box promoter

**Plate 6c: S 2 X- Box promoter released with *Hind*III + *Pst*I**

M : 100 bp DNA ladder

1-2: S 2 X- Box promoter

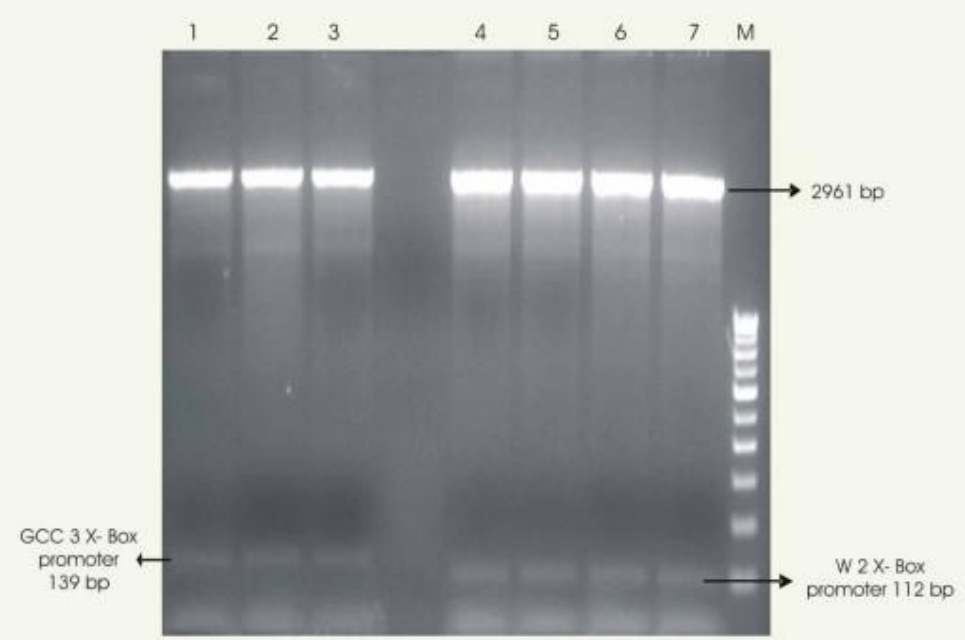


**Plate 6a. W 2X- Box and GCC 3X – Box promoters**

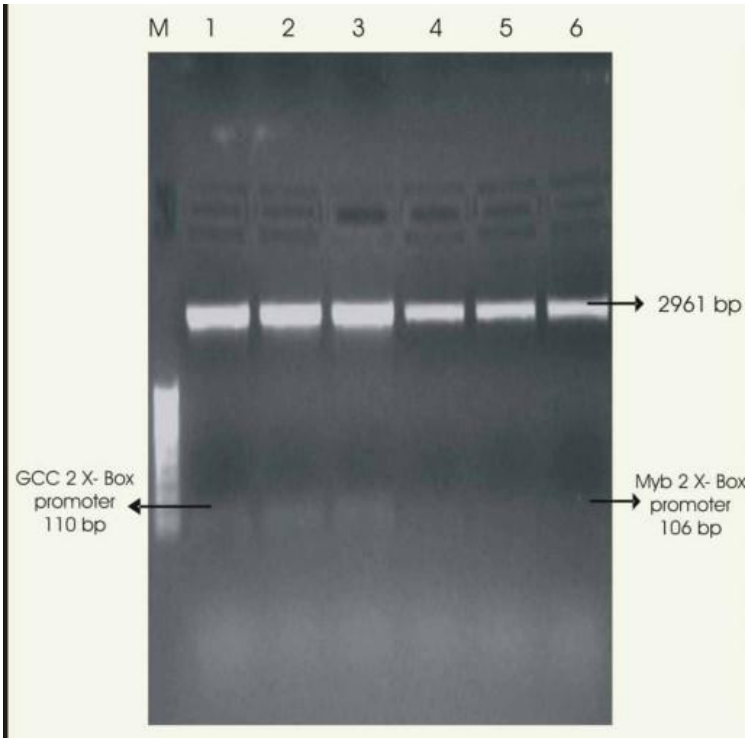


**Plate 6b. GCC 2X – Box and Myb 2X- Box promoters**

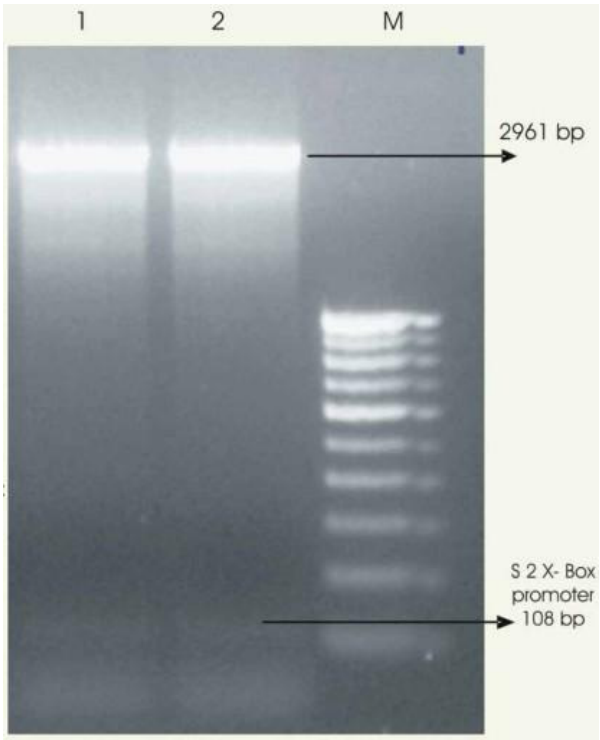


**Plate 6c. S 2 X- Box promoters**

**Plate 6: Restriction (*Hind*III + *Pst*I) confirmation of synthetic promoters in pGA4**

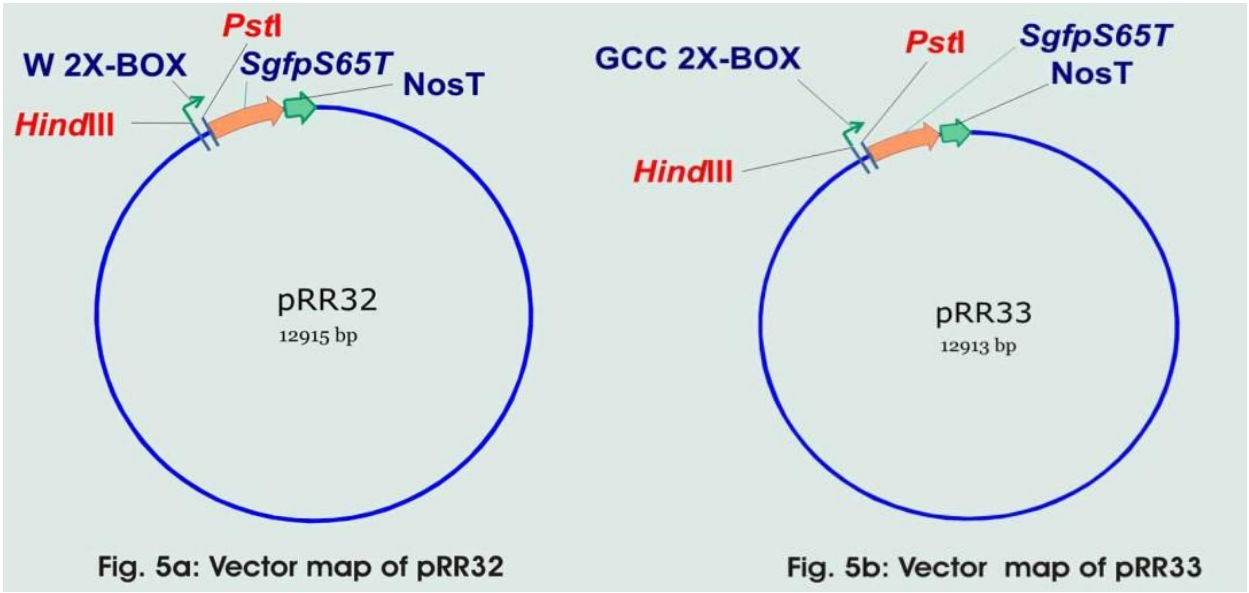


Fig 5a. Vector map of pRR32

Fig 5b. Vector map of pRR33

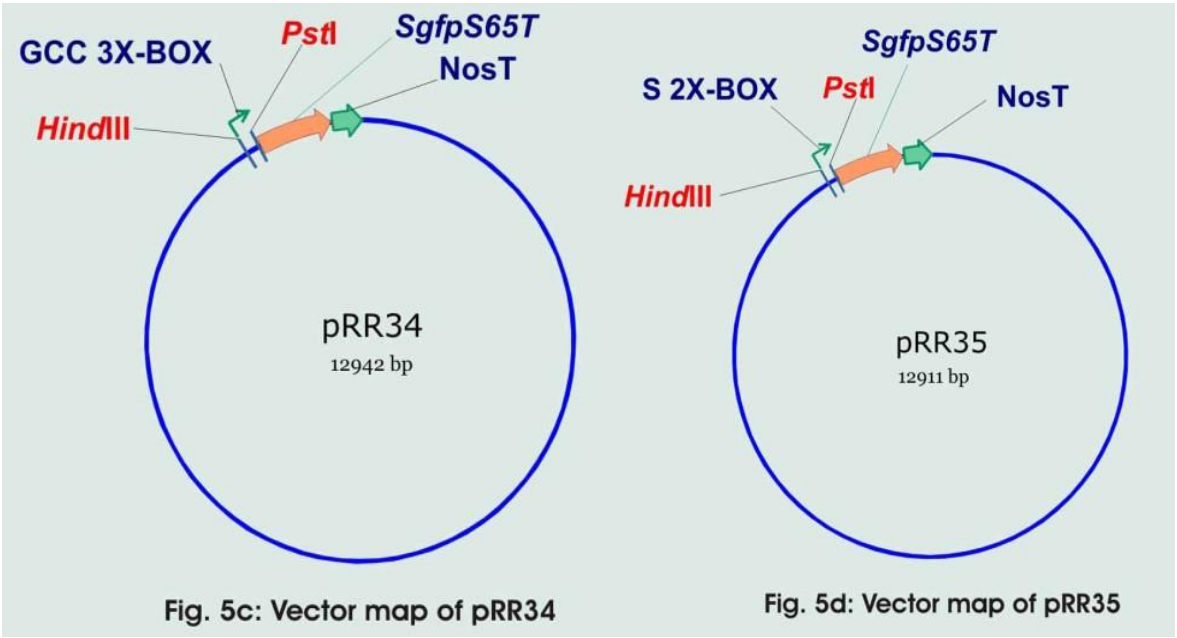


Fig 5c. Vector map of pRR34

Fig 5d. Vector map of pRR35

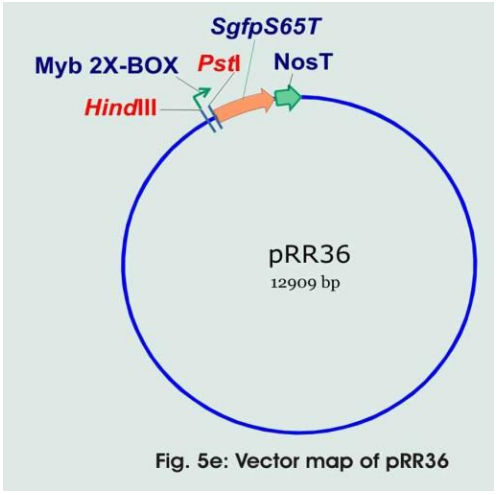


Fig 5e. vector map of pRR36

**Fig 5.** Vector map of recombinant promoter-probe vector containing synthetic promoters

**Plate 7: Restriction (*Hind*III + *Eco*RI) confirmation of recombinant Promoter-probe vectors**

**Plate 7a: W 2 X- Box, GCC 3 X- Box and S 2 X- Box promoters released from pRR32, pRR34 and pRR35 respectively**

M: λ DNA /*Eco*RI/*Hind*III Double digest

1-2: pRR32

3-4: pRR34

5: pRR35

6: pRR21 (control- promoter-probe vector)

7: pRR35

**Plate 7b: GCC 2X- Box and Myb2X-Box promoters released from pRR33 and pRR36 respectively**

M: λ DNA /*Eco*RI/*Hind*III Double digest

1-2: pRR33

3: pRR36

4: pRR21 (control- promoter-probe vector)

5: pRR36

**Plate 8: PCR confirmation of recombinant promoter-probe vectors in *Agrobacterium* clone (RB20_GFP_F/R primers)**

M: λ DNA /*Eco*RI/*Hind*III Double digest

1: pRR32

2: pCAMBIA1305.1 (-ve control)

3: pRR33

4: pRR34

5: pRR35

6: pRR36

7: pRR20 (+ve control)

