Studies on various strategies to develop transgenic Castor tolerant to *Botrytis* grey mold using defense regulatory genes

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K.B.Durga Bhavani
(06LPPH04)

Department of Plant Sciences, School of Life Sciences
University of Hyderabad, Hyderabad-500 046
Andhra Pradesh, India

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University of Hyderabad
(A Central University by an Act of Parliament)
Department of Plant Sciences, School of Life Sciences
P.O. Central University, Gachibowli, Hyderabad-500 046

Certificate

This is to certify that I, **K.B.Durga Bhavani** has carried out the research work in the present thesis entitled "**Studies on various strategies to develop transgenic castor tolerant to *Botrytis* grey mold using defense regulatory genes"** and submitted for the degree of **Doctor of Philosophy** was accomplished for the full period prescribed under Ph.D. ordinances of the University, under the supervision of Dr. V. Dinesh Kumar, Principal Scientist, Directorate of Oilseeds Research, Rajendranagar, Hyderabad and under the co-supervision of Dr. P.B. Kirti, Professor, Department of Plant Sciences, School of Life Sciences, University of Hyderabad, Hyderabad. I declare to the best of my knowledge that no part of this thesis was earlier submitted in part or in full, for the award of any research degree or diploma of any university.

K.B.Durga Bhavani
(06LPPH04)

Dr. V. Dinesh Kumar
(Supervisor)

Prof. P.B. Kirti
(Co-supervisor)

Head
Dept of Plant Sciences

Dean
School of Life Sciences

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Abbreviations and Acronyms

bp	:	base pair
BSA	:	bovine serum albumin
BAP	:	benzyl amino purine
BLAST	:	Basic Local Alignment Search Tool
$CaCl_2$	:	calcium chloride
CAMBIA	:	Center for the Application of Molecular Biology to International Agriculture
cDNA	:	complementary DNA
CTAB	:	cetyl trimethyl ammonium bromide
DNA	:	deoxy ribo nucleic acid
dNTP	:	deoxy nucleotide tri-phosphate
DOR	:	Directorate of Oilseeds Research
dpi	:	days post infection
dsRNA	:	double stranded RNA
EDTA	:	ethylene diamine tetra acetic acid
g	:	gram
gus	:	glucuronidase
h	:	hours
HCl	:	hydrochloric acid
HEPES	:	N- (2-Hydroxyethyl) piperazine-1-ethanesulfonic acid
$HgCl_2$	:	mercuric chloride
hpt	:	hygromycin phosphate transferase
IBA	:	indole butyric acid
IPTG	:	isopropyl-β-D-thiogalactopyranoside
kDa	:	kilo Dalton
KCl	:	potassium chloride
KOD	:	*Thermococcus kodakaraensis*
*lac*Z	:	β-galactosidase
LA	:	Luria agar
LB	:	Luria broth

Leu	:	leucine
M	:	molar
MDR	:	multi-drug resistant
MCS	:	multiple cloning site
$MgCl_2$	:	magnesium chloride
mg	:	milligram
min	:	minutes
ml	:	millilitre
mM	:	millimolar
mRNA	:	messenger RNA
MS	:	Murashige and Skoog
NAA	:	naphthalene acetic acid
NaCl	:	sodium chloride
NaOH	:	sodium hydroxide
NCBI	:	National Center for Biotechnology Information
ng	:	nano gram
OD	:	optical density
O/N	:	overnight
PCR	:	polymerase chain reaction
Pfu	:	*Pyrococcus furiosus*
pH	:	pussancea hydrogen (potential hydrogen)
RES	:	restriction enzyme sites
RNAi	:	RNA interference
rpm	:	revolutions per minute
rRNA	:	ribosomal RNA
SDS	:	sodium dodecyl sulphate
Taq	:	*Thermophilus aquaticus*
$T_{10}E_1$	:	10 mM of Tris.Cl, 1 mM of EDTA
T_{10}	:	10 mM of Tris.Cl
TAE	:	Tris.Cl, acetic acid, EDTA
TDZ	:	thiadiazuron
TE	:	Tris.Cl, EDTA
T_m	:	temperature of melting
tRNA	:	transfer ribonucleic acid

Tris.Cl	:	Tris (hydroxy methyl) aminomethane hydro chloride
U	:	units
UTR	:	untranslated region
V	:	volts
X-Gal	:	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
X-Gluc	:	5-bromo-4-chloro-3-indolyl-β-D-glucuronide
°C	:	degree Celsius
µg	:	micro gram
µl	:	microlitre
µM	:	micro Molar

CHAPTER I

INTRODUCTION

Castor (*Ricinus communis* L.), a member of Euphorbiaceae or spurge family (2n=20) is one of the industrially important non-edible oilseed crops cultivated worldwide. India ranks first in both area and production of castor in the world. The commercial importance of castor is due to the occurance of industrially valued ricinoleic acid at a very high percent in seed oil. . Castor oil has various applications in industrial sectors like paintings and coatings, polyurethane coating, plastics, transport, cosmetics, textiles and leathers. It also has importance in conventional medicines where it is used as a laxative in combination with other medicines. In India, castor is cultivated under irrigated as well as rainfed agro-ecosystem catering to most of the global demand for castor oil. During 2011-12 the production was about 2.3 million tonnes with an average productivity of 1400 kg/ha and accounted for over 60% of the entire global production. Gujarat, Rajasthan and Andhra Pradesh are the major castor producing states. In Andhra Pradesh and other states in southern and central India, castor is cultivated mostly as rainfed crop with low input management

Though the productivity of castor in India has increased up to 1400 kg/ha (2011-12), there is still scope for increasing the productivity. The crop is affected by several abiotic and biotic production constraints. Severe outbreak of pests and diseases cause more than 25% yield loss in castor. Among the 150 pathogens infecting castor, the necrotrophic fungus *Botrytis ricini* is a major biotic constraint and it causes Botrytis, commonly referred to as grey mold or grey rot. The inflorescence is highly susceptible to this infection and thus leads to significant yield loss. Godfrey (1923) described the symptoms of castor grey mold in the form of small bluish spots on panicles, leaves, stems and they become water soaked, wilted and densely covered with luxuriant, external tan to grey fungal growth. The disease is very severe during spike formation and capsule development stage under prolonged monsoon/cyclonic rains. Affected portions break-off at the point of infection due to the destruction of the tissues or necrosis. Temperature below 22°C during night and continuous rain are the favourable conditions for rapid disease spread within 72 hrs of infection.

Suitable management strategies for tackling this disease during the commercial cultivation of castor are still unavailable. Absence of characterized sources of genetic resistance in the released varieties /hybrids or in the available germplasm (more than 3000 lines) rules out the possibility of developing resistant cultivars against this disease. Thus, conventional breeding techniques have limited scope in developing Botrytis resistant castor lines. Developing transgenic castor line(s) resistant/tolerant to the *Botrytis* grey rot could be the solution for saving castor from this disease. The biotechnological studies in castor is in its infancy and there are no such studies with respect to imparting *Botrytis* tolerance. Therefore it is essential to formulate strategies and approaches based on those have been adopted to develop *Botrytis* resistant lines in other crops or model plant systems. Expression of anti-fungal genes (eg. *PDF1.2, BOS1*, etc) or defence regulatory genes (*WRKY1, WRKY2, PAD3*, *ERF1*, *BIK1*, etc) in dicots like *Arabidopsis thaliana* has been shown to impart tolerance against the necrotrophic fungi *Botrytis cinerea, Alternaria spp, Phytohthora spp, etc.* But, the levels of tolerance imparted by these genes have been partial. Expression of these genes in castor could lead to lines tolerant to grey mold. Considering the rapidity with which grey mold spreads in castor, it can be surmised that simultaneous expression of multiple genes, each with an ability to impart partial tolerance against necrotrophic fungus, would provide better tolerance against this disease. Hence, stacking the expression of multiple genes is expected to provide increased levels of tolerance.

Stacking of multiple genes to increase the cumulative expression could be achieved through various approaches. There are different ways to obtain transgenic plants expressing multiple transgenes. One frequently chosen option is to introduce each transgene individually via separate transformation events and to cross the different single transgene expressing lines to pyramid them (Zhu *et al*., 1994; Bizily *et al*., 2000). A drawback of this method is that transgenes are inserted at different loci with different expression levels due to position effect, and selecting plants carrying all the genes in subsequent breeding cycles could be complicated.

A second possibility is to introduce the different transgenes *via* sequential single-gene transformation steps. The drawback of this method is that different selectable markers are to be used for every transformation step, which is complicated by the fact that there is only a limited choice of selectable markers.

The third option would be to produce multiple proteins from one transcription unit by separating the distinct coding regions by internal ribosomal entry site (IRES), which allow ribosomes to reiterate translation at internal positions within an mRNA species.

The fourth possibility is based on the production of multiple proteins by proteolytic cleavage of a polyprotein precursor encoded by a single transcription unit. The poty viral system has been exploited to co-produce different proteins in plant. A disadvantage of this system is that a viral protease needs to be produced, together with the proteins of interest and that may cause cleavage of endogenous proteins and hence may lead to undesired side-effects. Another approach was followed by Halpin *et al*. (1999) who linked the coding regions of two different genes by a viral sequence encoding a 20-amino acid-long sequence, called 2A sequence. The 2A sequence originates from the foot-and-mouth disease virus, where it mediates co-translational cleavage at its own carboxy terminus in an enzyme-independent type of reaction. The limitation of this method is the attachment of 19 amino acids of the 2A sequence to the carboxy terminus of the first protein of interest. Moreover, the 20^{th} amino acid of the 2A sequence remains attached to the amino-terminus of the second protein of the polyprotein precursor.

Fifth strategy is the introduction of different transgenes as linked expression cassettes, each with a promoter and terminator, within a single transformation vector (Slater et al., 1999). It has been observed, however, that the presence of multiple copies of the same promoter within a transgenic plant often results in transcriptional silencing of the transgenes (Matzke and Matzke, 1998). To avoid this problem, different promoters for each of the expression cassettes in the construct could be used.

In line with this premise, the present investigation was carried out with the aim of testing the cumulative effects of three genes *BIK1 (Botrytis Induced Kinase1), ERF1 (Ethylene Response Factor1)* and *AtEBP (Arabidopsis thaliana Ethylene Responsive Element Binding Protein)* in imparting resistance against *Botrytis*. Two of the three target genes, *BIK1* and *ERF,* are involved in the signal pathway elicited by necrotrophic fungi, and *AtEBP* is an anti-apoptotic gene that negatively regulates cell death. These genes were cloned under the inflorescence

specific promoters, that natively drive *AtACS4, AtACS5 and AtACS7,* from *Arabidopsis that* have been reported to have elevated expression in the inflorescence in innate as well as heterologus systems. Three independent gene cassettes were to be developed using the chosen genes and later these cassettes were to be cloned within a T-DNA in an iterative way.

Based on this background, the present investigation was carried out with the following objectives:

1. Phylogenetic analysis of *Botrytis ricini* to understand its relatedness with other *Botrytis* species.
2. Characterization of *Arabidopsis thaliana* promoters *ACS4*, *5* and *7* in tobacco using (*gus*) *uidA* as reporter gene.
3. Development of gene constructs using the three inflorescence specific promoters *ACS4, 5 and 7,* and three genes *ERF1, BIK1* and *AtEBP* from *Arabidopsis thaliana.*
4. To develop multi-gene cassette vector by stacking the three individual gene cassettes within one T-DNA.
5. Transformation of model plant tobacco and target plant castor using the developed constructs.
6. Validation of transgene expression and the imparted resistance against *Botrytis* in the obtained transgenic plants.

CHAPTER 2

REVIEW OF LITERATURE

Castor is one of the important commercial non-edible oilseed crops of India earning more than 2500 crores every year to the national exchequer. Castor belongs to the family Euphorbiaceae with spurges and croton forming very large genera (Hutchinson, 1964). Castor (*Ricinus communis* L. 2n=20) whose origin is believed to be Abyssinia is industrially important for production of non-edible oil. It is distributed throughout the tropics and subtropics, and is well adapted to the temperate regions. The major castor producing countries are India, China, Brazil, USSR and Thailand, while the major importing countries are the USA, the USSR, the EEC and Japan. India is the leading producer of castor in the world (FAOSTAT, 2008) with an annual production of about 1.1 Mt. India accounts for nearly 73% of the world's production of castor (FAOSTAT, 2008). India has virtual monopoly in world castor oil market earning foreign exchange of Rs. 800 to Rs. 1200 crores annually by export of castor oil and its derivatives, castor seed and cake.

2.0.Economic importance of castor

Castor is important as a source of vegetable and medicinal oil and has numerous benefits to humanity. The oil has many industrial uses; dehydrated castor oil is used in the paint and varnish industry, manufacture of a wide range of sophisticated products like nylon fibers, jet engine lubricants, hydraulic fluids, plastics, artificial leather, manufacture of fiber optics, bullet proof glass and bone prostheses and as an antifreeze for fuels and lubricants utilized in aircraft and space rockets (Scarpa and Guerci, 1982; Ogunniyi, 2006). Castor oil is unique among vegetable oils because of the presence of ricinoleic acid, a hydroxy fatty acid up to 90%. The presence of long (18 Carbon) fatty aliphatic carbon chain, a carboxyl group, a hydroxyl group, a double bond and the proximity of the latter two offer exciting possibilities of many chemical reactions and production of a variety of oleo chemicals. The demand for castor oil and its derivatives continues to be high and India is likely to continue to be the major player in world castor seed production as Brazil and China being the only competitors, turning to soybean and coffee. India is yet to leverage the potential of its monopoly as mostly raw oil and only a few value added products are exported.

Castor cake is a by product of milling industry, which accounts for 60%of the crushed seed. It is rich in protein (25-40%), sugar (25%) and minerals (20%). Castor cake is very useful organic manure (N-6.0%, P_2O_5-2.5%, K_2O-2.5%). Presence of many toxic constituents render castor cake non-edible and make it less useful. The most toxic constituent is ricin, which is present to the extent of up to 200-mg/l00 g of cake. The cake is also very rich in allergens (upto13% of the cake) because of which its cultivation is avoided in some countries of Europe and USA. Further, castor cake is a good source of vitamins like thiamine, beta-carotene and tocopherols. The detoxified castor cake can be an excellent animal feed. Protein isolates obtained from castor cake are useful for manufacture of surfactants, fibers, plastics and wall distempers. The toxic constituents in castor provide nematicidal and termiticidal properties due to which castor cake is used as organic manure in plantation crops.

2.1.*Botrytis* infection in castor

Castor is one of the important commercial non-edible oilseed crops of India. India is the leading producer of this crop in the world and with an income of more than Rs.1000 crores to the national exchequer. Though, success has been achieved in increasing the productivity of the crop, of late *Botrytis* also called Grey mold disease caused by *Botrytis ricini* has become a serious threat to castor cultivation, especially in peninsular India, where the crop is devastated during cyclonic and incessant rains. The yield losses could be more than 80% when the weather conditions are favorable for the disease. The causal organism *Botrytis ricini* is a necrotrophic fungus and it promotes the death of host cells and tissues and survives on the dead tissues. Basically the infection comes on the spike, which is the economical part of the crop leading to severe yield losses. The spread of the disease is very fast and within a narrow window of time of 72 hrs the fungus grows on the entire inflorescence and causes the death of the entire inflorescence leading to major yield loss. *B.ricini* has a very narrow host range and is known to infect only castor (Raoof *et al*., 2003).