**Plate 9: PCR confirmation of pCAMBIA1305.1 in *Agrobacterium* clone (*npt*II primers)**

M: λ DNA /*Eco*RI/*Hind*III Double digest

1 & 2: Amplification of *nptII* (794bp) from pCAMBIA1305.1

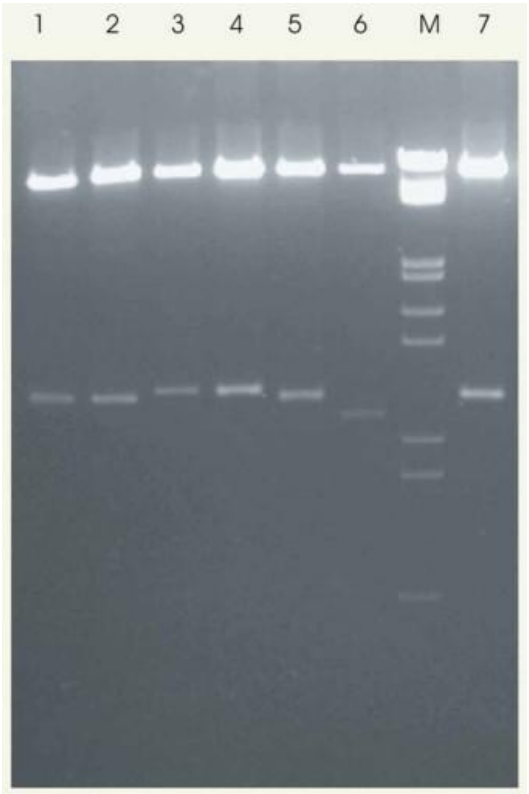


**Plate 7a. W2X-Box, GCC3X-Box and S2 X- Box promoters Released from pRR32, pRR34 and pRR35, respectively**

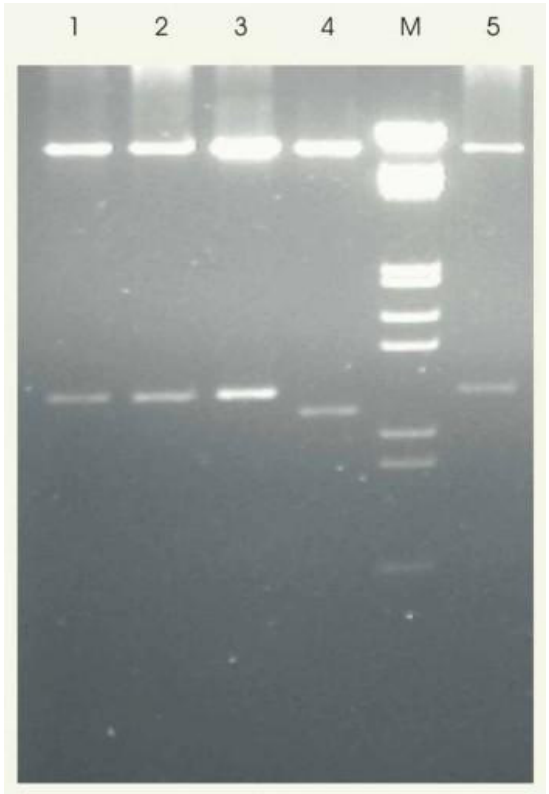


**Plate 7b. GCC 2X- Box and Myb 2X- Box promoters released from pRR33 and pRR36**

**Plate 7. Restriction (*Hind*III + *Eco*RI) confirmation of recombinant promoter-probe vectors**

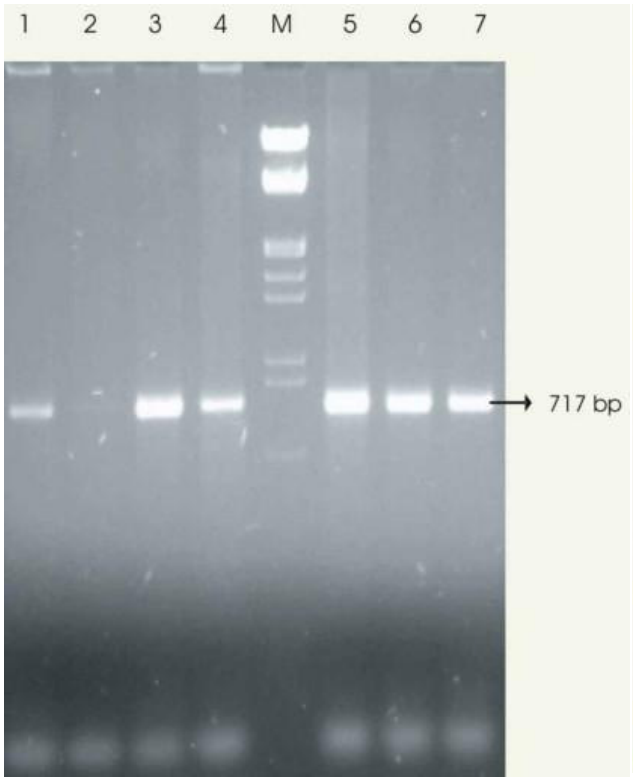


**Plate 8. PCR confirmation of recombinant promoter-probe vectors in *Agrobacterium* clone (RB20_GFP_F/R primers)**

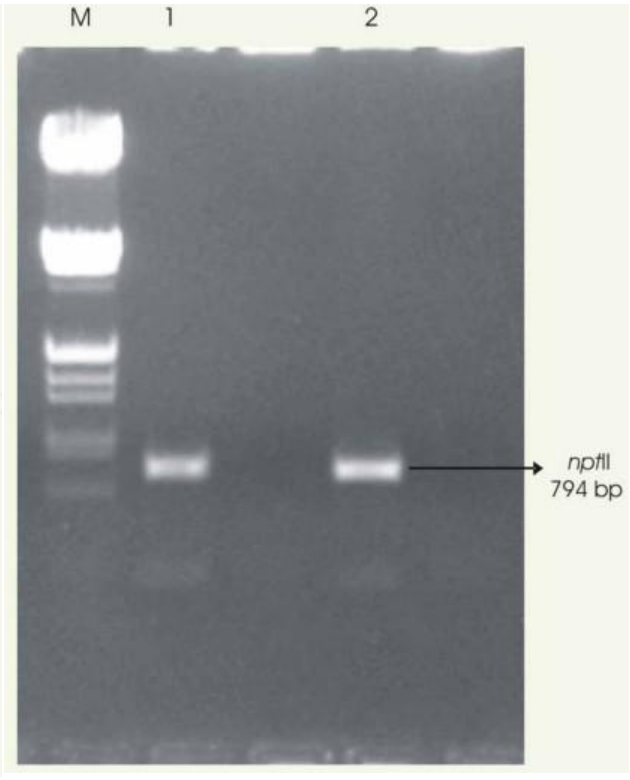


**Plate 9. PCR confirmation of pCAMBIA1305.1 in *Agrobacterium* clone (*npt*II primers)**


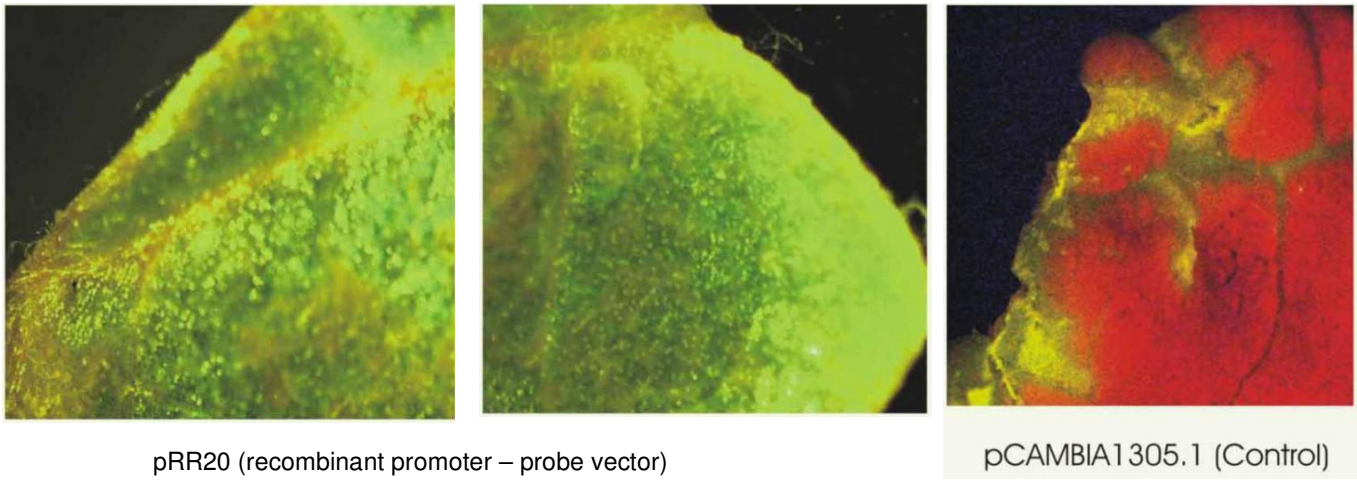


**Plate 10a. Leaf discs 15 days after co-cultivation showing sgfpS65T expression with pCAMBIA 1305.1 as control**

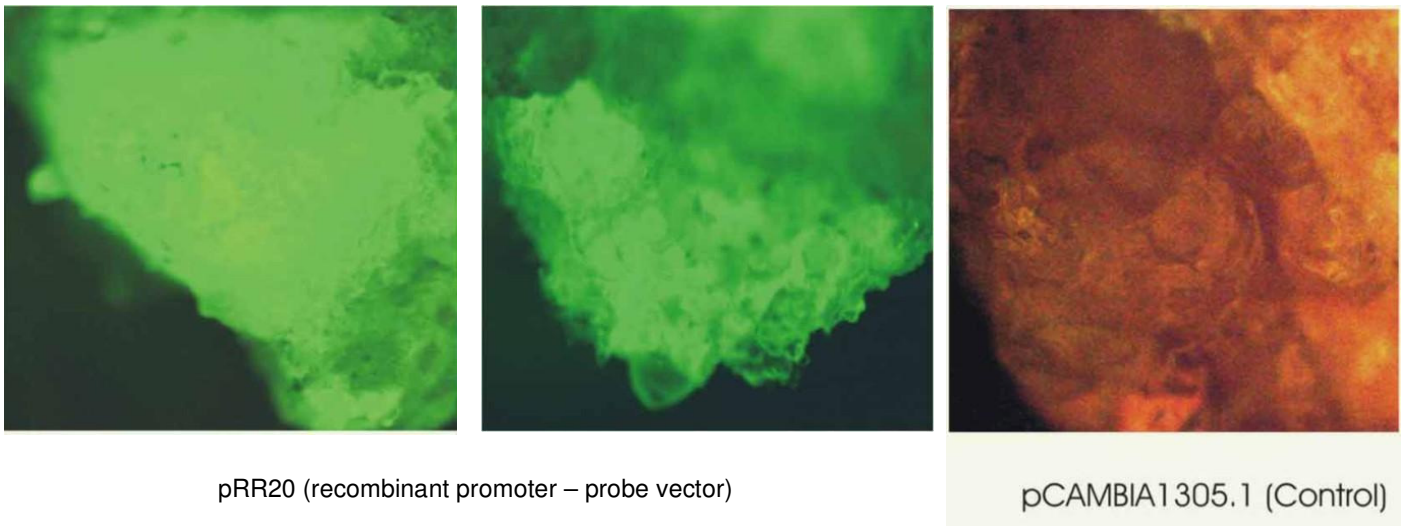


**Plate 10. Visualization of SgfpS65T expression**

At 15 days after co-cultivation, since no calli were formed, only the co-cultivated leaf discs were observed for SgfpS65T expression. Of the 30 leaf discs observed, 19 showed SgfpS65T. *De novo* calli formed from all SgfpS65T-positive leaf discs also showed fluorescence when observed 30 days after co-cultivation. No significant difference was observed for the intensity of SgfpS65T expressed in leaf discs and calli (Plate 10)

# 5 DISCUSSION

One of the major challenges in a plant genetic engineering program is to design a transformation-cassette that would enable the precise control of transgene activity. The choice of promoter to confer constitutive, spatial and/or temporal transgene expression is one of the key determinants used in plant biotechnology applications. In recent years, a wide range of different promoters from plant, viral and bacterial origin have been characterized and used extensively in regulated transgene expression systems in plant cells (Yoshida and Shinmyo, 2000; Lessard *et al*., 2002). Several plant genetic engineering strategies have incorporated the use of strong constitutive promoters in the study of gene and transcription