The first occurrence of this disease in USA was directly linked to castor seeds imported from Bombay (now Mumbai), India, even though until that time, such disease had not been described in that country (Godfrey, 1923). In his work, Godfrey (1923) did a detailed account of the destructive potential of the gray mold of castor under favourable condition. By attacking mainly reproductive

organs of the castor plant, gray mold disease is implicated in direct losses of yield whatever the level of infection. In India, today the major castor producer, gray mold is found in few states and is regarded as troublesome only in Andhra Pradesh and Tamil Nadu, in the South, where the weather conditions are more favourable for disease development where in 1987, an epidemic outbreak of gray mold occurred (Dange et al., 2005). Gray rot appeared for the first time as an epidemic in 1996 in castor-growing regions of Andhra Pradesh, India (Annual Progress Report, Castor, 1996). Since then it has reoccurred every year in farmers' fields causing severe damage and it is currently the major castor disease in this region. All the existing castor varieties and hybrids are susceptible to gray rot and non-genetic measures have failed to control it.

2.2.*Botrytis* infection and host range

However, *Botrytis cinerea* a necrotrohic fungus belonging to the same genus *Botriotinia* (*Botrytis*) has a very wide host range and is also known to infect castor (Godfrey, 1923). There has been a lot of studies carried out with respect to epidemiology, infection process, plant-pathogen interactions etc. with regard to *Botrytis cinerea* and plants. Owing to the recent information available with regard to *B.ricini*, the information available for *B.cinerea* has been reviewed here.

The necrotrophic fungi *Botrytis cinerea* has been extensively studied and the disease mechanism is also deduced to some extent. *Botrytis cinerea* causes serious losses in more than 200 crop species worldwide. It has the most destructive effect on mature or senescent tissues of dicotyledonous hosts, but it usually gains entry to such tissues at a much earlier stage in crop development and remains quiescent for a considerable period before rapidly rotting tissues when the environment is favorable. Therefore, serious damage is caused following harvest and the subsequent storage would lead to aggrevated risk of damage. However, *B. cinerea* also causes massive losses in some field- and greenhouse-grown horticultural crops prior to harvest, or even at the seedling stage in some hosts. *B. cinerea* is difficult to control because it has different modes of attack, diverse host range for infection and survival, and it can survive as mycelia and/or conidia or for extended periods as sclerotia in crop debris. For these reasons the use of any single control measure is unlikely to succeed and a more detailed understanding of the host–pathogen interaction, the microenvironment in which the fungus operates and its microbial competitors on the host is essential.

The current cost of bringing a new fungicide or biological control agent to market is so high that only major crops attract sufficient interest by agribusiness. The major *Botrytis* species and the diseases they cause have been up-dated (Elad *et al*., 2004). Because of the worldwide importance of this fungus and the availability of molecular genetic tools to study the organism, it has become the most extensively studied necrotrophic fungal pathogen. Its entire genome sequence is also available for analysis and this should provide a new insight into the biology, evolution and opportunities for control of this organism.

2.3.Mode of infection of *B.cinerea*

Botrytis cinerea Pers.:Fr., anamorph of *Botryotinia fuckeliana* (de Bary) Whetzel infects more than 200 species of dicot plants in temperate and subtropical regions (Williamson *et al*., 2007, Zang *et al*., 2007). It preferentially attacks wounded or senescing fruits and flowers producing a grey rot. *B. cinerea* attacks stems, leaves and seeds (Muckenschnabel *et al*., 2002; Williamson *et al*., 2007) in a non-host-specfic, non-organ-specifc manner. As a necrotrophic pathogen, it induces plant cell death to establish infection and to start a parasitic life cycle. Its pathogenicity factors are lytic enzymes, reactive oxygen species (ROS), toxins, and phytohormones. The compatible interactions are characterized by mycelium proliferating in the infected plant tissues, water-soaked, macerated lesions followed by the appearance of grey masses of conidia (grey mould). The incompatible interactions, however show local mycelial outgrowth restricted within dead and eventually desiccated plant tissue.

2.3.1.Host Range

Droby and Lichter (2004) have proved a comprehensive list of post harvest rots caused by *B. cinerea.* These range from grey mould on different plant organs, including flowers, fruits, leaves, shoot and soil storage organs (i.e. carrot, sweet potato), although the fungus is not regarded as a true root pathogen or one causing soil-borne diseases. Vegetables (i.e. cabbage, lettuce, broccoli, beans) and small fruit crops (grape, strawberry, raspberry, and blackberry) are most severely affected. With increasing international trade in cold-stored produce this fungus has attained great importance

because it can grow effectively over long periods at just above freezing temperatures in products such as kiwifruit, apples (‘dry eye rot’) and pears. Similarly, the important trade in cut flowers is adversely affected by this pathogen; rose and gerbera flowers are particularly prone to damage. Culture of plants out-of-season in heated or unheated greenhouses and under plastic tunnels used increasingly to supply fruits, vegetables, herbs and flowers in northern latitudes greatly increases the risk of infection, especially in tomato, cucumber and sweet pepper. There are important field crops that sustain serious damage due to grey mould. Most notable are the heavy losses in chickpeas and other protein-rich legumes that support millions of rural families in India, Bangladesh and Nepal (Pande *et al*., 2002). French bean (*Phaseolus vulgaris*) in cases of severe incidence sustains almost complete loss. Most legumes suffer attack by *B. cinerea* to some extent. Sunflower is an important oil crop that can be infected severely. In tree nurseries, conifer seedlings are vulnerable to grey mould. Thus, the host range of *B.cinerea* is wide and the losses incurred are very high.

2.4.The phylogenetic analysis among *Botrytis* species

The genus *Botrytis* contains 22 recognized species and one hybrid. The classification of this species has been largely based on morphological characters and, to a minor extent, on physiology and host range. The genus *Botrytis* comprises one generalist, *B. cinerea*, infecting over 200 eudicot hosts, especially senescing or otherwise weakened or wounded plants (MacFarlane 1968). All other species are considered specialists with a narrow host range. They infect only one or a few closely related species within the same plant genus (Mansfield 1980). To investigate whether closely related *Botrytis* species are pathogenic on closely related plant species, the phylogenies of fungi and their angiosperm hosts (APG 2003) were compared for possible cospeciation. An attempt to understand the relatedness among these species, polygenetic analysis study was done based on the DNA sequence data of three nuclear protein-coding genes(*RPB2, G3PDH,* and *HSP60*) and compared with the traditional classification (Staats *et al*., 2005). The polygenetic study revealed the classical species delineation.

The evolutionary events were identified by tracing the sexual reproduction and the host range, important fitness traits. The hybrid status of *B.allii* (*B.byssoidea* and *B.aclada*) was confirmed. It was concluded that the genus *Botrytis* can be divided into two clades, radiating after the separation of *Botrytis* from other *Sclerotiniaceae* genera. The above study was based on

individual gene trees and the combined tress which show that two clades (Clade 1 contains four species that all colonize exclusively eudicot hosts, whereas clade 2 contains 18 species that are pathogenic on either eudicot (3) or monocot (15) hosts. It has been proposed that the host shifts have occurred during *Botrytis* speciation, possibly by the acquisition of novel pathogenicity factors. Loss of sexual reproduction has occurred at least three times and is supposed to be a consequence of negative selection.

2.5.Chemical methods for resistance against *Botrytis*

The chemical control of *Botrytis* spp., and *B.cinerea* the casual agent of grey mould on many crops, have been studied extensively. The mode of action of these fungicides is mainly achieved by affecting the fugal respiration, the oldest ones are multi-site toxicants (eg. diclofluanid, thiram); newer ones are uncouplers (e.g. fluazinam), inhibitors of mitochondrial complex II (e.g. boscalid) or complex III (e.g. strobilurins). Within anti-microtubule botryticides, negative-cross resistance can occur between benzimidazoles (e.g. carbendazim) and phenylcarbamates (e.g. diethofencarb), a phenomenon determined by a mutation in the gene encoding beta-tubulin. Aromatic hydrocarbon fungicides (e.g. dicloran), dicarboximides (e.g. iprodione, procymidone, vinclozolin) and phenylpyrroles (e.g. fludioxonil) affect the fungal content of polyols and resistance to these various compounds can be associated with mutations in a protein histidine kinase, probably involved in osmoregulation. However, dicarboximide-resistant field strains of *B. cinerea* are sensitive to phenylpyrroles. Anilinopyrimidines (e.g. cyprodinil, mepanipyrim, pyrimethanil) inhibit methionine biosynthesis but their primary target site remains unknown. Hydroxyanilide fenhexamid, which inhibits the 3-keto reductase involved in sterol C4-demethylations, is a powerful botryticide. Monitoring conducted in French vineyards revealed the presence of multi-drug resistant (MDR) strains, a phenomenon probably determined by over-production of ATP-binding cassette transporters. The summary of the results obtained with different chemicals in controlling the Botrytis infection is given in table below.

Table2.0. Chemicals used for control of Botrytis infection and the control achieved by them

S.No	Chemical Compound	Crop	% of Resistance
1.	Enantiopure alcohol derivatives of 4_-chlorophenyl cyclopropyl ketone	Grapes	65%
2.	Benzyl 4_-chlorophenyl ketone	Grapes	50%
3.	Potassium metabisulphite ($K_2S_2O_5$),	Grapes	60%
4.	Fludioxonil	Tomato	77%
5.	BoscaLid	Tomato	77%
6.	Pyrimethanil	Tomato	49.35%
7.	Diethofencarb	Tomato	49.35%
8.	Carbendazim	Tomato	49.35%
9.	Procymidone	Tomato	>49.35%
10.	Thiophanate-methyl	Tomato	>49.35%
11.	Triazole fungicides		50%

Though, the chemical methods have remained as the only source of check against *Botrytis*, the complete resistance is not yet achieved due to the interference of environment, the weather conditions prevailing during infection and the MDR exhibited by *Botrytis spp*. As, this fungi has a very short window period for infection, the use of these chemicals on the crop is time driven especially under the conducive conditions for disease development. Hence, other novel approaches, like the transgenic approach which deals with the infection at the molecular level of the various defense regulatory pathways is a necessary and powerful tool to answer this problem.

2.6.Gene Silencing Approaches to Control *Botrytis* through RNAi

It is well established that critical and crucial genes are required as the part of pathogen for infecting and establishing on the susceptible host plant. Therefore, it is logical to expect that if the pathogen is mutated for those genes, they will be with more reduced efficiency of infection. This has also been established by isolation of such mutants in the pathogen. However, identifying such crucial genes is a critical step in an endeavor to control the fungus through this approach. In such case, gene silencing approaches could come in handy.

Gene silencing has become a powerful tool for reverse genetics; by refining methodologies, silencing can serve to investigate and determine gene function quickly and efficiently. In contrast to gene knockout by homologous recombination, gene silencing is a dominant trait, which makes it feasible to perform gene function analysis in organisms with a diploid genome or in haploid fungi with multinucleate thallus, such as *B. cinerea*. In plant-pathogenic fungi, successful gene silencing was first reported in *Magnaporthe grisea*, *Magnaporthe orzae* (Kadotani *et al*., 2003, 2004) and *Venturia inaequalis* (Fitzgerald *et al*., 2004).

Although gene silencing is normally involved in genome defense, researchers are able to manipulate gene expression of endogenous- and trans-genes through the generation of dsRNA molecules complementary to the target gene, making it an invaluable tool for gene discovery and function (Nakayashiki*et al*., 2005). It is believed that the benefits observed from gene silencing may be demonstrated for the plant pathogen *Botrytis cinerea*, a necrotrophic fungus capable of infecting over 200 plant species worldwide (Williamson*et al.,* 2007). Unlike many plant pathogens, *B. cinerea* is prominent all year round and causes infection under a broad range of environmental conditions (Jarvis *et al*., 1997). Because *B. cinerea* is resilient against natural plant defensive measures and various fungicides (Elad *et al.*, 1992; Katan *et al.*, 1982; Leroux *et al.*, 1985), new target genes affecting pathogenicity must be uncovered before additional forms of control can be developed. To develop a gene silencing system in *B. cinerea*, the endogenous gene superoxide dismutase (*bcsod1*), an H_2O_2-generating enzyme (Rolke *et al*., 2004) was first reported. Superoxide dismutases (SODs) belong to a family of enzymes that neutralize the effect of active oxygen species (AOS). Specifically, SOD catalyzes the conversion of O^{2-} into H_2O_2 (Fridovich *et al.*, 1998). As a result, silencing efficiencies can be assessed or compared *in vitro* and *in vivo* for better

understanding regarding SOD production levels (Patel *et al*., 2008). The RNAi technology may be used in screening unknown genes from the recently sequenced *B.cinerea* genome (Table.2.1)

Paraquat plate-based assays served to determine initial SOD silencing frequencies. Gene knockout mutants exhibited minimal growth with paraquat, whereas transformants containing silencing constructs exhibited varying degrees of growth reduction with paraquat supplementation (ranging from similar to the wild type to 71% reduction in colony radius), signifying a range of SOD silencing levels. Knockout mutants and silenced transformants exhibited growth similar to the wild type without paraquat supplementation. Overall, plate-based analysis indicated that silencing was feasible using sense and antisense silencing constructs. Antisense-based constructs yielded a higher percentage of transformants (~71%) with silenced phenotypes compared with sense-based constructs (~ 27%). The increase in observed antisense silencing frequencies may be attributed to the fact that complementary gene sequences promote the formation of RNA–RNA complexes between antisense and sense endogenous RNA molecules (Boulcombe., 1996; Wesley *et al.*, 2001).

The analysis of the *ArgininoSuccinate Synthase (ASS1)* gene and its deliberate or accidental silencing has been completely studied, along with a targeted disruption of the gene. The knowledge relating to the mycoviruses *Botrytis* Virus F (BVF) and *Botrytis* Virus X (BVX) found within these fungi are also being exploited to disrupt the natural biochemical functioning of the fungi (Bailey *et al.*, 2010).

These studies have shown that gene silencing does have the potential to be a powerful tool for the analysis of gene function in *Botrytis*, shown that nutrients in plants can be limiting to the development of fungi containing specific autotrophies, opening the possibility of further analysis to discover which nutrients are actually utilized during infection and can be studied in this manner to develop resistance against *Botrytis spp.* These studies can further explain the gene networks to be taken into considerations to overcome the fungal attack and its survival in the host tissues.

Table. 2.1.: *Botrytis cinerea* Genome Statistics

	Size	Chrs	%GC	Genes	tRNAs	rRNAs
B. cinerea	42.66 Mb	N/A	43.06	16,448	0	--

Table header definitions

- **Size** length of complete genome sequence, calculated by adding lengths of all scaffolds total
- **Chrs** number of chromosomes
- **%GC** GC content of scaffolds
- **Genes** number of predicted protein-coding genes in genome
- **tRNAs** number of predicted tRNA genes in genome
- **rRNAs** number of predicted rRNA genes in genome

2.7.Utility of transgenics against necrotrophic fungi *Botrytis*

The ability of *Botrytis* species to infect living host plants may result from a combination of at least four factors:

(1) Possession of pathogenicity factors (e.g., toxins and cell-wall degrading enzymes) that confer the ability to kill and invade plant tissue (*Prin*s *et al*., 2000).