factor (TF) function as well as for conferring transgene expression for crop improvement and bio-pharmaceutical applications. The well-described cauliflower mosaic virus (CaMV) 35S promoter (Odell *et al*., 1985; Benfey and Chua, 1990) confers high-level gene activity and has been used most commonly in plant transgene expression studies. The way forward for the study and design of inducible transgene expression cassettes was laid by research investigating the modification of the 35S promoter using the core-region (essential to initiate transcription), combined with dissected *cis*-regulatory elements, as well as early analysis of other dicot- and monocot-promoters (Gilmartin *et al*., 1990; Puente *et al*., 1996). From these initial attempts it has become apparent that the use of a synthetic and regulatory module can be tuned. To suit specific application and driven by the core-transcriptional initiation region of a constitutive promoter will prove invaluable in future genetic engineering programs. A few exciting studies have described how changes in promoter architecture, and the targeted design of *cis*-motif context can improve the control of spatial and temporal gene activity, regulate multiple transgenes, and overcome drawbacks such as homology-dependent gene silencing in plant cells (Bhullar *et al*., 2003; Sawant *et al*., 2005; Chaturvedi *et al*., 2006). Results from these research efforts underscore the value of using synthetic promoters to assist in elucidating synergistic regulatory interactions, the role of individual *cis*-motifs and in biotechnological applications (Venter, 2007).

Though there are classical methods to identify and isolate promoter sequences, the developments in gene- and promoter trapping methods and transcriptome analysis have become attractive. Gene- and promoter-tagging/trapping approach relies on the random insertion of promoterless reporter genes, or reporter genes with a minimal promoter, upstream of endogenous genes. Successful tagging of a regulatory sequence is assessed by detection of reporter gene expression in the transformants (Topping and Lindsey, 1995; Springer, 2000). This method has been used to tag promoters in *Arabidopsis*, rice, potato, tobacco etc (Salgueiro *et al*., 2002; Johnson *et al*., 2005).

Currently, numerous reports are available on transcriptional profiling via differential display, microarray, SAGE and MPSS in several crops. The data generated being enormous, the best alternative is to identify those transcripts which are stress-induced to multi-folds (as measured by Realtime-PCR) and to look at their corresponding genomic regions to walk into upstream promoter sequences. Using transcriptome analyses, a number of genes have been reported to be induced by environmental stresses, and their products are thought to function in stress tolerance and response.

Promoter analysis of abiotic stress-induced genes could recognize the presence of core elements in many of the promoter sequences. These elements include; dehydration-responsive element (DRE) with the core sequence A/GCCGAC (Yamaguchi-Shinozaki and Shinozaki, 1994), CRT (C-repeat) and LTRE (low-temperature responsive element) in cold-inducible genes (Baker *et al*., 1994) and ABA responsive elements (ABREs) (Ingram and Bartels, 1996; Shinozaki and Yamaguchi-Shinozaki, 2000). Similarly, the promoter regions of pathogen-inducible genes contained *cis*-acting elements such as W1, W2, GCC, JERE, S, Gst1, and D boxes.

An effort was made in this study to design synthetic promoters containing pathogen-inducible *cis*-acting regulatory elements such as W, GCC, S and Myb boxes, and to construct and functionally validate a promoter-probe vector with improved *gfp* (*SgfpS65T*) for use in promoter analysis.

## 5.1 Pathogen inducible synthetic promoters

*cis*-acting elements located in the promoter act as the control regions of plant genes. They are known to contribute to one or more aspects of a complex expression profile. One strategy to overcome this complexity is to produce synthetic promoters containing only defined individual elements, thereby reducing expression profile complexity (Salinas *et al*., 1992). Because of the modular nature of plant promoters, synthetic promoters can be constructed by putting together building blocks containing one or more elements.

In this study, five novel pathogen-inducible promoters were designed and were synthesized. Of the five, three synthetic promoters namely, W 2X, GCC 2X and S 2X contained only 2 copies of W, GCC and S box, respectively. Because the earlier efforts with synthetic promoters indicate that tetramers of W, GCC and S boxes showed inducibilities of 5-30 fold, whereas four copies of box S had a remarkably high inducibility (400 fold), which was attributable to an almost complete lack of expression in the absence of pep25 (oomycete-derived peptide elicitor) (Rushton *et al*., 2002). Although box S is very similar in sequence to the GCC, JERE, and DRE boxes, they appear to direct different patterns of gene expression. Among three S-related boxes, GCC is stronger than box S but showed greatly reduced inducibility by pep25, indicating an increase in the background expression.

However, all these boxes were tried with high copy number (4 and 8) could have lead to significantly high background expression even in the absence of pathogen or elicitor (Rushton *et al*., 2002). Therefore, in the present study, the copy number of W, GCC and S boxes was restricted to only two. However, GCC 3X was designed to contain 3 copies of GCC box in tandem to check the effect of an additional copy on pathogen inducibility.

Myb 2X promoter was designed by employing 2 copies of the element belonging to MREs which are known to be pathogen-inducible (Lois *et al*., 1989; da Costa e Silva *et al*., 1993; Feldbrugge *et al*., 1997; Jung *et al*., 2006). Though, functional studies with H box (belonging to MRE) indicated that it cannot function to a high level alone, it is active in combination with a G-Box (Faktor *et al*., 1997b; Faktor *et al*., 1997a). Till now no synthetic promoters have been tested using these elements. Therefore, it will be interesting to extend synthetic promoters to include other pathogen-inducible elements such as MREs and *as-1*–like elements. In this study, the Myb core element (TAACTG) along with a 15bp spacer was taken from the pathogen inducible promoter region of pepper lipid transfer protein III (CALTPIII) (Jung *et al*., 2006).

Spacing between individual *cis*-acting elements and/or between these elements and the pre-initiation complex also can have a profound effect, but spacing is difficult to predict (Wray, 1998). Optimal spacing, like the optimal number of elements, therefore needs to be determined experimentally. It is the length but not the sequence of the spacing between two boxes that would have a significant effect on the strength as well as inducibility of the promoter. However, the present study did not focus on varying the length/sequence of the spacing between the elements as well as element and TATA box. They were cloned into promoter-probe vector, confirmed by restriction digestion and sequencing.

All five synthetic promoters contained CaMV 35S promoter -46 region as the minimal promoter providing TATA box. W, GCC, S and Myb boxes which are the binding sites for various transcription factors require a minimal promoter for transcription initiation. Minimal promoters do not show any promoter activity in the absence of enhancer sequences (Benfey *et al*., 1989). Being located in considerable distance from the transcription start point, the enhancers resemble those of promoters for their components, in that they consist of a variety of modular elements. The elements of an enhancer, however, are organized in a closely packed array and function alike those in promoter. Therefore, W, GCC, S and Myb boxes designed in this study might be even regarded as the enhancer elements that could increase the concentration of activators in the vicinity of the minimal promoter. Enhancers are found to increase the probability of a promoter to be active but not the level of expression (Walters *et al*., 1995). Previous studies with synthetic pathogen-inducible promoters contained either CaMV 35S promoter -46 region (Eulgem *et al*., 1999) or parsley chalcone synthase (*Chs*) promoter fragment (Rushton *et al*., 1996; Rushton *et al*., 2002).

## 5.2 Promoter-probe vector

These vectors have a promoterless reporter gene, encoding an easily assayable protein located downstream of one or more restriction sites. Known promoter sequences or uncharacterized segments of genomic DNA can be ligated into these restriction sites, and the expression of the reporter gene can then be quantified under various conditions. Initially the efforts were made to construct promoter-probe vectors to be used in *E. coli*. However, with the availability of synthetic promoters in plants, the development of promoter-probe vectors in binary vector background would be useful. In an earlier attempt, *gus* reporter gene was made use in validating the pathogen inducible synthetic promoters (Rushton *et al*., 2002). Since GFP permits both qualitative and quantitative assays (Albano *et al*., 1996; Scholz *et al*., 2000), an attempt was made to construct a binary vector based promoter-probe vector (pRR21) for use in plants. pRR21 was constructed by PCR cloning of *SgfpS65T* gene amplified from pKU352NA, and sub-cloning into pUbiCasER- to get a fusion of *SgfpS65T* and *Nos*-Ter, which was released with *Bam*HI and *Eco*RI to clone into pACAMBIA1305.1. pRR21 released fragments of ~717 and 988bp upon restriction with *Bam*HI + *Kpn*I and *Bam*HI + *Eco*RI, respectively, indicating that pRR21 carried *SgfpS65T* + *Nos*-Ter fusion. PCR of pRR21 with *SgfpS65T*-specific primers also yielded a product of ~717bp. Sequencing of pRR21 with M13 primers showed intact open reading frame of *SgfpS65T.* Hence, it was regarded as a promoter-probe vector with promoterless *SgfpS65T* reporter gene containing terminator signal (*Nos*-Ter) at its 3' end. The multiple cloning site (MCS) in pRR21 includes the sites for *Hind*III, *Pst*I, *Sal*I, *Xba*I and *Bam*HI which can be used to clone novel promoter sequences for functional validation. The novel synthetic promoters (five) and CaMV 35S promoter (as a control) were cloned into pRR21 at *Hind*III + *Pst*I and *Hind*III + *Bam*HI, respectively. They were confirmed by restriction digestion and sequencing. These recombinant promoter-probe vectors need to be tested for pathogen inducibility.

## 5.3 Functional validation of promoter-probe vector

Novel promoters are normally validated by cloning them in fusion with a reporter gene in a suitable promoter-probe vector that can be later used for either transient or stable *in planta* expression. However, important prerequisites for a reporter of gene expression requires signal to accurately reflect protein concentrations, and that the assay is not involve costly external agents or substrates. Green fluorescent protein (GFP) meets these two criteria (Albano *et al*., 1996), and has been employed as a quantitative reporter of heterologous protein production (Albano *et al*., 1998; Daabrowski *et al*., 1999; Matthew P. DeLisa, 1999). GFP is able to autocatalytically form a fluorophore without external agents other than oxygen (Chalfie *et al*., 1994). This feature has made GFP one of the most widely used proteins as a marker for protein localization (Tsien, 1998).

Chromophore mutation, S65T in the wild GFP is an improved version (*SgfpS65T*) with 35-fold increased fluorescence compared to wild-type when excited at 488 nm (Cormack *et al*., 1996). The promoter-probe vector constructed in this study employed *SgfpS65T*. Coding sequence of the *SgfpS65T* cloned from pKU352NA was fused with *Nos*-Ter, and this expression cassette was put under the control of CaMV 35S in pRR21 for functional validation to make sure that this promoter-probe vector is good enough to analyze the pathogen-inducibility of novel promoters designed and synthesized in this study. Transient expression of SgfpS65T was observed in tobacco leaf discs co-cultivated with LBA4404 strain of *A. tumefaciens* carrying pRR21, and also in calli originated from such leaf discs.