(2) The ability to avoid or counteract plant resistance mechanisms.

(3) The ability to survive outside host-plant tissue under less favorable (e.g., low humidity, UV irradiation) conditions.

(4) The ability to reproduce and disperse.

The above four factors have guided many researchers to develop several transgenic plants which show varied levels of tolerance against this devastating fungi *Botrytis,* that causes major yield loss in many of the commercial crops.There is a lot of information available on the strategies adopted by

several researchers to develop tolerance in other crops against *Botrytis cinerea*. Partial resistance against *Botrytis* has been reported in either *Arabidopsis* or in tomato using transgenic approach by expressing genes like antiapoptotic genes from human/ animal/ nematode (Dickman *et al.,* 2001), a kinase gene *BIK1* involved in *Botrytis* resistance in *Arabidopsis (*Veronese *et al.,* 2006), the transcription factor like *ERF1* (Glazebrook 2005). Also, expression of defensin genes like *PAD3, PDF1.2*, etc are shown to be up-regulated in the plants exhibiting resistance to necrotrophic fungi (Glazebrook , 2005).

The well-known gene-for-gene mechanism mediated by resistance genes (R genes) operates against the biotrophs but no such unique mechanism operates against the necrotorphs. This ceases the opportunity to tackle necrotrophic fungi using resistance genes. It has been well documented that the resistance/tolerance against the necrotrophs is mediated by ethylene and/or JA induced pathway and many genes are involved in imparting tolerance though individually they may confer only some degree of tolerance. Therefore, though *per se Botrytis* resistant transgenic plants have not been produced, there are indications that such a plant could be produced if more genes implicated in resistance against the necrotrophic fungi are pyramided into one genotype. Advent of novel techniques and strategies for stacking of the candidate genes into one plant could make this a possibility (Halpin 2005).

2.8.Resistance through breeding

Cross pollination in castor bean fields probably occurs very extensively. It would require years of work to develop pure strains and then to select and breed for desirable qualities combined with resistance before permanent results could be secured (Godfrey, 1923). Based on this information, it is clear that only minor progress has been achieved toward the development of resistant cultivars during the last century. As a result, breeding programs have failed to develop a resistant cultivar or hybrid until today.

Breeding for resistance against *B. cinerea* has been difficult and unrewarding in most crops, but recently there has been substantial advance in conventional breeding for grey mould resistance in tomato. The approaches taken for this plant may serve as a useful model in other plant families. Wild *Solanum* species closely related to cultivated tomato *Solanum lycopersicum* were found to display

partial resistance in leaves and/or stems. *S. habrochaites* genotype LYC4 was used for introgressing resistance to grey mould into *S. lycopersicum.* Three quantitative trait loci (QTLs) for resistance were identified in a segregating F2 population . Seven additional QTLs were detected in an introgression line population consisting of 30 individual lines, each containing different well-defined segments of *S. habrochaites* LYC4 chromosomes in the genetic background of *S. lycopersicum* (Finkers *et al*., 2007b). One of the genotypes obtained in these studies contained several QTLs and displayed a reduction of grey mould disease parameters as high as 85% compared with the susceptible parent (Finkers *et al*., 2007a). As these studies were performed under rather high disease pressure, such partial resistance levels may possibly confer absolute resistance in normal greenhouse cultivation where lower disease pressure prevails. The QTLs for resistance to grey mould offer excellent perspectives for improved disease control in tomato. The mechanisms underlying the increased resistance remain to be unravelled and the introgression line population offers an excellent tool to study resistance mechanisms governed by the individual QTLs.

With increasing understanding of the underlying mechanisms of genetic resistance it will be possible to use gene transfer techniques to enhance the host response to infection, without loss of other important plant characteristics required by agribusiness and the consumer.

2.9.Biocontrol agents against *Botrytis* spp.

Antagonists that act against the germination and growth of necrotrophic fungi *Botrytis spp.* were identified on segments of dead onion leaves for their ability to suppress sporulation of the pathogens under controlled alternating moisture conditions representative for the field situation (Kohl *et al*., 1995b). Sclerotia as resting structures of several important plant-pathogenic fungi are an attractive target for biocontrol. Antagonists have been selected directly on sclerotia by assessing their ability to kill sclerotia of pathogens such as *Sclerotinia sclerotiorum* (Jones and Stewart, 2000), *Sclerotium cepivorum* (Clarkson *et al*., 2002) or *Botrytis cinerea* (Kohl and Schlosser, 1989). Post-harvest decay of fruit caused by pathogens such as *Monilinia fructicola*, *Botrytis cinerea*, *Penicillium spp*. or *Rhizopus stolonifer* can potentially be controlled by antagonists applied to fruit surfaces before or after harvest (Sharma *et al*., 2009). Such antagonists could be selected on wound-

inoculated fruits incubated under controlled conditions close to those applied in commercial stores (Smilanick, 1994).

2.10.Current status of research and development in the development of Botrytis tolerant castor line(s), (both international and national status)

Concerted efforts have not been made in castor for developing *Botrytis* tolerant material either through conventional methods or through biotechnological means. However, there is a lot of information available on the strategies adopted by several researchers to develop tolerance in other crops against *Botrytis cinerea*, a related fungus that is known to affect more than 200 plant species. Studies so far have basically concentrated on understanding the mechanism of infection by this fungus and an insight has been obtained in this regard (Diaz *et al.,* 2002; Kars and van Kan 2004; Glazebrook, 2005; van Kan 2006). Several reports of using transgenic approach to understand the mechanism of disease with respect to *B. cinerea* do exist (Lincoln *et al.,* 2002; Iris *et al.,* 2003; Xu *et al.,* 2004; Qin *et al.,* 2006).

In the oilseed crop castor though a large number of germplasm lines and breeding material have been screened using both field and laboratory techniques developed at the Directorate of Oilseeds Research, high degree of tolerance to the disease has not been observed in any material so far. However, a few germplasm lines with some degree of tolerance have been identified. Some of the promising accessions identified to be resistant to *Botrytis ricini* include RG2752 (Annual Reports of DOR, 2002-03, 2003-04). Management of this disease is also difficult because of the narrow window of time taken for the disease development from the time of infection i.e 48 hrs.

This situation demands utilizing biotechnological tools for tackling the problem. Though, most of the earlier reports on development of transgenic plants against fungal diseases were limited to the tolerance to biotrophic fungi, of late there are successful examples of transgenic plants raised against necroptrophic fungi also.

2.11.Rationale for selection of genes to impart tolerance in castor against *B. ricini*

The approach in which antiapoptotic genes from human/ animal/ nematode have been expressed has given promising results (Dickman *et al.,* 2001). Another recent report of identification of a gene **(*BIK1*)** involved in *Botrytis* resistance in *Arabidopsis (*Veronese *et al.*, 2006), has opened a new avenue for transgenic approach to tackle this disease. Induced general responses regulated by the plant-hormones ethylene (ET) and jasmonate (JA) are reported to be required for defense responses to necrotrophic pathogens in some plant species and expressing the transcription factor like ***ERF1*** has shown promising results against the necrotrophic fungi (Glazebrook 2005, Qin *et al.,* 2006). Also, the use of heterologous expression systems as a means of producing defensins for study has also been achieved (Lay and Anderson, 2005). Isolation and characterization of promoters *AtACS4, AtACS5* and *AtACS7*, from *Aradidopsis thaliana* which show high expression in inflorescence (Table..) have been reported recently (Wang *et al.,* 2005).

Thus, though *per se Botrytis* resistant transgenic plants have not been produced, there are indications that such a plant could be produced if more genes implicated in resistance against the necrotrophic fungi are pyramided into one genotype.

Different approaches in stacking the genes include

- **Co-transformation**: The vectors harbouring the gene cassettes will be used for transformation simultaneously (*Agrobacterium*-mediated or Biolistic).
- **Re-transformation**: Transgenic cassettes are stacked one after the other iteratively into transgenic plants by repeated transformations.
- **Crossing the plants through breeding**: Transgenic plants harbouring different gene cassettes will be crossed and the progeny plants are selected for the presence of gene cassettes
- **Multiple gene cassettes in a single T-DNA:** Multiple gene cassettes will be cloned within a single T-DNA so that all of them will be transferred into the plan simultaneously.

Advent of novel techniques and strategies for stacking of the candidate genes into one plant could make this a possibility (Halpin, 2005). Use of 2A oligopeptide sequences for expressing multiple proteins from a single poly-protein has been demonstrated to be a very useful tool in achieving simultaneous expression of many proteins (Francois *et al.,* 2004).In India, so far there are no efforts made to develop transgenic plants in castor against *Botrytis* disease.

- ***Ethylene Response Factor 1 (ERF1)***

Ethylene is a gaseous plant hormone that affects myriad developmental processes and fitness responses, including germination, flower and leaf senescence, fruit ripening, leaf abscission, root nodulation, programmed cell death, and responsiveness to stress and pathogen attack (Bleecker *et al.,* 2000; Johnson *et al*., 1998). Briefly, ethylene is perceived by a family of membrane associated receptors, including ETR1/ETR2, ETHYLENE RESPONSE SENSOR1 (ERS1)/ERS2 and EIN4 in Arabidopsis. Ethylene binds to its receptors via a copper co-factor, which is probably delivered by the copper transporter RAN1. Genetic studies predict that hormone binding results in the inactivation of receptor function. In the absence of ethylene, therefore, the receptors are hypothesized to be in a functionally active form that constitutively activates a Raf-like serine/threonine (Ser/Thr) kinase, CTR1, which is also a negative regulator of the pathway (Kieber *et al*., 1993). EIN2, EIN3, EIN5, and EIN6 are positive regulators of ethylene responses, acting downstream of CTR1. EIN2 is an integral membrane protein whose function is not understood. EIN5 and EIN6 have not yet been characterized at the molecular level. The nuclear protein EIN3 is a transcription factor that regulates the expression of its immediate target genes such as *ETHYLENE RESPONSE FACTOR1 (ERF1)* (Solano *et al*.,1998). *ERF1* belongs to a large family of APETALA2-domain-containing transcription factors that bind to a GCC-box present in the promoters of many ethylene inducible, defense-related genes. Thus, a transcriptional cascade that is mediated by EIN3/EIN3-like (EIL) and ERF proteins leads to the regulation of ethylene controlled gene expression.

Depending on the number of domains, the superfamily is divided into AP2 and EREBP subfamilies (Riechmann *et al.,* 2000). Members of the AP2 subfamily have two domains in tandem while

members of EREBP subfamily contain only one (Weigel, 1995; Okamura *et al.*, 1997; Sakuma *et al.*, 2002). Notably, all of the characterized AP2 subfamily members are found to be development-related (Nakano *et al.*, 2006). In contrast, EREBPs play roles in response to phytohormone, pathogen attack and environmental stresses such as cold, drought and high salt. Based on their function and conserved amino acids of their AP2/EREBP domain, presently known EREBPs have been further assigned into the ethylene responsive factor (ERF) or dehydration-responsive element-binding protein (DREB) subgroup. Amino acids 15 and 20 in the domain of DREBs are V (Val) and E (Glu), respectively, in contrast to A (Ala) and D (Asp) in ERFs. This divergence is supposed to explain the functional differences between the two subgroups. DREB1 from Arabidopsis are implicated in responses to abiotic stresses (Liu *et al.*, 1998), and ERFs function mainly in biotic stress-resistant responses (McGrath *et al*., 2005), examples being ERF1, ERF2, ERF3 and ERF4 from Arabidopsis thaliana (Solano *et al*., 1998; Berrocal-Lobo *et al*., 2002).

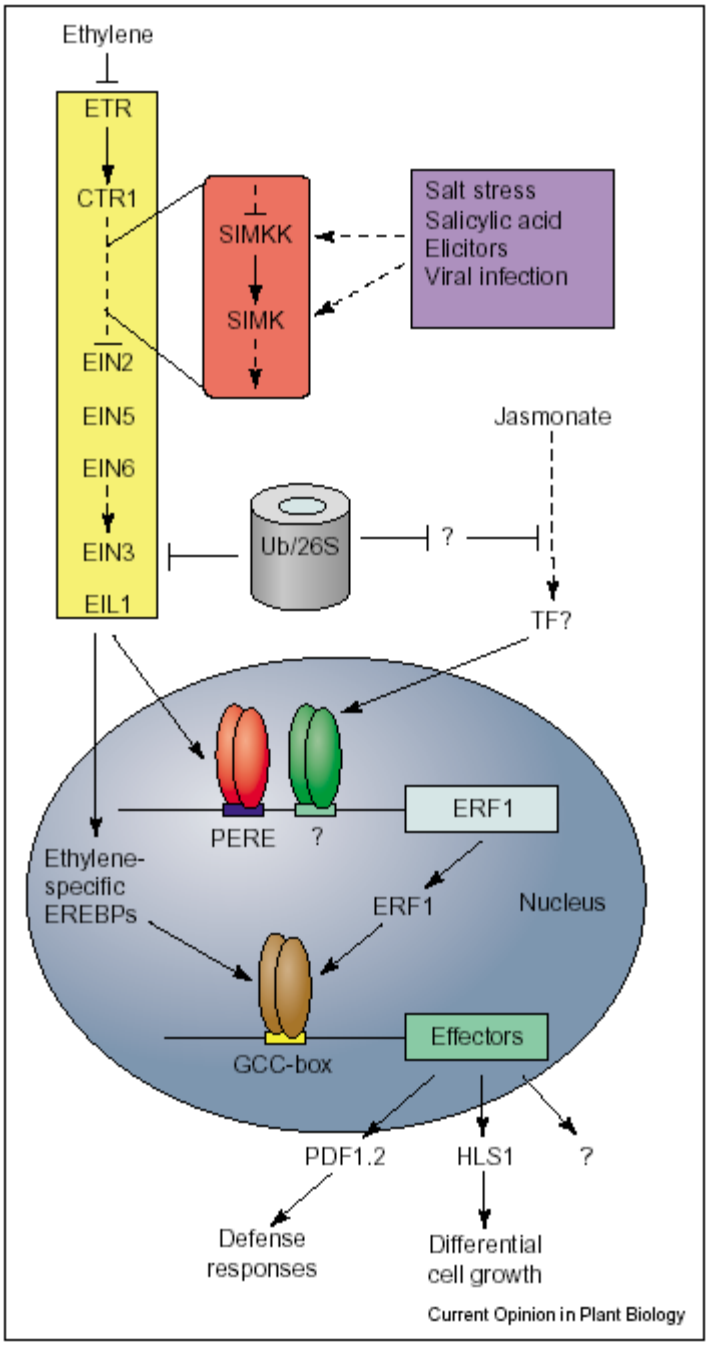


Fig 2.0. **A model for the ethylene response pathway in the regulation of gene expression.** The primary ethylene signaling pathway components (indicated in yellow) are required for all known ethylene responses and, to date, none have been found to respond to signals other than ethylene. Branch points in the ethylene response pathway may lie downstream of EIN3/EIL1. These genes encode effector proteins that are needed to execute a wide variety of ethylene responses, from disease resistance to differential cell growth. '?' represents an unknown factor or element. Arrows and t-bars represent positive and negative effects, respectively. Solid lines indicate effects that occur through direct interaction whereas dotted lines indicate effects that have not yet been shown to occur through direct interaction.

Different approaches used have included a functional study of the activity of the receptor His kinase, the determination of the ethylene receptor signaling complex at the endoplasmic reticulum and of the regulation of CONSTITUTIVE TRIPLE RESPONSE1 (CTR1) activity by these receptors, the identification of a unique MITOGEN-ACTIVATED PROTEIN KINASE (MAPK) cascade, the cloning and characterization of numerous ETHYLENE INSENSITIVE3 (EIN3)/EIN3-like (EIL) transcription factors from many plant species, and the integration of the ethylene and jasmonate response pathways via the ETHYLENE RESPONSE FACTOR (ERF) family of transcription factors. The elucidation of the biochemical mechanisms of ethylene signal transduction and the identification of new components in the ethylene response pathway in Arabidopsis are providing a framework for understanding how all plants sense and respond to ethylene. Ethylene gas is perceived by a family of ER-associated receptors (ETR). Ethylene binding is proposed to inhibit receptor function. CTR1 is proposed to be activated by the unoccupied receptors via physical interaction with them, and is inhibited upon binding of ethylene by the receptor. A MAPK module, consisting of SIMKK and SIMK, is proposed to act downstream of CTR1, although the biochemical consequence of this MAPK pathway is not evident. Because many biotic and abiotic stimuli activate the SIMKK/SIMK pathway, it remains to be determined whether their activation is dependent upon the functions of the ethylene receptors and CTR1. Downstream components in the ethylene pathway include several positive regulators (EIN2, EIN5, EIN6 and the transcription factors EIN3 and EIL1). The level of EIN3 protein is controlled by ethylene, possibly via the proteosome (Ub/26S).

Several EREBP transcription factors are known to be immediate targets of EIN3/EIL1, which can bind to a primary ethylene response element (PERE) in the promoters of *EREBP* genes. One EREBP, called *ERF1*, is also involved in JA mediated gene regulation. It is likely that an as yet unidentified JA regulated transcription factor (TF) may also bind to the promoter of *ERF1* to activate its expression. Therefore, the promoter of *ERF1* might function to integrate signals from both the ethylene and JA signaling pathways. Other EREBPs may act in a similar manner to integrate the actions of ethylene with developmental signals and/or other hormone signals. Many EREBP proteins are known to regulate gene expression through interaction with a cis-element called the GCC-box, which is found in several ethylene-responsive genes including *PDF1.2* and *HOOKLESS1 (HLS1*).

Ethylene signaling in plant growth and development:

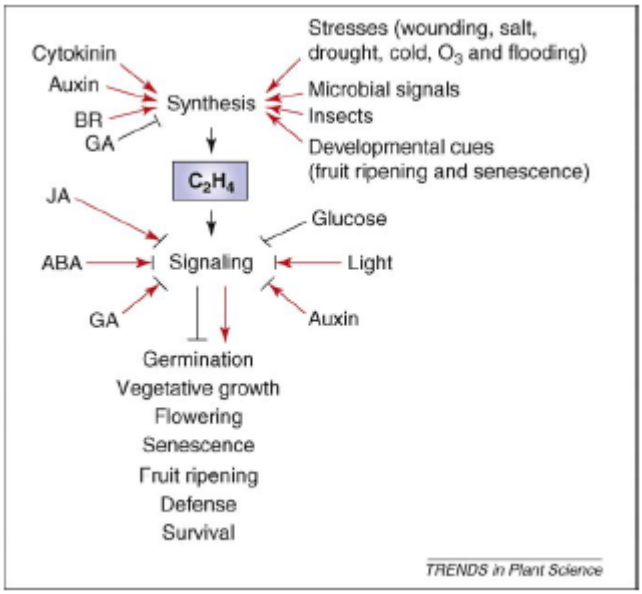


Fig 2.1. The ethylene regulatory network. Ethylene biosynthesis and signaling are regulated by multiple plant hormones, as well as by developmental and environmental signals, to modulate important plant processes from germination through to senescence. Both activation and inhibition can be observed in the relationships between ethylene and other signals in different tissues and cell types under diverse physiological, developmental and environmental conditions Abbreviations: ABA, abscisic acid; BR, brassinosteroid; JA, jasmonic acid.

ERF1 and related transcription factors conferring GCC element binding activities induce the expression of the secondary response genes in ethylene-dependent transcription cascades. These gene products are eventually involved in modulating plant survival, defense and growth. Further studies have shown that signaling components of the linear pathway are highly conserved in tomato (*Lycopersicon esculentum*), tobacco (*Nicotiana tobacum*), rice (*Oryza sativa*) and maize (*Zea mays*).

Although ERF subfamily transcription factors are generally considered to be mediators of ethylene-related responses, they include members that respond to abiotic stresses, such as drought and high salinity, and can confer tolerance to these stresses by over expression in transgenic plants. Genome-

wide expression analyses of AP2/ERF family genes in poplar (Populus trichocarpa), tomato and rice reveal that many ERF subfamily genes are also induced by low or high temperature, dehydration or high salinity. Although the functions of abiotic stress-inducible ERF subfamily genes in abiotic stress responses are largely unknown, they are expected to be involved in gene regulation under stress conditions in both ethylene-dependent and -independent ways.

Recently, it has been reported that osmotic stress causes cell-cycle arrest, and ethylene signalling is important for this process. When Arabidopsis plants are exposed to mild (non-lethal) osmotic stress, ethylene production is facilitated and ethylene-signalling-related genes, including a number of ERF genes (B-1 subgroup: ERF11; B-3 subgroup: ERF1, ERF2, ERF5 and ERF6), are induced. Although genes downstream of these ERFs in this process are unknown, this observation suggests
that one of the important roles of ERFs is in the aspect of plant adaptation to abiotic stress conditions.

2.12.ERF1 expression against *Botrytis sps*

ERF genes show a variety of stress-regulated expression patterns. Regulation by disease-related stimuli, such as ethylene (ET), jasmonic acid (JA), salicylic acid (SA), and infection by virulent or avirulent pathogens, has been shown for several *ERF* genes. However, some *ERF* genes are also induced by wounding and abiotic stresses. At present, it is difficult to assess the overall picture of ERF regulation in relation to phylogeny because different studies have concentrated on different *ERF* genes, treatments and time points. The advent of the Arabidopsis whole-genome microarray will result in more easily comparable data. Significantly, several ERF TFs that confer enhanced disease resistance when overexpressed, such as *ERF1*, *Pti4*, and *AtERF1*, are transcriptionally regulated by pathogens, ET, and JA. *ERF1* is induced synergistically by ET and JA, and induction by either hormone is dependent on an intact signal transduction pathway for both hormones, indicating that ERF1 may be a point of integration for ET and JA. At least four other ERFs are also induced by JA and ET, implying that other ERFs are probably also important in ET/JA signal transduction. Several of the genes in subgroup 1, including AtERF3 and AtERF4, are thought to act as transcriptional repressors, and these two genes were found to be induced by ET, JA, and an incompatible pathogen. The net transcriptional effect on these pathways may be balanced between the activation and the repression of target genes. Posttranscriptional regulation of *ERF* genes by

phosphorylation may be a significant form of regulation. *Pti4* is phosphorylated specifically by the Pto kinase, and this phosphorylation enhances Pti4's binding to its target sequence. Recently, the *OsEREBP1* gene of rice was shown to be phosphorylated by the pathogen-induced mitogen-activated protein kinase (MAPK) *BWMK1*. This phosphorylation enhanced the binding of *OsEREBP1* to the GCC box, suggesting that the phosphorylation of ERF proteins may be a common theme. A potential MAPK phosphorylation site has been noted in AtERF5.

To study the down stream defense gene expression in *ERF* gene family transgenics many experiments have led to the understanding of the defense mechanism elicited by various hosts in response to the *Botrytis* attack. *ERF1* is thought to function as a transcription factor, thus implying that a transcriptional cascade is involved in ethylene signaling. *ERF1*, like *EIN3*, is a positive regulator of ethylene signaling. In fact, over expression of *ERF1* leads to the constitutive activation of many ethylene responses.

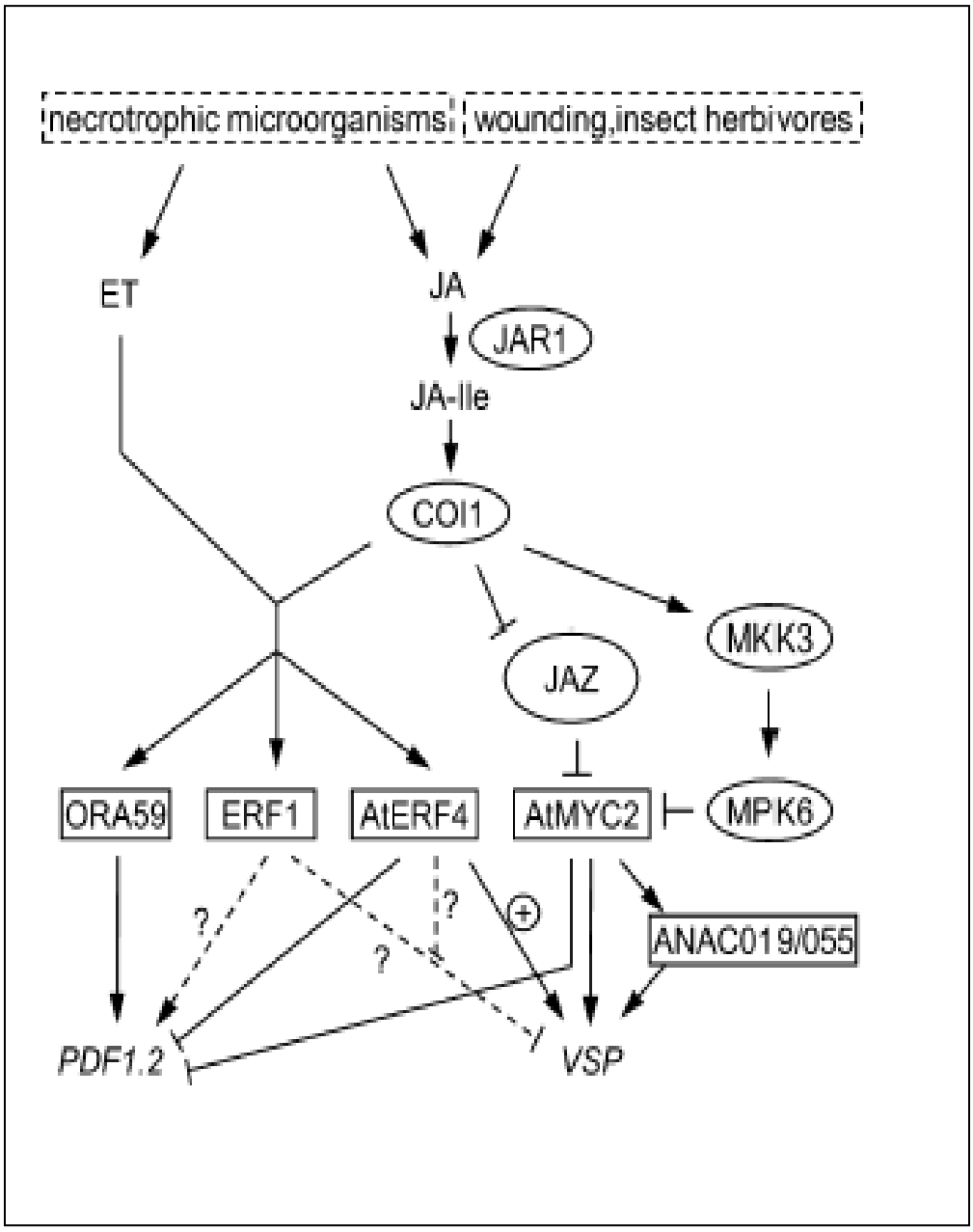


Fig 2.2. Role of transcription factors in the stress-responsive network involving the JA and ET signaling pathways. Different types of biotic or abiotic stress, including wounding, attack by herbivorous insects and infection with necrotrophic pathogens, induce the synthesis of JA and related oxylipins. JAR1 converts JA into the biologically active JA-Ile. Some stress signals such as infection with necrotrophic pathogens simultaneously induce ET biosynthesis. JAs induce the expression of several genes encoding transcription factors, including ORA59, ERF1, AtERF4, AtMYC2 and ANAC019/055, via COI1, an F-box protein that is the receptor for JA-Ile. Binding of JA-Ile results in COI1-mediated degradation of JAZ repressors via the ubiquitin/proteasome pathway, thereby releasing AtMYC2 from repression. The bHLH-type transcription factor AtMYC2 positively regulates the expression of wound-responsive genes (e.g. VSP) and represses other genes, including PDF1.2. The NAC transcription factors ANAC019 and ANAC055 function downstream from and parallel with AtMYC2 to co-stimulate the expression of wound-responsive genes. JAs also activate MPK6 through MKK3. Protein phosphorylation by activated MPK6 affects AtMYC2 activity negatively. The JA and ET signals cooperate to induce the expression of genes encoding the AP2/ERF-domain transcription factors ORA59, ERF1 and

AtERF4. ORA59 is the key regulator of JA/ET-responsive genes including PDF1.2, whereas the role of ERF1 in gene regulation remains unclear and awaits analysis of a knockout mutant (indicated by dashed lines and question marks). Conversely, AtERF4 represses the induction of JA/ET-responsive genes including PDF1.2. AtERF4 also enhances the JA-induced expression of AtMYC2 target genes including VSP (circled plus), possibly by repressing the negative effect of ET executed by ERF1 (dashed bar line and question mark).

The triple response of *ERF1*-overexpressing plants is, however, suggesting that other factors may be required for a full response. The overexpression of *ERF1* gene lead to induction of other defese related genes like PDF1.2. The plants analyzed showed an enhanced resistance to *Botrytis spp.* In general and especially to *B.cinerea* – grey mould.

Indeed, a large number of *EREBP* genes exists in *Arabidopsis* and other species, but the expression of only a fraction of them is regulated by ethylene. Not all ethylene-responsive *EREBPs* encode transcriptional activators, some encode repressors of transcription. Given the myriad of responses to ethylene, it seems logical to expect that some ethylene-related phenotypes result from both the activation of some genes and repression of others. Furthermore, a number of ethylene responsive genes possess neither the EIN3-binding element nor the GCC-box in their promoters. This observation indicates that transcription factors that are unrelated to either *EIN3* or *ERF1*, and which have a different binding specificity, may also be involved in the later steps of ethylene signaling. Identification of these molecules will be crucial to complete our understanding of ethylene-mediated gene regulation.

In tomato, the product of an ethylene-inducible *EREBP*, *PTI4,* can be phosphorylated by the serine/threonine kinase PTO, and the resulting phosphorylated version of PTI4 has a greater affinity for the GCC-box than does unphosphorylated PTI4. Consistent with this observation, the concomitant overexpression of *PTO* and *PTI4* in tomato leads to the induction of pathogenesis-related (PR) genes that have a GCC-box in their promoters.

The rice *OsBIERF3* encodes a protein belonging to the ethylene-responsive element binding protein (EREBP) transcription factor family and was shown to be induced by treatments with chemical inducers of disease resistance response, infection with the blast fungus, *Magnaporthe grisea*, and by

treatments with some abiotic stress conditions, e.g. salt and cold (Cao.Y*et al*., 2006) Elevated level of defense-related *PR-1a* gene expression was also detected in the transgenic tobacco plants.