Previous reports indicate that the expression of GFP could be seen after 2-4 days after co-cultivation (Maximova *et al*., 1998). However, in the present study, co-cultivated leaf discs were checked after 15 days of co-cultivation. Nineteen of the 30 leaf discs were found to be positive for SgfpS65T. *De novo* calli formed from these 19 leaf discs also showed SgfpS65T expression. However, high intensity of SgfpS65T was observed in both leaf discs and calli (Plate 10) indicating the utility of SgfpS65T as a reporter in the promoter-probe vector (pRR21) for function validation of novel pathogen-inducible promoters.

The future line of work would include testing the pathogen inducibility of these synthetic promoters cloned into promoter-probe vector, by developing stable transformants and observing for SgfpS65T expression upon pathogen infection or exposure to pathogen

elicitors. The promoter-probe vector constructed in this study does not carry any intron upstream of *SgfpS65T* gene, which might lead to leaky expression in prokaryotes. However, intron sequences from monocotyledonous and dicotyledonous origin can be used to abolish reporter gene expression in *A. tumefaciens* but permit expression in selected eukaryotic systems using the eukaryotic specific splicing mechanism. The successful insertion of an intron into a reporter gene might provides a suitable system to reduce the number of false-positives in transgenic plant production (Maas *et al*., 1997) if the selection is solely based on the observation at explant level.

# 6. SUMMARY AND CONCLUSIONS

Pathogen-inducible plant promoters contain multiple *cis*-acting elements, only some of which may contribute to pathogen inducibility. Therefore, defined synthetic promoters containing two and three copies of only a single type of element were designed and synthesized. A novel promoter-probe vector was constructed with *SgfpS65T* reporter gene, and its expression under CaMV 35S was checked in tobacco. The synthetic promoters were cloned into promoter-probe vector for promoter analysis in further analysis. The results obtained in this study are summarized below.

- For the construction of the promoter-probe vector, an improved version of *gfp i.e., SgfpS65T* was used as reporter gene. This gene was amplified from pKU352NA with specific primers containing *Bam*HI and *Kpn*I. sites
- Amplified *SgfpS65T* product of ~717 bp was cloned into pUbi1casER$^-$ at *Bam*HI and *Kpn*I to secure *Nos*-Ter downstream of *SgfpS65T* in the resulting vector pRR19, which was confirmed by the restriction, *SgfpS65T*-specific PCR and sequencing.
- pRR19 was restricted with *Bam*HI + *Eco*RI to release the *SgfpS65T* + *Nos*-Ter fusion, which was cloned into the plant transformation vector pCAMBIA1305.1 at the same restriction sites to get the promoter-probe vector (pRR21).
- pRR21 transferred *E. coli* DH5α, was confirmed by restriction, *SgfpS65T*-specific PCR and sequencing. Restriction digestion with *Bam*HI + *Kpn*I and *Bam*HI + *Eco*RI released 717bp (*SgfpS65T*) and 988bp (*SgfpS65T* + *Nos*-Ter) fragments, respectively. Also *SgfpS65T*-specific PCR yielded an amplicon of 717bp.
- Five pathogen-inducible promoters containing W, GCC, S and Myb boxes were designed. The synthetic promoters are W 2 X-BOX(W-box repeated two times), GCC 2 X- BOX (GCC-box repeated two times), GCC 3 X- BOX (GCC-box repeated three times), S 2 X-BOX (S-box repeated two times), Myb 2 X-BOX( *myb*-sequence repeated two times).
- In these promoters, elements were separated by the spacer sequences as in the original promoter, and a minimal promoter (CaMv 35S -46 region) was placed at the downstream.
- These synthetic promoters were synthesized and cloned into pGA4 vector at *Kpn*I and *Sac*I sites by GENEART, Regenburg, Germany.
- They were released from the pGA4 vector with *Hind*III + *Pst*I sites, and cloned into the promoter-probe vector (pRR21) to get recombinant promoter-probe vectors pRR32, pRR33, pRR34, pRR35 and pRR36. They were confirmed by restriction (*Hind*III + *Eco*RI) and sequencing.
- For functional validation of promoter-probe vector, a PCR product (423bp) corresponding to CaMV 35S promoter from the pWbvec8 was cloned into pRR21 at *Hind*III and *Bam*HI sites
- The recombinant clone (pRR20) having CaMV 35S promoter was confirmed by the CaMV 35S-specific PCR, restriction digestion and also by sequencing.
- All the recombinant promoter-probe vectors were mobilized into *Agrobacterium* by tri-parental mating and confirmed by *SgfpS65T*-specific PCR.
- To validate the expression cassette of CaMV 35S + *SgfpS65T* + *Nos*-Ter, tobacco leaf discs were co-cultivated with *A. tumafaciens* strain LBA4404 carrying pRR20.
- Expression of *SgfpS65T* was observed in co-cultivated leaf discs and *de novo* calli at 15 and 30 days after co-cultivation, respectively. Expressed SgfpS65T from the recombinant promoter-probe vector was high enough to use it as a reporter gene for promoter analysis.

# REFERENCES


Albano, C. R., Randers-Eichhon, L., Chang, Q., Bentley, W. E. and Rao, G., 1996, Quantitative measurement of green fluorescent protein expression. *Biotechnol. Tech.*, **10** (12): 953-958.

Albano, C. R., Randers-Eichhorn, L., Bentley, W. E. and Rao, G., 1998, Green fluorescent protein as a real time quantitative reporter of heterologous protein production. *Biotechnol. Prog.*, **14** (2): 351-354.

Arnone, M. I. and Davidson, E. H., 1997, The hardwiring of development: organization and function of genomic regulatory systems. *Development*, **124** (10) : 1851-1864.

Bagga, R., Michalowski, S., Sabnis, R., Griffith, J. D. and Emerson, B. M., 2000, HMG I/Y regulates long-range enhancer-dependent transcription on DNA and chromatin by changes in DNA topology. *Nucl. Acids Res.,* **28** (13) : 2541-2550.

Baker, S. S., Wilhelm, K. S. and Thomashow, M. F., 1994, The 5'-region of *Arabidopsis thaliana* cor15a has *cis*-acting elements that confer cold-, drought- and ABA-regulated gene expression. *Plant Mol. Biol.*, **24** (5): 701-713.

Baliga, N. S., 2001, Promoter analysis by saturation mutagenesis. *Biol. Proced. Online*, **3** (1): 64-69.

Barton, M. C., Madani, N. and Emerson, B. M., 1997, Distal enhancer regulation by promoter derepression in topologically constrained DNA *in vitro*. *Proc. Nat. Acad. Sci. USA.,* **94** (14): 7257-7262.

Benfey, P. N., Ren, L. and Chua, N. H., 1989, The CaMV 35S enhancer contains at least two domains which can confer different developmental and tissue-specific expression patterns. *EMBO J.*, **8** (8): 2195-2202.

Benfey, P. N. and Chua, N. H., 1990, The cauliflower mosaic virus 35S promoter: combinatorial regulation of transcription in plants. *Science*, **250** (4983): 959-966.

Berk, A. J., 1999, Activation of RNA polymerase II transcription. *Curr. Opin. Cell Biol.*, **11** (3): 330-335.

Bhullar, S., Chakravarthy, S., Advani, S., Datta, S., Pental, D. and Burma, P. K., 2003, Strategies for development of functionally equivalent promoters with minimum sequence homology for transgene expression in plants: *cis*-elements in a novel DNA context versus domain swapping. *Plant Physiol.,* **132** (2): 988-998.

Bustos, M. M., Begum, D., Kalkan, F. A., Battraw, M. J. and Hall, T. C., 1991, Positive and negative *cis*-acting DNA domains are required for spatial and temporal regulation of gene expression by a seed storage protein promoter. *EMBO J.*, **10** (6): 1469-1479.

Casadaban, M. J. and Cohen, S. N., 1980, Analysis of gene control signals by DNA fusion and cloning in *Escherichia coli*. *J. Mol. Biol.*, **138** (2): 179-207.

Chakravarthy, S., Tuori, R. P., D'ascenzo, M. D., Fobert, P. R., Després, C. and Martin, G. B., 2003, The tomato transcription factor Pti4 regulates defense-related gene expression via GCC box and non-GCC box *cis*-elements. *Plant Cell,* **15**(12): 3033-3050.

Chalfie, M., Tu, Y., Euskirchen, G., Ward, W. W. and Prasher, D. C., 1994, Green fluorescent protein as a marker for gene expression. *Science*, **263** (5148): 802-805.

Chaturvedi, C. P., Sawant, S. V., Kiran, K., Mehrotra, R., Lodhi, N., Ansari, S. A. and Tuli, R., 2006, Analysis of polarity in the expression from a multifactorial bidirectional promoter designed for high-level expression of transgenes in plants. *J. Biotechnol.*, **123** (1): 1-12.