The *ERF* genes, *BkERF1, BkERF2.1 and BkERF2.2,* were isolated from a medicinal plant *Bupleurum kaoi*. The deduced *BkERFs* contain a canonical nuclear localization signal and an ERF/AP2 DNA binding domain. It was revealed from their analysis that *BkERF1* and *BkERF2.1* were ubiquitously expressed at low levels in all parts of mature plants, and that *BkERF2.2* was expressed at moderate levels in vegetative tissues. Exogenous application of methyl jasmonate induced *BkERF1/2.1/2.2* transcripts. *BkERF2.2* transcript levels were slightly increased by addition of ethephon and salicylic acid. *BkERFs* were localized in the plant nucleus and functioned as transcriptional activators. In *B. kaoi* cells overexpressing *BKERFs*, inoculation with *Botrytis* cinerea increased expression of some defense genes which are associated with enhanced disease resistance. Similarly, overexpression of *BkERFs* in transgenic *Arabidopsis thaliana* resulted in elevated mRNA levels of the defense gene *PDF1.2*, and in enhanced resistance to *B. cinerea*.

Thus, all the above studies on the *ERF* family of genes have revealed the importance of these genes in elevating the defense response in plants on pathogen attack. *ERF1* gene plays a key role both in the normal growth and development and also protecting the plant against biotic and abiotic stress conditions.

2.12.Botrytis Induced Kinase 1(BIK1)

Necrotrophic pathogens are known to induce pathogen-associated molecular pattern –triggered immunity (PTI), involving the induction of jasmonate- (JA) and ethylene (ET)–dependent pathways that activate a different set of defense responses. These include the induction of camalexin biosynthesis and other responses that limit pathogen growth and the ability of the pathogen to cause host cell death. One of the central components of PTI signaling in *Arabidopsis thaliana* is *BOTRYTISINDUCED KINASE1 (BIK1),* which was identified as an early-induced gene in response to infection by *Botrytis cinerea*. *BIK1* required for resistance to the necrotrophic fungal pathogens B. *cinerea* and *Alternaria brassicicola* but suppresses defense against the (hemibiotrophic) bacterial pathogen *Pseudomonas syringae* (Veronese et al., 2006). *BIK1* encodes a receptor-like cytoplasmic kinase that mediates PTI signaling from multiple pathogen-associated molecular pattern receptors (Zhang et al., 2007).

Botrytis Induced Kinase (BIK1) is a crucial component of host response signaling required to activate the resistance responses to *Botrytis* and *A. brassicicola* infection in *Arabidopsis*. *BIK1* encodes a regulatory protein, specifically a protein kinase, predicted to be specific to Ser/Thr residues, that is similar to receptor-like cytoplasmic protein kinases and may act early in the disease response pathway. Additionally, the *BIK1* mutant phenotypes suggest that the *BIK1* kinase function cannot be fully complemented by other endogenous kinases. The *BIK1* gene transcript is strongly upregulated in response to challenge with *Botrytis* and a Reactive Oxygen Intermediate ROI-generating compound. The *BIK1* mutant is susceptible to *Botrytis* and *A. brassicicola* and shows attenuated expression of the plant defensin *PDF1.2* gene, but it is competent for the activation of SAR. However, *BIK1* plants exhibit increased resistance to a virulent bacterial pathogen that is based on SA-dependent and SA-independent pathways and independent of cell death lesions. Interestingly, *BIK1* plants accumulate increased levels of SA before and after *Botrytis* infection, suggesting that *BIK1* acts upstream of SA accumulation. *BIK1*is implicated as a negative regulator of SA accumulation and basal defense against virulent bacterial pathogens, because the *BIK1-null* mutant supported less bacterial growth and a total absence of disease symptoms.

The function of *BIK1* as a disease resistance factor may be to regulate normal levels of SA required for resistance to necrotrophs. Upon infection, *BIK1* may be activated to trigger the *Botrytis* and *A. brassicicola* resistance response, including the regulation of optimal levels of SA synthesis. In the absence of *BIK1*, this regulation is relieved and SA level increases, which may lead to suppression of the mechanisms required for necrotrophic resistance, consistent with previous reports (Penninckx *et al*., 1998; Gupta *et al*., 2000; Kunkel and Brooks, 2002; Spoel *et al*., 2003). Accordingly, *BIK1* contains increased SA levels, reduced *PDF1.2* expression, and distinct disease resistance responses to biotrophic and necrotrophic pathogens. These distinct responses are further substantiated by data from *BIK1 nahG* plants that show wild-type levels of resistance to *Botrytis* infection and expression of *PDF1.2*. The ET/JA functions independent of defense responses are normal, as *BIK1* plants exhibited wild-type sensitivity to plant hormones and lacked hormone-related phenotypes. The loss of resistance to necrotrophic pathogens but enhanced resistance to virulent pathogens suggests that *BIK1* modulates the crosstalk between different defense signaling pathways.
The role of SA in regulating responses to *Botrytis* and other necrotrophs appears to be complex and may be multidimensional, with both positive and negative regulatory roles.

Increased SA also suppresses the expression of JA-responsive genes (Spoel *et al*., 2003) normally required for full resistance to necrotrophic pathogens (Thomma *et al*., 1998, 1999). The function of *BIK1* as a positive regulator of resistance to *Botrytis* and a negative regulator of resistance to *Pseudomonas syringae* pv *tomato (Pst)* is dependent on normal levels of SA. When SA increases above a certain threshold level, it may trigger the suppression of mechanisms required for resistance to *Botrytis* and *A.brassicicola* while still promoting mechanisms for resistance to *Pst*. Alternatively, *BIK1* may suppress SA accumulation and basal resistance to *Pst,* and when removed, it may lead to the activation of defense responses that some necrotrophs (*Botrytis* and *A. brassicicola*) exploit for their benefit. Consistent with this notion, the hypersensitive cell death, a particularly strong defense response, promotes susceptibility to *Botrytis* (Govrin and Levine, 2000).

2.12.1.Localization of *BIK1* to the plasma membrane

Localization of *BIK1* to the plasma membrane suggested that *BIK1* may act as an early component of the plant defense response, either directly in pathogen recognition or early in the signaling cascade.

In response to *Botrytis* infection, the kinase activity of MPK3 and MPK6 increased, but no consistent differences were observed between *BIK1* and wild-type plants. *BIK1* may be activated by pathogen-derived signals or signals resulting from pathogen–plant interactions regardless of the strain. The kinase activity of *BIK1* may be required to transduce pathogen-derived signals to downstream molecules that are targeted for negative or positive regulation depending on the pathogen. In the case of *Botrytis*, host susceptibility positively correlated with the levels of H_2O_2 produced (Govrin and Levine, 2000).

Among defense responses associated with resistance to necrotrophs in *Arabidopsis*, in *BIK1*, camalexin accumulation was not affected, whereas PR-1 was expressed more strongly and the induced expression of PDF1.2 was reduced. The *BIK1* mutation represents a defect in a novel gene affecting disease responses and plant growth (Veronese et al., 2006). It is suggested that *BIK1* affects the signaling required for the activation of cellular mechanisms involved in plant development and the pathogen response, and they provide a novel avenue for further dissection of the components of disease response pathways.

The detailed analysis of the genetic, molecular, and biochemical function of *BIK1* was presented that showed it's regulating responses to bacterial and fungal pathogens. The authors used site-directed mutagenesis, biochemical assays, and mutant analysis to define specific residues essential for *BIK1* phosphorylation, *in vivo* kinase activity, and biological function. The results showed how differential phosphorylation in the kinase domain region might confer specificity to *BIK1* function. They have also shown that *BIK1* is required for seedling growth responses to ET and glucose, which are known to interact antagonistically. *BIK1* was found to be required for ET signaling, having a function similar to that of *EIN2*, a central transducer of ET signaling. Induction of immunity to *B. cinerea* required both *BIK1* and *EIN2* but did not involve salicylate responses and was antagonized by COI1 (a key component of JA signaling). By contrast, basal and induced *PTI* responses to virulent and nonpathogenic *P. syringae* strains were modulated by salicylate as well as by ET and JA responses. *BIK1* may be linked to promotion or suppression of disease, and provides insight to the complex interplay between plant growth and defense. Interestingly, although *BIK1* shares high sequence

similarity to many Arabidopsis receptor-like cytoplasmic kinases, its function in defense against necrotrophic infection and responses to ET appears to be unique.

2.13.Arabidopsis thaliana Ethylene responsive element Binding Protein (AtEBP)

A class of proteins that contain the AP2/EREBP domain are the ethylene-resonsive element binding proteins (EREBPs). The EREBPs bind the GCC box that confers ethylene responsiveness to a number of pathogenesis related (PR) gene promoters, thereby increasing the expression of the PR proteins which confer the defense against pathogen attack. The EREBP RNA levels are up-regulated by ethylene, thereby inducing the defense response. Further evidence roving the role of EREBs in eliciting the defense response has come from an analysis of a tomato resistance (R) gene, *Pto*.

Similar to the EREBPs are a second class of transcription factors that may play a role in the lant defense response are *ocs* element binding factor (OBF) proteins which bind to a 20-b DNA promoter sequences called the *ocs* elements. AtEBP (Arabidosis thaliana ethylene-responsive Element Binding Protein) is one of the OBF proteins which has been identified to bind to these *ocs* elements in *Arabidopsis* further induce the lant defense resonses. *AtEBP* gene expression is inducible by exogenous ethylene in both *Arabidopsis* and Tobacco plants (Buttner *et al*.,1997). *AtEBP* transcripts were seen to be increased in the *ctr-1* mutant, where ethylene-regulated pathways are constitutively active. Electrophoretic mobility-shift assays and DNase I footprint analysis revealed that AtEBP protein can specifically bind to the GCC box. The synergistic effects of the GCC box with the *ocs* elements would further up-regulate the expression of number of ethylene-induced PR gene promoters. These studies suggest that AtEBP proteins which belong to the EREBP transcription factors therefore be important in regulating the gene expression during the plant defense response.

2.14.Need for Promoters with elevated expression in the inflorescence

Ethylene biosynthesis in higher plants is regulated developmentally and environmentally. Ethylene, a two-carbon olefin, is a volatile hormone in higher plants. It has diverse regulatory functions in plant growth and development (Yang and Hoffman, 1984; Abele*et al*., 1992; Kende, 1993; Fluhr, 1996; Bleecker and Kende, 2000)**.** As a senescing hormone, it promotes leaf-yellowing, climacteric fruit ripening, flower and leaf abscission. As a stress hormone, it is involved in biotic and abiotic

stress responses of plants (Yang and Hoffman, 1984; Ge *et al.*, 2000). In addition to its stimulatory effect on seed germination and root growth (Tanimoto *et al.*, 1995; Clark *et al*., 1999; Petruzzelli *et al*., 2000), it also regulates gravitropism and phototropism (Harper *et al*., 2000; Lu *et al*., 2001, 2002). The production of ethylene in high er plants is from *S*-adenosyl-L-methionine (AdoMet) via the ACC-dependent pathway. The prevalent AdoMet is first converted to 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase and then to ethylene by ACC oxidase (Adams and Yang, 1979). Sustainable production of ethylene through this pathway is accomplished by replenishing AdoMet through the methionine cycle at the expense of ATP. The regulation of the rate of ethylene biosynthesis in higher plants is at the step of ACC formation (Yang and Hoffmann, 1984). The key enzyme that regulates the level of ethylene production, including most cases of stress ethylene production, is ACC synthase (*S*-adenosyl-L-methionine methylthioadenosine-lyase, EC 4.4.1.14; Boller *et al*., 1979; Yu *et al*., 1979). It is known that a higher level of ACC accumulation is stimulated by increasing chilling stress (Wang and Adams, 1982). Cadmium- and copper-elicited ethylene production was preceded or paralleled by enhanced ACC production (Yu and Yang, 1980). Wounding of winter squash mesocarp first triggers a rise in ACC synthase activity, followed by a surge in the ACC accumulation and ethylene production rate (Hyodo *et al*., 1985). IAA-induced ethylene biosynthesis (Abeles, 1966) is accompanied by an increase in ACC synthase activity and ACC content (Yu and Yang, 1979).

ACC synthase (ACS), the key enzyme in the ethylene biosynthesis pathway, is encoded by a multigene family (Theologis, 1992; Kende, 1993; Fluhr and Mattoo, 1996; Ge*et al.*, 2000; Wang *et al*., 2002). It has been reported that there are eight ACS genes in tomato (Rottmann*et al*., 1991; Yip*et al*., 1992; Lincoln *et al*., 1993; Olson *et al*., 1995; Oetiker *et al.,* 1997; Shiu *et al*., 1998), six in mung bean (Botella *et al.,* 1992; Yoon *et al*., 1997), and five in potato (Destefano-Beltran *et al*., 1995; Schlagnhaufer *et al.,* 1997). Complete sequencing of the rice genome (Yu *et al*., 2002) helped to identify five ACS genes from rice plants (van der Straeten *et al.,* 2001). The expression of ACC synthase genes in higher plants is regulated developmentally and environmentally (Kende, 1993; Fluhr and Mattoo, 1996). They are induced either in such specific tissues as in the hypocotyl, leaf, root, tuber, petiole, flower petal, pistil, stamen, and fruit or in response to such biotic and/or abiotic environmental factors as radiation, Cu^{2+}, Li^{+}, ozone, wounding, amino-oxyacetic acid, cyclohexamide, EIX, protein kinase inhibitor, anaerobiosis (flooding), ethylene, IAA,

benzyladenine, chilling, and pathogens (Ge *et al*., 2000; Wang *et al*., 2002). The expression of each member of the ACS multigene family is also differentially regulated in response to these factors. On the one hand, a member of the ACS gene family is able to respond to numerous different developmental and environmental signals (van der Straeten *et al*., 1990; Rottmann *et al*., 1991; Zarembinski and Theologis, 1993; Wang and Arteca, 1995; Clark *et al*., 1997; *et al*., 1997; Shiu *et al*., 1998; Arteca and Arteca, 1999; Ge*et al*., 2000). On the other hand, a single developmental cue or environmental factor is able to induce the co-ordinated expression of several ACS genes (Olson *et al*., 1991; Subramaniam *et al*., 1996; Schlagnhaufer *et al*., 1997).

The complete sequencing of the *Arabidopsis* (Salanoubat *et al*., 2000) genome has revealed that there are 10 *AtACS* genes. All *AtACS* genes, except *AtACS3*, are known to be active and differentially expressed during *Arabidopsis* growth and development (Yamagami *et al*., 2003). Among these active *AtACS* genes, *AtACS1* (At3g61510), *AtACS2* (At1g01480), *AtACS4* (At2g22810), *AtACS5* (At5g65800), and *AtACS6* (At4g11280) were best studied for their transcriptional activities with both RT-PCR and northern blot analysis (van der Straeten *et al*., 1992; Liang *et al*., 1992, 1996; Abel *et al*., 1995; Vahala *et al*., 1998; Miller *et al*., 1999; Smalle *et al*., 1999).

A GUS reporter-aided approach was adopted to elucidate the promoter activities of *AtACS4*, *AtACS5*, and *AtACS7* in *Arabidopsis* (Table 1.1). To that end, the 5′ flanking regions of the three *AtACS* genes were cloned in order to perform the GUS reporter-aided histochemical and fluorometric study. It was found that there was always a member of *AtACS* gene family that remains active at any stage of *Arabidopsis* growth and development. *AtACS5* had the highest promoter activity in the 2-week-old light-grown seedlings. At the reproductive stage, the expression *AtACS5* gene was localized in areoles, whereas *AtACS4* and *AtACS7* were localized in both areoles and veins. The *AtACS4* promoter became more active as plants grew older. All *AtACS* genes studied were expressed in root tissues. Treatment of these transgenic plants with hormones and stresses showed that *AtACS* genes were differentially regulated, suggesting that each *AtACS* gene had a unique expression programme during *Arabidopsis* growth and development (Wang *et al*., 2005).

Table 2.2.GUS reporter aided expression profiles of *Arabidopsis* promoters AtACS4, 5 and 7

Plant region examined for the GUS expression	AtACS4 (1.2Kb)	AtACS5(1.23Kb)	AtACS7(1.2Kb)
3 day old etiolated seedling	High	High	High
1 week old seeding	High	High	High
2 week old seedling	Moderate	Moderate	Moderate
4 week old seedling	Moderate/Less	Moderate/Less	Moderate/Less
Trichomes of leaves	Absent	High	Absent
Cauline leaves	Moderate	High	High
Flower stalk	High	Moderate	Moderate
Inflorescence stem	High	High	High
Siliques	High	High	High
False septum	High	High	High
Suspendors	High	High	High
Seeds	Moderate	Moderate	High

2.15.Multi cassette vector- A Challenge!

The simultaneous manipulation of multiple genes in transgenic plants presents a clear challenge for plant biologists and biotechnologists. Although several approaches can be used for the delivery of multiple genes into plant cells, the stacking of multiple expression cassettes onto a single binary plasmid seems to be the most straightforward method (Halpin *et al*., 2002). The availability of the various methods of multigene vector assembly systems will most likely facilitate the introduction of multiple genes into plant species (Tzfira *et al*., 2007). Nevertheless, several questions still remain open and technical issues need to be addressed before these systems, and others that may follow, can be routinely used for plant research and biotechnology.

While it has been shown that stable expression of four different genes can be achieved in transgenic plants, even when all genes are driven by the same constitutive promoter (Bohmert *et al*., 2002), further research is needed to determine whether such long constructs with repetitive elements remain stable in both bacteria and plant cells. Naturally, new strong constitutive promoters need to be identified to expand the user's choice of regulatory elements beyond the rather limited number of constitutive promoters that exist today.

The construction of large binary vectors also raises the issue of vector size limitations and the efficiency of delivering long T-DNA molecules into plant cells. These issues can be addressed, for example, by using vectors based on binary bacterial artificial chromosomes (Hamilton, 1997) or TAC (Liu *et al*., 1999), as these vectors possess the capacity to clone very large DNA fragments. Indeed, the Cre/LoxP-based vector system (Lin *et al*., 2003) utilizes TAC vectors as the acceptor plasmids for the plant expression cassettes and a Gateway-compatible TAC vector has also been constructed to support the transfer of long DNA fragments via the Multi Round Gateway cloning strategy (Chen *et al*., 2006). Similar vectors will need to be designed and constructed to support the use of rarecutter cloning strategies for the assembly of large multigene plant transformation vectors.

Finally, the modularity of the rare-cutter-based cloning pSAT system is limited only by the small number of commercially available rare-cutting restriction enzymes. Zinc finger nucleases (ZFNs) are

hybrid synthetic restriction enzymes that can be specifically designed to bind and cleave long (typically 24–30 bp) stretches of DNA sequences (Mani *et al*., 2005). ZFNs have been successfully used for various purposes of genome engineering in various organisms (Durai *et al*., 2005) ZFNs, which can be artificially assembled and used for cloning long DNA molecules (Tzfira *et al*., 2005) can facilitate the expansion of the pSAT system beyond its current capacity of seven expression cassettes (Tzfira *et al*.,2007). Thus, further research should be directed toward the development of novel ZFNs, as well as toward the establishment of protocols for their assembly, purification, and use for DNA cloning purposes.

2.16.Tissue culture studies

2.16.1.Tobacco- A Proof of Concept

The generation of genetically transformed plants is central to, and has indeed revolutionized, plant molecular biology. This is true for studies at both the fundamental and more applied levels of research. For researchers interested in unraveling the roles of specific genes in particular pathways of growth and development, the introduction into plants of foreign genes and gene promoters linked to reporter genes allows the detailed study of the temporal, spatial, and quantitative expression of plant genes and the activities of associated regulatory sequences.

It is not yet possible to transform many of the important crop species, and therefore so-called model plants species are used widely in transgenic research. A model plant species, for use in such studies, can be defined as one that can be efficiently and simply transformed with foreign DNA. Furthermore, the transformed cells or tissues must then be able to regenerate to produce fertile mature plants that produce transgenic seed.

Over the years, one particular dicot species that has emerged as an excellent model plant for transgenic studies is *Nicotiana tabacum* (Tobacco). One tobacco cultivar commonly used is *N. tabacum* cv. Petit Havana. However, the methods are also applicable to other cultivars, such as Samsun and Xanthl. The most efficient and technically most simple method of transforming tobacco is to infect leaf explants with disarmed strains of the naturally occurring soil-borne bacterium

Agrobacterium tumefaciens, which contains a disabled (nononcogenic) Tl plasmld. The gene construct to be transferred is integrated between the T-DNA borders of a binary vector, which is introduced into the *Agrobacterium*. Following inoculation, and under suitable culture conditions, the leaf explants will readily regenerate transgenic plantlets which can then be potted out and grown to maturity.

As castor is not that amenable for transformation Tobacco is used as a proof-of-concept to study the expression of the inserted transgenes and the tolerance imparted by these obtained transgenics against *Botrytis* infection. The gene constructs that show the maximum tolerance against *Botrytis* infection have been chosen to further transform castor explants to develop the castor transgenics tolerant to *Botrytis* and any necrotrophic fungi in general.

2.16.2Castor tissue culture

Earlier studies on tissue culture in Euphorbiaceae including castor were mostly with endosperm cultures (Thomas and Chaturvedi, 2008). The interest in endosperm culture was mainly due to the large endospermic seeds that enabled easy culturability. The objectives were to obtain triploid plants and to understand the metabolism of glyoxylate pathway. Triploids will be useful for obtaining trisomic lines for genetic mapping. This can be accomplished through endosperm culture or through chromosome doubling (in vitro and in vivo) and crossing the resultant autotetraploids with diploids (Gmitter *et al*.,1990; Sikdar and Jolly,1994; Chaturvedi *et al*., 2003). Attempts to grow endosperm tissue in cultures began in the 1930's and immature and mature endosperms of various angiosperm taxa have been successfully cultured (Johri *et al*., 1980). La Rue (1944) had reported organogenic differentiation in endosperm cultures of castor bean. However, extensive investigations by subsequent workers on maize and castor bean could not confirm La Rue's claim of achieving organogenesis.

The ability of cells from mature endosperm to divide was first demonstrated by Mohan Ram and Satsangi (1963) followed by successful establishment of tissue cultures from mature endosperm (Satsangi and Mohan Ram, 1965). White's medium supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D), kinetin, and yeast extract (YE) supported optimal growth of the endosperm tissues of

castor (Srivastava,1971a; Johri and Srivastava, 1972). In castor, the 'embryo factor' was an absolute necessity and mature endosperm required the initial association of an embryo for its proliferation (Satsangi and Mohan Ram, 1965; Johri and Bhojwani, 1965). Organogenesis, however, has not been achieved in this crop. Studies made by Brown *et al*. (1970) in *Castor* indicated that the cultured endosperm tissue provides possibilities for detailed studies on enzyme changes that take place during culture but does not appear to provide suitable material for the detailed study of the capabilities of the endosperm *in vivo.*

In castor, callus initiation and plantlet regeneration from vegetative explants are restricted to young seedling tissues (Athma and Reddy, 1983; Sarvesh *et al*., 1992; Ganesh Kumari *et al*., 2008; Table 1). Callus could be initiated from the seedling explants but the ability to regenerate plants has been limited. Athma and Reddy (1983), assessed the differences in callusing ability and organogenic potential of the various seedling explants and obtained shoots from the shoot tips and rhizogenesis from root explants, shoot and leaf tissues. Similarly, plant regeneration that has been reported to occur in the seedling explants was reported to involve pre-existing meristematic regions (Reddy *et al*., 1987b; Sangduen *et al*., 1987; Khumsub, 1988; Sujatha and Reddy, 1998; Sujatha and Sailaja, 2005; Malathi *et al*., 2006). Multiple shoot induction reported by Sangduen *et al*. (1987) and Khumsub (1988) was from embryo explants. Further, Reddy and Bahadur (1989b) reported the genotypic response for shoot tip multiplication in castor.

The shoot multiplication rates obtained from meristematic explants in most of these studies varied between 1 and 5.2 (Athma and Reddy, 1983; Sangduen *et al*., 1987; Reddy and Bahadur, 1989b; Molina and Schobert, 1995). A significant improvement of meristematic explant proliferation was the assessment of a wide range of cytokinins on different meristematic explants (Sujatha and Reddy, 1998). Shoot proliferation rates reported in this investigation were high and were 40 and 81.7 from the embryo axis and 46.7 and 22.0 from shoot tips on induction and subculture media, respectively. The study clearly revealed the differential effects of cytokinins in different meristematic explants and the carryover effect of thidiazuron (TDZ) in shoot proliferation cultures for 2–3 cycles. Meristematic explants responded differently with different types of cytokinins and TDZ gave the maximum number of shoots (37.8–40.0) from embryo axes, while benzyl adenine (BA) promoted highest number of shoots (46.7) from the shoot apex. Excessive proliferative ability

of castor meristematic tissues on medium supplemented with TDZ has been successfully used for genetic transformation of castor (Sujatha and Sailaja, 2005; Malathi *et al*., 2006). Thidiazuron was found to promote direct shoot regeneration from hypocotyls segments and callus mediated regeneration from seedling leaf tissues (Ganesh Kumari *et al*., 2008).

Callus mediated regeneration is reported from hypocotyls sections (Reddy *et al*., 1987a), young stem segments (Genyu, 1988), young leaves (Reddy and Bahadur, 1989a) and epicotyl/ cotyledons (Sarvesh *et al*., 1992). However, differentiation of callus into shoots and shoot buds was reported to be either occasional or low. Reddy and Bahadur (1989a) reported 3–4 buds per leaf explant but the overall caulogenic response was not described. In studies by Sarvesh *et al*. (1992), 20% of the cultures with shoot buds induced on B5 (Gamborg, 1968) medium supplemented with 2.5 mg l− 1 BA, 0.1 mg l− 1 naphthaleneacetic acid (NAA) and 0.4 mg l−1 gibberellic acid (GA3), on transfer to the same medium produced 6–8 shoots per callus. Sujatha and Reddy (2007) assessed the morphogenic competence of castor tissues on several basal media supplemented with a large number of growth regulators individually and in combination and in a broad range experiment according to De Fossard*et al*. (1974) having 81 combinations at three levels of minerals, sucrose +growth factors+amino acids besides growth regulators, which revealed low caulogenic response of castor explants for direct as well as callus mediated shoot regeneration.

In most of the tissue culture studies using seedling explants of castor, Murashige and Skoog (MS) basal medium has been found to be ideal except in callus mediated regeneration from epicotyl/cotyledonary tissues (Sarvesh *et al*., 1992; Sujatha and Reddy, 2007; Ganesh Kumari *et al*., 2008) where B5 basal media or B5 vitamins proved to be superior. Multiple shoots could be proliferated when cytokinin was supplemented singly, such as BA at 0.5−2.0 mg l−1 (Athma and Reddy, 1983), 1.0−2.0 mg l−1 (Reddy *et al*., 1986), 4.0 mg l−1 (Sangduen *et al*., 1987; Khumsub, 1988), 0.2 mg l−1 TDZ or 4.0 mg l−1 BA (Ahn *et al*., 2007) or in combination with an auxin [1.0 mg l−1 BA+0.5 mg l−1 NAA or 0.5 mg l−1 kinetin+1.0 mg l−1 NAA] (Reddy *et al*., 1986), 2.0 mg l−1 BA+0.8 mg l−1 NAA (Ganesh Kumari *et al*., 2008). Callus cultures of *R. communis* are usually initiated in the presence of both an auxin and cytokinin. The cytokinins, kinetin (2.0 mg l−1) and BA (1.0, 2.0 or 4.0 mg l−1) with NAA (1.0mg l−1) or indoleacetic acid (IAA) (0.5 mg l−1) were most often used (Table 1). GA3 (0.2 mg l−1) is sometimes added to shoot regeneration medium although

no requirement for GA3 has been demonstrated for regeneration (Sarvesh *et al*., 1992) while it proved effective in shoot elongation (Sujatha and Reddy, 1998; Ganesh Kumari *et al*., 2008). Root formation has been initiated when regenerated shoots were transferred to medium supplemented with auxin (IBA, NAA) alone (Athma and Reddy,1983; Genyu,1988; Sujatha and Reddy,1998; Ahn *et al*., 2007), in combination with cytokinin (Reddy and Bahadur, 1989a,b), on the shoot proliferation medium itself (Sangduen *et al*., 1987) or with silver nitrate (Ganesh Kumari *et al*., 2008).

Despite research efforts over the last three decades, whole plants still could not be regenerated with reproducible frequencies from friable callus cultures of castor. The sporadic appearance of shoots from callus cultures of castor implies that the calli contain at least a few morphogenic cells interspersed in several non-morphogenic tissues. Failure to isolate a competent cell line might result in its suppression by the overgrowth of non-competent cells. Alternatively, the occasional appearance of shoots could be owing to the activation of recalcitrant calli to undergo caulogenesis caused by a rare inductive stimulus resulting from the interaction between exogenous and endogenous conditions. Recent investigations showed stimulatory effect of amino acids on organogenesis in castor (Sujatha and Reddy, 2007; Ganesh Kumari *et al*., 2008). Incorporation of 0.1% lysine monohydrochloride in B5 medium supplemented with BA and indolebutyric acid (IBA) improved the morphogenic competence of castor tissues (Sujatha and Reddy, 2007). Ganesh Kumari *et al*. (2008) evaluated the influence of amino acids like alanine, glutamine, hypocot and serine and 15 mg l−1 glutamine showed the best response for multiple shoot proliferation and maximum number of shoots (22) per regenerating callus. The study of Ganesh Kumari *et al*. (2008) also revealed the beneficial effect of polyvinyl pyrrolidone (10–15 mg l−1) in controlling phenolic secretion.

Although there is considerable potential to improve the agronomic performance of castor using recombinant DNA and gene transfer techniques, the lack of a protocol for plant regeneration has restricted the development of transgenic cultivars. Until very efficient and reproducible system of plant regeneration is available, genetic transformation experiments would have to continue with meristem explants (Sujatha and Sailaja, 2005; Malathi *et al*., 2006). Recently, protocols for highly efficient shoot regeneration and somatic organogenesis have been reported (Ahn *et al*., 2007; Ganesh Kumari *et al*., 2008). The frequency of callus-mediated shoot regeneration reported by Ganesh

Kumari *et al*. (2008) is high (85%) but shoots appear 16 weeks after culture establishment. As genetic transformation involves several manipulations for gene introduction followed by selection for 2–3 subculture cycles, the efficiency of these regeneration systems for genetic transformation of castor needs to be established.