Chen, W., Provart, N. J., Glazebrook, J., Katagiri, F., Chang, H. S., Eulgem, T., Mauch, F., Luan, S., Zou, G., Whitham, S. A., Budworth, P. R., Tao, Y., Xie, Z., Chen, X., Lam, S., Kreps, J. A., Harper, J. F., Si-Ammour, A., Mauch-Mani, B., Heinlein, M., Kobayashi, K., Hohn, T., Dangl, J. L., Wang, X. and Zhu, T., 2002, Expression profile matrix of Arabidopsis transcription factor genes suggests their putative functions in response to environmental stresses. *Plant Cell,* **14** (3): 559-574.

Christensen, A. H., Sharrock, R. A. and Quail, P. H., 1992, Maize polyubiquitin genes: structure, thermal perturbation of expression and transcript splicing, and promoter activity following transfer to protoplasts by electroporation. *Plant Mol. Biol.*, **18** (4): 675-689.

Cormack, B. P., Valdivia, R. H. and Falkow, S., 1996, FACS-optimized mutants of the green fluorescent protein. *Gene,* **173** (1): 33-38.

Crameri, A., Whitehorn, E. A., Tate, E. and Stemmer, W. P. C., 1996, Improved green fluorescent protein by molecular evolution using DNA shuffling. *Nat. Biotechnol.*, **14** (3): 315-319.

Da Costa E Silva, O., Klein, L., Schmelzer, E., Trezzini, G. F. and Hahlbrock, K., 1993, BPF-1, a pathogen-induced DNA-binding protein involved in the plant defense response. *Plant J.,* **4** (1): 125-135.

Daabrowski, S., Brillowska, A. and Kur, J., 1999, Use of the green fluorescent protein variant (YFP) to monitor MetArg human Proinsulin production in *Escherichia coli*. *Protein Expr. Purif.*, **16** (2): 315-323.

De Boer, G.-J., Testerink, C., Pielage, G., Nijkamp, H. J. and Stuitje, A., 1999, Sequences surrounding the transcription initiation site of the Arabidopsis enoyl-acyl carrier protein reductase gene control seed expression in transgenic tobacco. *Plant Mol. Biol.*, **39** (6): 1197-1207.

De Pater, S., Greco, V., Pham, K., Memelink, J. and Kijne, J., 1996, Characterization of a zinc-dependent transcriptional activator from Arabidopsis. *Nucl. Acids Res.,* **24** (23): 4624-4631.

Deveaux, Y., Peaucelle, A., Roberts, G. R., Coen, E., Simon, R., Mizukami, Y., Traas, J., Murray, J. A. H., Doonan, J. H. and Laufs, P., 2003, The ethanol switch: a tool for tissue-specific gene induction during plant development. *Plant J.,* **36** (6): 918-930.

Diaz, E. and Garcia, J. L., 1990, Construction of a broad-host-range pneumococcal promoter-probe plasmid. *Gene,* **90** (1): 163-167.

Dorsett, D., 1999, Distant liaisons: long-range enhancer–promoter interactions in Drosophila. *Curr. Opin. Genet. Dev.*, **9** (5): 505-514.

Durrant, W. E., Rowland, O., Piedras, P., Hammond-Kosack, K. E. and Jones, J. D., 2000, cDNA-AFLP reveals a striking overlap in race-specific resistance and wound response gene expression profiles. *Plant Cell,* **12** (6): 963-977.

Dynan, W. S. and Tjian, R., 1985, Control of eukaryotic messenger RNA synthesis by sequence-specific DNA-binding proteins. *Nature,* **316** (6031): 774-778.

Ellis, J. G., Tokuhisa, J. G., Llewellyn, D. J., Bouchez, D., Singh, K., Dennis, E. S. and Peacock, W. J., 1993, Does the ocs-element occur as a functional component of the promoters of plant genes? *Plant J.,* **4** (3): 433-443.

Eulgem, T., Rushton, P. J., Schmelzer, E., Hahlbrock, K. and Somssich, I. E., 1999, Early nuclear events in plant defence signalling: rapid gene activation by WRKY transcription factors. *EMBO J.*, **18** (17): 4689-4699.

Faktor, O., Loake, G., Dixon, R. A. and Lamb, C. J., 1997a, The G-box and H-box in a 39 bp region of a French bean chalcone synthase promoter constitute a tissue-specific regulatory element. *Plant J.,* **11** (5): 1105-1113.

Faktor, O., Kooter, J. M., Loake, G. J., Dixone, R. A. and Lamb, C. J., 1997b, Differential utilization of regulatory *cis*-elements for stress-induced and tissue-specific activity of a French bean chalcone synthase promoter. *Plant Sci*., **124** (2): 175-182.

Featherstone, M., 2002, Coactivators in transcription initiation: here are your orders. *Curr. Opin. Genet. Dev*., **12** (2): 149-155.

Feldbrugge, M., Sprenger, M., Hahlbrock, K. and Weisshaar, B., 1997, PcMYB1, a novel plant protein containing a DNA-binding domain with one MYB repeat, interacts in vivo with a light-regulatory promoter unit. *Plant J.,* **11** (5): 1079-1093.

Fessele, S., Maier, H., Zischek, C., Nelson, P. J. and Werner, T., 2002, Regulatory context is a crucial part of gene function. *Trends Genet*., **18** (2): 60-63.

Gat, O., Inbar, I., Aloni-Grinstein, R., Zahavy, E., Kronman, C., Mendelson, I., Cohen, S., Velan, B. and Shafferman, A., 2003, Use of a promoter trap system in *Bacillus anthracis* and *Bacillus subtilis* for the development of recombinant protective antigen-based vaccines. *Infect. Immun*., **71** (2): 801-813.

Gatz, C., 1996, Chemically inducible promoters in transgenic plants. *Curr. Opin. Biotechnol*., **7** (2): 168-172.

Gatz, C. and Lenk, I., 1998, Promoters that respond to chemical inducers. *Trends Plant Sci*., **3** (9): 352-358.

Gidekel, M., Jimenez, B. and Herrera-Estrella, L., 1996, The first intron of the *Arabidopsis thaliana* gene coding for elongation factor 1ß contains an enhancer-like element. *Gene,* **170** (2): 201-206.

Gilmartin, P. M., Sarokin, L., Memelink, J. and Chua, N. H., 1990, Molecular light switches for plant genes. *Plant Cell,* **2** (5): 369-378.

Glazebrook, J., 2001, Genes controlling expression of defense responses in Arabidopsis--2001 status. *Curr. Opin. Plant Biol*., **4** (4): 301-308.

Gurr, S. J. and Rushton, P. J., 2005, Engineering plants with increased disease resistance: how are we going to express it? *Trends Biotechnol*., **23** (6): 283-290.

Hammond-Kosack, K. E. and Jones, J. D., 1996, Resistance gene-dependent plant defense responses. *Plant Cell,* **8** (10): 1773-1791.

Haseloff, J., Siemering, K. R., Prasher, D. C. and Hodge, S., 1997, Removal of a cryptic intron and subcellular localization of green fluorescent protein are required to mark transgenic Arabidopsis plants brightly. *Proc. Nat. Acad. Sci. USA.,* **94** (6): 2122-2127.

Heim, R., Prasher, D. C. and Tsien, R. Y., 1994, Wavelength mutations and posttranslational autoxidation of green fluorescent protein. *Proc. Natl. Acad. Sci. USA.,* **91** (26): 12501-12504.

Hooley, R., 1998, Auxin signalling. Homing in with targeted genetics. *Plant Cell,* **10** (10): 1581-1584.

Hooykaas, P. J. J. and Schilperoort, R. A., 1992, Agrobacterium and plant genetic engineering. *Plant Mol. Biol*., **19** (1): 15-38.

Ingram, J. and Bartels, D., 1996, The molecular basis of dehydration tolerance in plants. *Annu. Rev. Plant Physiol. Plant Mol Biol.,* **47**: 377-403.

Johnson, A. A. T., Hibberd, J. M., Gay, C., Essah, P. A., Haseloff, J., Tester, M. and Guiderdoni, E., 2005, Spatial control of transgene expression in rice (*Oryza sativa* L.) using the GAL 4 enhancer trapping system. *Plant J.,* **41** (5): 779-789.

Jung, H. W., Lim, C. W. and Hwang, B. K., 2006, Isolation and functional analysis of a pepper lipid transfer protein III (CALTPIII) gene promoter during signalling to pathogen, abiotic and environmental stresses. *Plant Sci*., **170** (2): 258-266.

Karlyshev, A. V., Pallen, M. J. and Wren, B. W., 2000, Single-primer PCR procedure for rapid identification of transposon insertion sites. *BioTechniques*, **28** (6): 1078-1082.

Kim, S. R., Choi, J. L., Costa, M. A. and An, G., 1992, Identification of G-Box sequence as an essential element for methyl jasmonate response of potato proteinase inhibitor II promoter. *Plant Physiol.*, **99** (2): 627-631.

Kirsch, C., Takamiya-Wik, M., Schmelzer, E., Hahlbrock, K. and Somssich, I. E., 2000, A novel regulatory element involved in rapid activation of parsley ELI7 gene family members by fungal elicitor or pathogen infection. *Mol. Plant Pathol.*, **1** (4): 243-251.