2.16.3.Genetic transformation of castor

- **Production of transgenic castor resistant to biotic stresses**

Transgenic research in castor has been undertaken for development of insect resistant and ricin free genotypes (Auld *et al*., 2001; Malathi *et al*., 2006). The other interest in castor is for production of epoxy oil which offers all the advantages of a premium oil based paint without the liberation of volatile pollutants (Mc Keon and Chen, 2001). The chemical structure of epoxy oil is very similar to that of castor oil and only minor modifications are needed to cue the castor plant to make epoxy oil instead of castor oil. Castor plant also has the ability to produce industrially useful fatty acids (az, 2006).

Recalcitrance for *in vitro* manipulations has been a major problem for undertaking plant transformation experiments in castor. Mc Keon and Chen (2003) obtained genetically engineered plants by employing the method of *Agrobacterium*-mediated transformation through vacuum infiltration of wounded flower buds (US Patent No 6.620.986). The first successful attempt to develop a stable transformation system for castor using vegetative explants has been described by Sujatha and Sailaja (2005). In this protocol, co-cultivated explants were initially subjected to expansion and proliferation on Murashige and Skoog (MS) medium with 0.5 mg l−1 thidiazuron followed by 3 cycles of selection on medium with 0.5 mg l−1 BA and increasing concentrations of hygromycin (20–40–60 mg l− 1). Selected shoot clusters were transferred to medium with 0.5 mg l−1 BA for proliferation and 0.2 mg l−1 BA for shoot elongation. Elongated shoots were rooted on half-strength MS medium with 2.0 mg l−1 NAA. With this protocol, primary transformants could be developed within 5 months from cultured embryo axes with an overall transformation efficiency of 0.08% (Fig. 2). As the protocol does not involve an intervening callus phase, no abnormal phenotypes are expected through this procedure. This protocol has been extensively used for development of transgenic lines of castor resistant to major foliage feeders through deployment of Cry1Ec, Cry1Aa and Cry1Ab genes (Sujatha and Sailaja, 2007). The same procedure with minor

modifications was used for production of semilooper resistant transgenic castor by incorporating Cry1Ab gene (Malathi *et al*., 2006). The transformation protocol, in this study, has been optimized with constructs harbouring hpt gene as selectable marker, while the construct harbouring insect resistance gene carried the herbicide resistance gene (bar) for selection of putative transformants.

Table 2.3.Morphogenic response from cultured tissues of castor (*R. communis* L.)

Explants	**Type of Morphogenic response**	**Medium used (mg l^{-1})**	**Frequency of morphogenesis**	**Reference**
Endosperm	Regeneration of roots	–	–	La Rue, 1944
Mature seed	Proliferation of endosperm	2, 4-D or Kn	–	Mohan Ram and Satsangi, 1963
Decoated mature seed	Proliferation of endosperm and establishment of tissue cultures; differentiation of tracheidal cells in callus	2,4-D+Kn+YE	–	Satsangi and Mohan Ram, 1965
Endosperm from germinated seed	Proliferation of endosperm (endosperm from dried seeds failed to grow)	2,4-D+Kn+YE	Callus −82%	Brown *et al*., 1970
Fresh decoated seed	No organogenesis	2,4-D+Kn+YE	–	Srivastava, 1971a
Fresh decoated seed	Proliferation of endosperm producing callus that could be continuously subcultured	2,4-D+Kn+YE	Callus −56%	Johri and Srivastava, 1972
Shoot, cotyledon, □ypocotyls, root, endosperm, embryo	Only callus except endosperm. Multiple shoots from embryo	BA 4.0	–	Khumsub, 1988
Cell suspensions	Nutrient transport and	—	—	Cho and Choi, 1990

	uptake			
Cotyledonary callus cultures	Xylogenesis	NAA 2.0+BA 0.5	–	Bahadur*et al*., 1991
Epicotyl and cotyledonary explants	Multiple shoots/roots	BA 2.5+NAA 0.1 – GA3 0.2 BA 2.0+NAA 1.0	96.5% callus with shoot buds	Sarvesh*et al*., 1992
Root, shoot and cotyledonary leaf	Shoots from pre-existing meristems, roots	BA 0.5-2.0 NAA 0.5 – roots	Callus – 90-98% Shoot regeneration - 25-30% with 1 shoot per explant	Athma and Reddy, 1983
Young stem	Bud differentiation	NAA 1.0+BA 1.0 or IAA 0.5+ BA 2.0 or 4.0 – shoot buds NAA 0.5 – roots	–	Genyu, 1988
Seedling explants	Shoots from pre-existing Meristems	BA 2.0	Shoot proliferation – 25-30%	Athma and Reddy, 1989
Leaf	Adventitious buds	Kn 2.0+IAA 1.0	–	Reddy and Bahadur, 1989a
Shoot apex	Multiple shoots	Kn 2.0+IBA 1.0	79.1% with a mean of 5.2 shoots	Reddy and Bahadur, 1989b
Hypocotyl, leaf and shoot tips	Shoot tip proliferation	BA 1.0 or 2.0, NAA 1.0+ Kn 0.5; NAA 0.5+BA 1.0	–	Reddy *et al*., 1986
Shoot tip callus	Shoot buds	Kn 2.0+NAA 1.0	–	Reddy *et al*., 1987b
Seed and seedling	Shoot bud	BA 4.0	–	Sangduen *et al*., 1987

explants	proliferation			
Shoot tip	Multiple shoots	BA 0.25	79% with 4.4 shoots per explant	Molina and Schobert, 1995
Embryo axis and shoot tips	Shoot proliferation	TDZ 0.5-10.0	100% shoot proliferation with 81.7 shoots from embryo axis and 22.0 shoots from shoot tips	Sujatha and Reddy, 1998
Hypocotyl from zygotic embryo axis	Adventitious shoots	TDZ 0.25 or BA 4.5	24.2 shoots per explant	Ahn *et al*., 2007
Hypocotyl	Adventitious shoots	BA 2.0+IBA 0.5-1.0+0.1% lysine monohydrochloride	22.3-25.0% shoot bud induction	Sujatha and Reddy, 2007
Seedling explants – cotyledons, hypocotyls, epicotyl, leaf	Callus mediated organogenesis	BA 2.0+NAA 0.8 – callus TDZ 2.5+NAA 0.4+glutamine 15	85.0% callus induction with a shoot mean of 22 shoots per callus	Ganesh Kumari *et al*., 2008

CHAPTER III

MATERIALS AND METHODS

The aim of the present study was to develop transgenic castor tolerant to *Botrytis spp* using a transgenic approach. As enumerated in Chapter 2, there are several reports of developing transgenic plants with partial tolerance to necrotrophic fungi by deploying single genes. But there are no reports of deploying two or more genes to study the cumulative effect in controlling fungal disease(s). In the resent investigation, we chose to study the cumulative effect of three genes in imparting tolerance against *Botrytis*. Based on the literature available, three defense regulatory genes, *BIK1, ERF1* and *AtEBP* from *Arabidopsis*, were selected for the development of gene constructs. As *Botrytis ricini* basically infects the inflorescence in castor, three promoters, *ACS4, 5* and *7*, known to drive elevated expression in the inflorescence parts in *Arabidopsis* (Wang *et al*., 2005) were chosen for driving the expression of selected genes. With this premise, a multigene cassette with three individual gene cassettes, a double gene cassette and three individual gene cassettes were to developed.

Also, as the expression pattern of the chosen promoters had not been clearly demonstrated in a heterologous system, suitable gene cassettes using a reporter gene system were to be developed for validating the expression pattern of these promoters in tobacco. With this hypothesis and rationale, the investigation was carried out at the Directorate of Oilseeds Research, Hyderabad, with the following objectives

(a) Phylogenetic analysis of *Botrytis ricini* to understand its relatedness with other *Botrytis* species.

(b) Characterization of *Arabidopsis thaliana* promoters *ACS4, 5* and *7* in tobacco using (*gus*) *uidA* as reporter gene.

(c) Development of gene constructs using three inflorescence specific promoters *ACS4, 5, 7* and three genes *ERF1, BIK1* and *AtEBP* from *Arabidopsis thaliana.*

(d) To develop multi-gene cassette vector by stacking the three individual gene cassettes within one T-DNA.

(e) Transformation of model plant tobacco and target plant castor using the developed constructs.

(f) Validation of transgene expression and the imparted resistance against *Botrytis* in the obtained transgenic plants.

Materials used in the present study are mentioned here

3.1 Materials

3.1.1 Plant Material

Castor (*Ricinus communis* L.) cultivar DCS-9 (Jyoti) and 48-1 (Jwala) from the Directorate of Oilseeds Research (DOR), Hyderabad and tobacco cultivar *Nicotiana tabacum* cv. tabacum were used in the present study.

Table 3.1.Equipments/Instruments

Equipment	**Company/Suppliers**
Autoclave	Sanyo, Lab-tech, Japan
Bio-imaging system	Syngene, Alpha innotech, USA
Centrifuges, 4 ºC	Heraeus, Germany
Centrifuge, RT	Eppendorf, Germany
Dry bath	Genei, USA
Electrophoresis units	Biorad, Consort , USA
Electroporator	Eppendorf, USA
Freezer (-20 ºC)	Sanyo, Japan
Hot air oven	JSR, Korea
Incubators	JSR, Korea
Laminar air flows	Klenzaids, Klenz Flo, Chennai
Ligation bath	Genei, USA
Microwave oven	Kenstar, LG, Bajaj, India
Microscopes	Leitz/Leica, Deutschland
Milli Q unit	Millipore, USA
Nanodrop	GE Healthcare, USA
PCR	Eppendorf, USA
pH meter	Eutech, USA
Pippetman	Eppendorf, Axygen, USA
Real Time PCR	ABI, USA
Spectrophotometer	Genway, USA
UTL freezer (-80 ºC)	Thermo Electron Corporation, USA
Vacuum pump	Millipore, USA
Vortex mixer	Genei,
Water bath	Haake, USA
Weighing Balance	Mettler, USA

3.1.3 Media

3.1.3.1 Plant tissue culture

Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) was used as the basal medium for tissue culture and transformation of castor as well as tobacco. For the preparation of medium, readymade media devoid of hormones (Himedia, India) was used for making the required working medium (by mixing them in required proportions in distilled water to obtain the desired concentrations). The composition of MS medium is given in Table 3.1. The basal MS medium with full strength or half strength of the components with suitable hormones, antibiotics/selection agents were used at various stages of transformation and regeneration.

3.1.3.2.Culture and maintenance of bacterial clones and strains

Luria Bertani (LB) liquid medium and solidified medium (LA) were prepared from the products supplied by Himedia Company and used for culturing *E. coli* strains. YEP broth [0.5% yeast extract, 0.5% bacteriological peptone, 0.3% $(NH_4)_2SO_4$, 0.3% KH_2PO_4, 2% glucose] was used for culturing as well as maintaining *Agrobacterium* strains. Keto-Lactose medium (Lactose 1%, yeast extract 0.1% and bacto agar 2%) was used to confirm the *Agrobacterium* clones.

3.1.4.Hormones

Details of the hormones used in the present investigation are given in Table 3.2.

3.1.5.Antibiotics / Herbicide

Details of the antibiotics used in the present investigation are given in Table 3.3.

Table 3.1.Composition of MS medium

Stock	Constituents	Actual conc. (mg l^{-1}) × magnificant	Vol. of stock solution (ml)	Vol. of stock solution used (ml/l)
A	**Macronutrients**			
	NH_4NO_3	1650 × 10	500	50
	KNO_3	1900 × 10		
	$MgSO_4.7H_2O$	370 × 10		
	KH_2PO_4	170 × 10		
B	**Calcium Chloride**		100	10
	$CaCl_2.2H_2O$	440 × 10		
C	**Micro-nutrients**			
	KI	0.83 × 50	100	2
	H_3BO_3	6.20 × 50		
	$MnSO_4.4H_2O$	22.3 × 50		
	$ZnSO_4.7H_2O$	8.6 × 50		
	$Na_2MoO_4.2H_2O$	0.25 × 50		
	$CuSO_4.5H_2O$	0.025 × 50		
	$CoCl_2.6H_2O$	0.025 × 50		
D	**Iron-EDTA**		100	10
	$Na_2EDTA.2H_2O$	37.3 × 10		
	$FeSO_4.7H_2O$	27.8 × 10		
E	**Vitamins**			
	Glycine	2.0 × 100	100	1
	Nicotinic Acid	0.5 × 100		
	Pyridoxine-HCl	0.5 × 100		
	Thiamine-HCl	0.1 × 100		
F	**Myo-Inositol**		100	10
	Myo-Inositol	100 × 10		
	Sucrose	30,000	Added directly	Added directly
	Agar	7,000		
	pH	5.8		

Table 3.2.Details of the hormones used for tissue culture

Hormone	Purpose	Working concentration	Stock	Supplier
Benzyl amino purine (BAP)	For shoot multiplication and elongation	0.2-0.5 mg/l	1 mg/ml	Sigma
Naphthalene acetic acid (NAA)	For shoot induction and root induction	0.2 mg/l	1 mg/ml	Sigma
Thidiazuron (TDZ)	For shoot induction	0.2 mg/l	1 mg/ml	Sigma
Kinetin	For elongation	1-2 mg/l	1 mg/ml	Sigma
Indole-3 butyric acid (IBA)	For root induction	1-2 mg/l	1 mg/ml	Sigma

Table 3.3.Details of the antibiotics/herbicide used for tissue culture

Antibiotic/ hormone/ herbicide	Purpose	Working concentration (mg/ml)	Stock (mg/ml)	Supplier
Ampicillin	For selection of ampicillin resistant *E. coli* strains	100	100	USB
Kanamycin	For selection of binary vector	50	50	Duchefa
Streptomycin	Marker for helper plasmid of *Agrobacterium*	25	25	Synbiotics
Rifampicin	Marker for chromosome of *Agrobacterium*	25	25	Duchefa
Hygromycin	Transgenic plant selection	5-20	20	Duchefa
Cefotaxime	Elimination of *Agrobacterium*	250	250	Alkem

3.1.6.Chemicals and consumables

The chemicals used in the present study were of molecular biology grade and procured from Amersham Biosciences, Bangalore Genei, Duchefa, Genetix, Himedia Chemicals, InVitrogen, Life Technologies, Fermentas, Promega Life Science, Sigma, SD Fine Chemicals, USB, Qiagen and Qualigens fine chemicals. Consumables like glassware, plastic ware, scalpel blades, forceps, scalpels, filter papers, pipette tips, eppendorf tubes, etc., were obtained from Axygen, Borosil and Tarsons. All standard solutions, buffers and reagents were prepared according to the procedures given by Sambrook and Russel (2001).

3.1.7.Vectors and Strains

Salient features of the commercial vectors used in the present study are given in Table 3.4. The physical maps and the features of pTZ57R T/A, pRT100, pCAMBIA1300 and pCAMBIA1381z vectors are shown in Figures 3.1, 3.2 and 3.3, respectively. Standard bacterial strain DH5α of *Escherichia coli* was used for maintenance of all the recombinant plasmids. The developed recombinant vectors were mobilized into *Agrobacterium tumefaciens* strain LBA4404, which was further used for plant transformation.