Knight, H. and Knight, M. R., 2001, Abiotic stress signalling pathways: specificity and cross-talk. *Trends Plant Sci.*, **6** (6): 262-267.

Konyecsni, W. M. and Deretic, V., 1988, Broad-host-range plasmid and M13 bacteriophage-derived vectors for promoter analysis in *Escherichia coli* and *Pseudomonas aeruginosa*. *Gene,* **74** (2): 375-386.

Lam, E., Benfey, P. N., Gilmartin, P. M., Fang, R. X. and Chua, N. H., 1989, Site-specific mutations alter in vitro factor binding and change promoter expression pattern in transgenic plants. *Proc. Natl. Acad. Sci. USA.,* **86** (20): 7890-7894.

Larkin, J. C., Oppenheimer, D. G., Pollock, S. and Marks, M. D., 1993, Arabidopsis GLABROUS1 gene requires downstream sequences for function. *Plant Cell,* **5** (12): 1739-1748.

Lessard, P. A., Kulaveerasingam, H., York, G. M., Strong, A. and Sinskey, A. J., 2002, Manipulating gene expression for the metabolic engineering of plants. *Metab. Eng.,* **4** (1): 67-79.

Lewin, B., 1994, Chromatin and gene expression: constant questions, but changing answers. *Cell,* **79** (3): 397-406.

Lewin, B., 2007, *Gene IX*. New York : Oxford University Press,

Logemann, E., Parniske, M. and Hahlbrock, K., 1995, Modes of expression and common structural features of the complete phenylalanine ammonia-lyase gene family in parsley. *Proc. Natl. Acad. Sci.*, **92** (13): 5905-5909.

Lois, R., Dietrich, A., Hahlbrock, K. and Schulz, W., 1989, A phenylalanine ammonia-lyase gene from parsley: structure, regulation and identification of elicitor and light responsive *cis*-acting elements. *EMBO J.*, **8** (6): 1641-1648.

Maas, C., Simpson, C. G., Eckes, P., Schickler, H., Brown, J. W. S., Reiss, B., Salchert, K., Chet, I., Schell, J. and Reichel, C., 1997, Expression of intron modified NPT II genes in monocotyledonous and dicotyledonous plant cells. *Mol. Breed.*, **3** (1): 15-28.

Maleck, K., Levine, A., Eulgem, T., Morgan, A., Schmid, J., Lawton, K. A., Dangl, J. L. and Dietrich, R. A., 2000, The transcriptome of *Arabidopsis thaliana* during systemic acquired resistance. *Nat. Genet.,* **26** (4): 403–410.

Matthew P. Delisa, J. L. G. R. W. A. W. W. E. B., 1999, Monitoring GFP-operon fusion protein expression during high cell density cultivation of *Escherichia coli* using an on-line optical sensor. *Biotechnol. Bioeng.*, **65** (1): 54-64.

Maximova, S. N., Dandekar, A. M. and Guiltinan, M. J., 1998, Investigation of *Agrobacterium*-mediated transformation of apple using green fluorescent protein: high transient expression and low stable transformation suggest that factors other than T-DNA transfer are rate-limiting. *Plant Mol. Biol.*, **37** (3): 549-559.

Mcdowell, J. M. and Woffenden, B. J., 2003, Plant disease resistance genes: recent insights and potential applications. *Trends Biotechnol.*, **21** (4): 178-183.

Mcelroy, D., Zhang, W., Cao, J. and Wu, R., 1990, Isolation of an efficient actin promoter for use in rice transformation. *Plant Cell,* **2** (2): 163-171.

Menkens, A. E., Schindler, U. and Cashmore, A. R., 1995, The G-box: a ubiquitous regulatory DNA element in plants bound by the GBF family of bZIP proteins. *Trends Biochem. Sci.*, **20** (12): 506-510.

Miller, W. G., Leveau, J. H. and Lindow, S. E., 2000, Improved gfp and inaZ broad-host-range promoter-probe vectors. *Mol. Plant-Microbe Interact*, **13**: 1243-1250.

Nakashima, K. and Yamaguchi-Shinozaki, K., 2006, Regulons involved in osmotic stress-responsive and cold stress-responsive gene expression in plants. *Physiol. Plant.*, **126** (1): 62-71.

Nikaido, H. and Vaara, M., 1985, Molecular basis of bacterial outer membrane permeability. *Microbiol. Rev.*, **49** (1): 1-32.

Nikolov, D. B., Chen, H., Halay, E. D., Hoffman, A., Roeder, R. G. and Burley, S. K., 1996, Crystal structure of a human TATA box-binding protein/TATA element complex. *Proc. Natl. Acad. Sci. USA.,* **93**: 4862-4867.

Nikolov, D. B. and Burley, S. K., 1997, RNA polymerase II transcription initiation: A structural view. *Proc. Nat. Acad. Sci. USA.,* **94** (1): 15-22.

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** (6005): 810-812.

Ohme-Takagi, M. and Shinshi, H., 1995, Ethylene-inducible DNA binding proteins that interact with an ethylene-responsive element. *Plant Cell,* **7** (2): 173-182.

Okamuro, J. K., Szeto, W., Lotys-Prass, C. and Jofuku, K. D., 1997, Photo and hormonal control of meristem identity in the *Arabidopsis* flower mutants apetala2 and apetala1. *Plant Cell,* **9** (1): 37-47.

Palmieri, M. C., Sell, S., Huang, X., Scherf, M., Werner, T., Durner, J. and Lindermayr, C., 2008, Nitric oxide-responsive genes and promoters in *Arabidopsis thaliana*: a bioinformatics approach. *J. Exp. Bot,* **59**: 177-186.

Petersen, M., Brodersen, P., Naested, H., Andreasson, E., Lindhart, U., Johansen, B., Nielsen, H. B., Lacy, M., Austin, M. J. and Parker, J. E., 2000, *Arabidopsis* MAP kinase 4 negatively regulates systemic acquired resistance. *Cell,* **103** (7): 1111-1120.

Prasher, D. C., Eckenrode, V. K., Wade, W. W., Prendergast, F. G. and Cormier, M. J., 1992, Primary structure of the *Aequorea victoria* green-fluorescent protein. *Gene* (Amsterdam), **111** (2): 229-233.

Ptashne, M., 1988, How eukaryotic transcriptional activators work. *Nature,* **335** (6192): 683-689.

Ptashne, M. and Gann, A., 1997, Transcriptional activation by recruitment. *Nature,* **386** (6625): 569-577.

Puente, P., Wei, N. and Deng, X. W., 1996, Combinatorial interplay of promoter elements constitutes the minimal determinants for light and developmental control of gene expression in Arabidopsis. *EMBO J.*, **15** (14): 3732-3743.

Raventos, D., Jensen, A. B., Rask, M. B., Casacuberta, J. M., Mundy, J. and San Segundo, B., 1995, A 20 bp *cis*-acting element is both necessary and sufficient to mediate elicitor response of a maize PRms gene. *Plant J.,* **7** (1): 147-155.

Riechmann, J. L., Heard, J., Martin, G., Reuber, L., Jiang, C., Keddie, J., Adam, L., Pineda, O., Ratcliffe, O. J., Samaha, R. R., Creelman, R., Pilgrim, M., Broun, P., Zhang, J. Z., Ghandehari, D., Sherman, B. K. and Yu, G., 2000, Arabidopsis transcription factors: genome-wide comparative analysis among eukaryotes. *Science*, **290** (5499): 2105-2110.

Roa-Rodriguez, C. and Cambia. 2003, Promoters used to regulate gene expression. CAMBIA,

Ronald, S. L., Kropinski, A. M. and Farinha, M. A., 1990, Construction of broad-host-range vectors for the selection of divergent promoters. *Gene,* **90** (1): 145-148.

Rushton, P. J., Torres, J. T., Parniske, M., Wernert, P., Hahlbrock, K. and Somssich, I. E., 1996, Interaction of elicitor-induced DNA-binding proteins with elicitor response elements in the promoters of parsley PR1 genes. *EMBO J.*, **15** (20): 5690-5700.

Rushton, P. J. and Somssich, I. E., 1998, Transcriptional control of plant genes responsive to pathogens. *Curr. Opin. Plant Biol.*, **1** (4): 311-315.

Rushton, P. J., Reinstadler, A., Lipka, V., Lippok, B. and Somssich, I. E., 2002, Synthetic plant promoters containing defined regulatory elements provide novel insights into pathogen-and wound-induced signalling. *Plant Cell,* **14** (4): 749-762.

Salgueiro, S., Matthes, M., Gil, J., Steele, S., Savazzini, F., Riley, A., Jones, H. D., Lazzeri, P. A. and Barcelo, P., 2002, Insertional tagging of regulatory sequences in tritordeum; a hexaploid cereal species. *Theor. Appl. Genet.,* **104** (6): 916-925.

Salinas, J., Oeda, K. and Chua, N. H., 1992, Two G-box-related sequences confer different expression patterns in transgenic tobacco. *Plant Cell,* **4** (12): 1485-1493.

Sambrook, J. and Russell, D. W., 2001, *Molecular Cloning: A Laboratory Manual,* Cold Spring Harbor Laboratory Press, New York, USA.

Sawant, S. V., Kiran, K., Mehrotra, R., Chaturvedi, C. P., Ansari, S. A., Singh, P., Lodhi, N. and Tuli, R., 2005, A variety of synergistic and antagonistic interactions mediated by *cis*-acting DNA motifs regulate gene expression in plant cells and modulate stability of the transcription complex formed on a basal promoter. *J. Exp. Bot.*, **56** (419): 2345-2353.

Schena, M., Lloyd, A. M. and Davis, R. W., 1991, A steroid-inducible gene expression system for plant cells. *Proc. Natl. Acad. Sci. USA.*, **88** (23): 10421-10425.

Schenk, P. M., Kazan, K., Wilson, I., Anderson, J. P., Richmond, T., Somerville, S. C. and Manners, J. M., 2000, Coordinated plant defense responses in Arabidopsis revealed by microarray analysis. *Proc. Natl. Acad. Sci. USA.*, **97** (21): 11655-11660.

Scholz, O., Thiel, A., Hillen, W. and Niederweis, M., 2000, Quantitative analysis of gene expression with an improved green fluorescent protein. *Eur. J. Biochem.,* **267** (6): 1565-1570.

Seki, M., Narusaka, M., Abe, H., Kasuga, M., Yamaguchi-Shinozaki, K., Carninci, P., Hayashizaki, Y. and Shinozaki, K., 2001, Monitoring the expression pattern of 1300 Arabidopsis genes under drought and cold stresses by using a full-length cDNA microarray. *Plant Cell,* **13** (1): 61-72.

Shinozaki, K. and Yamaguchi-Shinozaki, K., 2000, Molecular responses to dehydration and low temperature: differences and cross-talk between two stress signalling pathways. *Curr. Opin. Plant Biol.*, **3** (3): 217-223.

Silhavy, T. J. and Beckwith, J. R., 1985, Uses of lac fusions for the study of biological problems. *Microbiol. Rev.*, **49** (4): 398-418.

Simons, R. W., Houman, F. and Kleckner, N., 1987, Improved single and multicopy lac-based cloning vectors for protein and operon fusions. *Gene,* **53** (1): 85-96.

Singh, K. B., Foley, R. C. and Oñate-Sánchez, L., 2002, Transcription factors in plant defense and stress responses. *Curr. Opin. Plant Biol.*, **5** (5): 430-436.

Smale, S. T. and Kadonaga, J. T., 2003, The RNA polymerase II core promoter. *Annu. Rev. Biochem.*, **72** (1): 449-479.

Somssich, I. E. and Hahlbrock, K., 1998, Pathogen defence in plants - a paradigm of biological complexity. *Trends Plant Sci.*, **3** (3): 86-90.

Springer, P. S., 2000, Gene traps tools for plant development and genomics. *Plant Cell*, **12** (7): 1007-1020.

Stewart and Stewart, C., 2001, The utility of green fluorescent protein in transgenic plants. *Plant Cell Rep.,* **20** (5): 376-382.

Strittmatter, G., Gheysen, G., Gianinazzi-Pearson, V., Hahn, K., Niebel, A., Rohde, W. and Tacke, E., 1996, Infections with various types of organisms stimulate transcription from a short promoter fragment of the potato gst1 gene. *Mol. Plant. Microbe Interact.*, **9** (1): 68-73.

Sundaresan, V., Springer, P., Volpe, T., Haward, S., Jones, J. D., Dean, C., Ma, H. and Martienssen, R., 1995, Patterns of gene action in plant development revealed by enhancer trap and gene trap transposable elements. *Genes Dev.*, **9** (14): 1797-1810.

Tjian, R. and Maniatis, T., 1994, Transcriptional activation: A complex puzzle with few easy pieces. *Cell,* **77** (1): 5-8.

Topping, J. F. and Lindsey, K., 1995, Insertional mutagenesis and promoter trapping in plants for the isolation of genes and the study of development. *Transgenic Res.*, **4** (5): 291-305.

Tsien, R. Y., 1998, The green fluorescent protein. *Annu. Rev. Biochem.*, **67** (1): 509-544.

Urao, T., Yamaguchi-Shinozaki, K., Urao, S. and Shinozaki, K., 1993, An Arabidopsis myb homolog is induced by dehydration stress and its gene product binds to the conserved MYB recognition sequence. *Plant Cell,* **5** (11): 1529-1539.

Venter, M., 2007, Synthetic promoters: genetic control through *cis* engineering. *Trends Plant Sci.*, **12** (3): 118-124.

Walters, M. C., Fiering, S., Eidemiller, J., Magis, W., Groudine, M. and Martin, D. I., 1995, Enhancers increase the probability but not the level of gene expression. *Proc. Natl. Acad. Sci. USA.*, **92** (15): 7125-7129.

Wang, Z., Yang, P., Fan, B. and Chen, Z., 1998, An oligo selection procedure for identification of sequence-specific DNA-binding activities associated with the plant defence response. *Plant J.,* **16** (4): 515-522.

Wasserman, W. W., Palumbo, M., Thompson, W., Fickett, J. W. and Lawrence, C. E., 2000, Human-mouse genome comparisons to locate regulatory sites. *Nat. Genet.,* **26**: 225-228.

Wray, G. A., 1998, *Promoter logic. Science*, **279** (5358): 1896-1902.

Yamaguchi-Shinozaki, K. and Shinozaki, K., 1994, A novel *cis*-acting element in an Arabidopsis gene is involved in responsiveness to drought, low-temperature, or high-salt stress. *Plant Cell*, **6** (2): 251-264.

Yang, Y., Shah, J. and Klessig, D. F., 1997, Signal perception and transduction in plant defense responses. *Genes Dev.*, **11** (13): 1621-1639.

Yokoe, H. and Meyer, T., 1996, Spatial dynamics of GFP-tagged proteins investigated by local fluorescence enhancement. *Nat. Biotechnol.*, **14** (10): 1252-1256.

Yoshida, K. and Shinmyo, A., 2000, Transgene expression systems in plant, a natural bioreactor. *J Biosci Bioeng*, **90** (4): 353-362.

Zhang, H., Huang, Z., Xie, B., Chen, Q., Tian, X., Zhang, X., Zhang, H., Lu, X., Huang, D. and Huang, R., 2004, The ethylene-, jasmonate-, abscisic acid-and NaCl-responsive tomato transcription factor JERF1 modulates expression of GCC box-containing genes and salt tolerance in tobacco. *Planta*, **220** (2): 262-270.

Zhang, S. H., Broome, M. A., Lawton, M. A., Hunter, T. and Lamb, C. J., 1994, atpk1, a novel ribosomal protein kinase gene from Arabidopsis. II. Functional and biochemical analysis of the encoded protein. *J. Biol. Chem.*, **269** (26): 17593-17599.

Zheng, Z., Mosher, S. L., Fan, B., Klessig, D. F. and Chen, Z., 2007, Functional analysis of Arabidopsis WRKY25 transcription factor in plant defense against *Pseudomonas syringae*. *BMC Plant Biol*, **7**: 2.

# DESIGNING PATHOGEN INDUCIBLE SYNTHETIC PROMOTERS AND FUNCTIONAL VALIDATION OF A NEW EUKARYOTIC PROMOTER– PROBE VECTOR

RAVEENDRA G. M. 2008 Dr. SUMANGALA BHAT
Major Advisor

## ABSTRACT

In this study, an attempt was made to design pathogen inducible promoters, and construct and functionally validate a new promoter-probe vector. W 2 X, GCC 2 X, GCC 3 X, S 2 X, Myb 2 X pathogen inducible promoters contained various pathogen-responsible *cis*-regulatory elements in multiple copies fused to a minimal promoter (-46 region of CaMv 35S promoter) at 3', with a spacer sequences in between. They were synthesized at GENEART, Germany.

A promoter-probe vector (pRR21) was constructed in binary vector (pCAMBIA 1305.1) background with an improved version (*SgfpS65T*) of GFP and Nos-ter. pRR21 carried a multiple cloning site with target sequences for *Bam*HI, *Xba*I, *Sal*I, *Pst*I and *Hind*III to clone any DNA fragment whose promoter activity is to be checked. Complete sequence and feature (annotation) information of pRR21 has been deposited at GenBank of NCBI with an accession number (EU760495). This promoter-probe vector constructed with *SgfpS65T*. because pRR21 is in a binary vector background, it is expected to work in many plant systems.

Promoter-probe vector was functionally validated by PCR cloning CaMV 35S promoter from pWBVec8 into the multiple cloning site of pRR21 to get pRR20. Similarly, all five synthetic pathogen inducible promoters were cloned into pRR21. The recombinant promoter-probe vectors were transferred into *Agrobacterium* by tri-parental mating. Of the 30 tobacco leaf discs co-cultivated with *Agrobacterium* carrying pRR20, 19 showed *SgfpS65T* expression when observed under the fluorescent microscope at wavelength of 480-520 nm. De novo calli formed from all *SgfpS65T* positive leaf discs also showed fluorescence. Functional validation and expression analysis of pathogen inducible synthetic promoter is to be carried out.