Table 3.4.Salient features of the commercial vectors used

Plasmid	Size (bp)	Multiple cloning site (MCS)	Bacterial selection marker	Blue/ white selection (*lac*Z)	Purpose of use
pTZ57R T/A	2886	*Eco*RI,*Sac*I,*Kpn*I, *Bsp*681, *Mph*I, *Xba*I, *Bam*HI, *Sma*I, *Sma*I, *Apa*I, *Sal*I, *Eco*I, *Pae*AI, *Hin*dIII	Ampicillin	Present	Initial cloning of housekeeping genes, polyA, ACS4, 5, 7 promoters, and *BIK1, ERF1* and *AtEBP* coding sequence.
pRT100	3340	*Xho*I, A*pa*I, *Nco*I, *Sst*I, *Kpn*I, *Sma*I, *Bam*HI, *Xba*I	Ampicillin	Absent	Isolation of the polyA terminator fragment.
pCAMBIA 1300	8958	*Eco*RI, *Sac*I, *Kpn*I, *Sma*I,*Bam*HI, *Xba*I, *Sal*I, *Pst*I, *Sph*I, *Hin*dIII	Kanamycin	Present	For cloning the gene cassettes developed for tolerance against *Botrytis sps.*
pCAMBIA1381z	11226	*Xmn*I, *Bst*XI, *Eco*RI, *Nco*I, *Sma*I, *Bam*HI, *Sal*I,*Pst*I,*Hind*III,*Bgl* II, *Spe*I, *Nhe*I, *Pml*I, *Bst*EII	Kanamycin	Absent	For validation of the localized expression of promoters ACS4, 5 and 7 using GUS as the reporter.

3.2.Methods

The main objectives of the present investigation included isolation of gene fragments, development and confirmation of several constructs. The common procedures adopted during this process are discussed below.

3.2.1.Common Molecular Biology procedures followed:

3.2.1.1.Primer designing

In the present investigation several primer sets were designed and used for developing the gene constructs. Some primers available already at Directorate of Oilseeds Research, Hyderabad were also used.

The primers were designed with appropriate restriction enzyme sites (RES) included at the 5` while designing the primers (for the isolation of genes, promoters and terminator). Care was taken to add only those RES which were not present in the promoter(s)/gene(s) sequence(s) but present in the multiple cloning site (MCS) of the binary vector employed for cloning. All the primers used in the present study along with their details are given in Table 3.5. The T_m values of primers were calculated using the formula $T_m = 2\ (A+T) + 4\ (G+C)$ (Sambrook and Russell, 2001).

A set of primers with extension of the subsequent exon were used in the overlap primer extension PCR for isolation of *BIK1* and *AtEBP* genes. Also a different set of primers were designed and synthesized for use in real time PCR. These primers are listed in table 3.5.1.

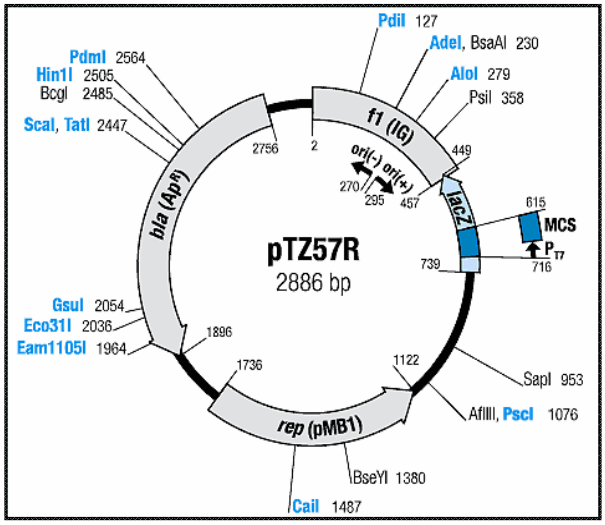


Fig: 3.1.Schematic diagram of T/A cloning vector (MBI Fermentas)

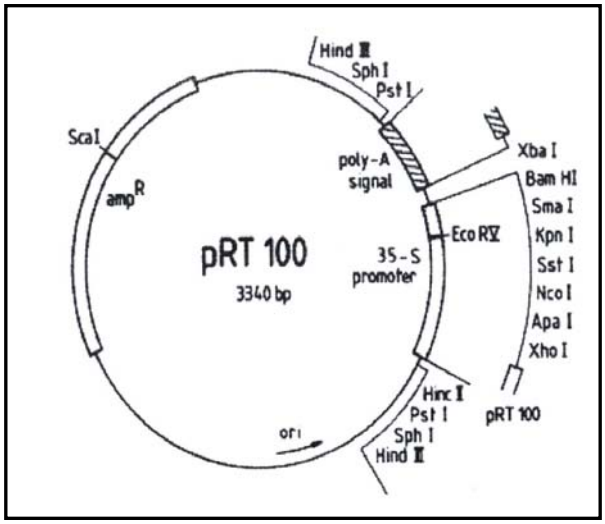


Fig: 3.2.Schematic diagram of pRT100 vector

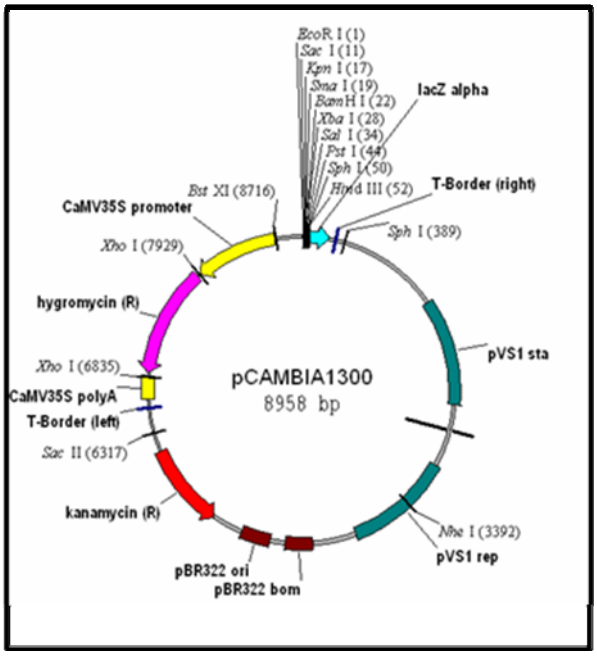


Fig: 3.3.Schematic diagram of pCAMBIA1300 vector

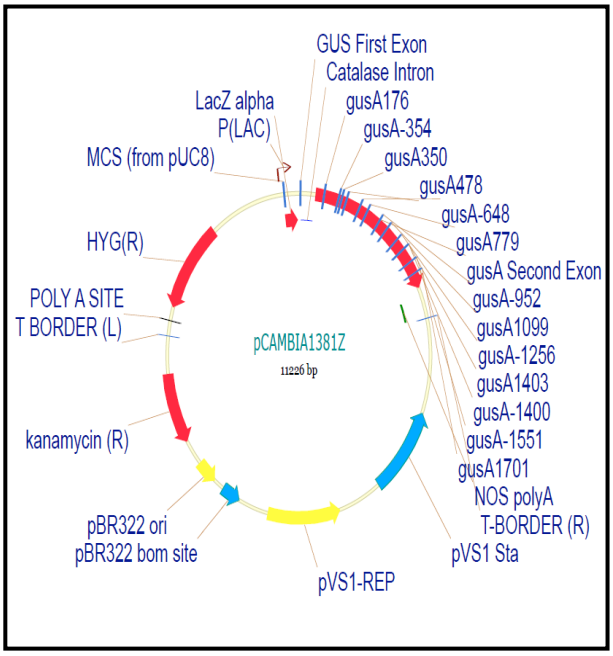


Fig: 3.4.Schematic diagram of pCAMBIA1381Z vector

Table 3.5.Sequence of the primers used, their melting temperature, purpose for which they were used and the PCR product size realized

Primer	Sequence	Tm (^{0}C)	Purpose of primers	Size of PCR product (bp)
G3PDH FP	ATT GAC ATC GTC GCT GTC AAC GA	58	Isolation of *G3PDH* gene from *B.ricini*	990
G3PDH RP	ACC CCA CTC GTT GTC GTA CCA	58		
RPB2 FP	GAT GAT CGT GAT CAT TTC GG	58	Isolation of *RPB2* gene from *B.ricini*	1100
RPB2 RP	CCC ATA GCT TGC TTA CCC AT	58		
HSP60 FP	CAA CAA TTG AGA TTT GCC CAC AAG	58	Isolation of *HSP60* gene from *B.ricini*	1000
HSP60 RP	GAT GGA TCC AGT GGT ACC GAG CAT	58		
Sl_Poly A FP	TGT CGA CGT CCG CAA ATC ACC AGT C	58	Isolation of PolyA terminator fragment from pRT 100 plasmid using three different RP with restriction enzymes specific to cloning into pCAMBIA 1300	250
P_Poly A RP	TCT GCA GGT CAC TGG ATT TTG GTT TTA G	58		
P_E_Poly A RP	TCT GCA GAA TTC GTC ACT GGA TTT TGG TTT TAG	58		
P_S_Poly A RPI	TCT GCA GAG CTC GTC ACT GGA TTT TGG TTT TAG	58		
B_AMV_*ERF*1 FP	TAG ATC T_AMV_GA TCC ATT TTT AAT TCA GTC	58	Isolation of *ERF1* gene from *Arabidopsis thaliana*	250
Sl_*ERF1* RP	TGT CGA CTC ACC AAG TCC CAC TAT TTT C	58		
B_AMV_*BIK1*_Exon1_FP	TAG ATC T_AMV_GGT TCT TGC TTC AGT TC	58	Isolation of *BIK*_Exon1 fragment from *Arabidopsis thaliana*	105
*BIK1*_Exon1_RP	ATA AAG ATC GCT GCT CTT ACC ATT GTG GAA GAT GTC TGC TTT GAC TCG AGA ACT GAA GCA AGA ACC	58		
*BIK1*_Exon2_FP	GTA AGA GCA GCG ATC TTT ATG G	58	Isolation of *BIK*_Exon2	308

*BIK1*_Exon2_RP	CCA AGT AAT TGA TCT CTG TCA GCC ATT CAC GGT GAC CTT G	58	fragment from *Arabidopsis thaliana*	
*BIK1*_Exon3_FP	GGT CAC CGT GAA TGG CTG ACA GAG ATC AAT TAC TTG GG	58	Isolation of *BIK*_Exon3 fragment from *Arabidopsis thaliana*	136
*BIK1*_Exon3_RP	CTT AAA ATA TGC ACC TCT TCT GAA TAG ATG ATT CTC AAG	58		
*BIK1*_Exon4_FP	ATC ATC TAT TCA GAA GAG GTG CAT ATT TTA AGC C	58	Isolation of *BIK*_Exon4 fragment from *Arabidopsis thaliana*	143
*BIK1*_Exon4_RP	GTT TTG CGT TGT AGT CCG CAT CAA GTA AGA TGT TC	58		
*BIK1*_Exon5_FP	CAT CTT ACT TGA TGC GGA CTA CAA CGC AAA ACT TTC	58	Isolation of *BIK*_Exon5 fragment from *Arabidopsis thaliana*	124
*BIK1*_Exon5_RP	GCT TCT TGC ATT CAA GTG ACC TGA TGA CAT GTA CTC AGG	58		
*BIK1*_Exon6_FP	GCC TGA GTA CAT GTC ATC AGG TCA CTT GAA TGC AAG AAG C	58	Isolation of *BIK*_Exon6 fragment from *Arabidopsis thaliana*	428
Sl_*BIK1*_Exon6_RP	TGT CGA CCT ACA CAA GGT GCC TGC C	58		
B_*AtEBP*_Exon1_FP	TGG ATAC CAT GTG TGG CGG TGC TAT TA	60	Isolation of *AtEBP*_Exon1 fragment from *Arabidopsis thaliana*	160
*AtEBP*_Exon1_RP	GCC TCC TCT TTC ACG TTA ACT TGG TTG GTG GGA TG	60		
*AtEBP*_Exon2_FP	CTC CAT CCC ACC AAC CAA GTT AAC GTG AAA GAG GAG	60	Isolation of *AtEBP*_Exon2 fragment from *Arabidopsis thaliana*	587
Sl_*AtEBP*_Exon2_RP	TGT CGA CTT ACT CAT ACG ACG CAA TGA	60		
At ACS4 FP	GTT ACT TTT CAA ATC TTC CCT C	58	Isolation of AtACS4 promoter fragment from *Arabidopsis thaliana*	1200
At ACS4 RP	TTT CTT TTG TTC TTG TTT TTT TT	58		
At ACS5 FP	AGG GAG CAT AAA TGG TCC	58	Isolation of AtACS5 promoter fragment from *Arabidopsis thaliana*	1200
At ACS5 RP	TTC TCT GTT TTT AAA GTC AAG AG	58		

At ACS7 FP	AGT GTA AAT GGA TAG CCA CCC	58	Isolation of AtACS7 promoter fragment from *Arabidopsis thaliana*	1200
At ACS7 RP	TTT TCT TAG AGC TTC GAA CCT G	58		
M13 FP	GTA AAA CGA CGG CCA GT	56	For PCR confirmation of the cloned fragments in InsT/A cloning vector	Variable
M13 RP	GGA AAC AGC TAT GAC CAT G	58		
Actin FP	CTT GAC GGA AAG AGG TTA TT	58	Used as an endogenous control in tobacco plants	450
Actin RP	GAT CCT CCA ATC CAG ACA CT	58		
*hpt*II Forward Primer	CAA AAT CCC ACT ATC CTT CGC	62	For confirmation of the hygromycin phosphotransferase II gene conferring hygromycin resistance in transgenic plants	1094
*hpt*II Reverse Primer	GCA GTT CGG TTT CAG GCA GGT	66		
*hpt*II Forward Primer	CTA TTT CTT TGC CCT CGG ACG	58	For confirmation of the *hygromycin phosphotransferase II* gene conferring hygromycin resistance in transgenic plants	350
*hpt*II Reverse Primer	ATG AAA AAG CCT GAA CTC ACC G	58		
*uid*A Forward Primer	GGT GGG AAA GCG CGT TAC AAG	62	For confirmation of *uidA* gene in transgenic plants	1200
*uid*A Reverse Primer	GGT TAC GCG TTG CTT CCG CCA	68		

Note:

- T_m was calculated excluding restriction enzyme sites
- AMV refers to the 5` UTR sequence taken from alfalfa mosaic virus and added at the 5` end of forward primers of the three defense regulatory genes and the sequence included is **5` TGG ATC CGT TTT TAT TTT TAA TTT TCT TTC AAA TAC TTC CAT CAA AAA AAA AA ATG GCT 3`**

3.6.List of primers used for the real time experiments:

Primer	Sequence	Tm (^{0}C)	Purpose of primers	Size of PCR product (bp)
Nt_*CHN50* FP	GTCTCCCTGGATTTGGTGTCA	54	To check the upregulated expression in the tobacco transgenics	50
Nt_*CHN50* RP	CTGTAAAACCCAATGCGATCCT	53		
Nt_*ERF1* FP	TAGAATGCGCGGTTCAAAGG	52		50
Nt_*ERF1* RP	TCAGGCGATGCTCGTCTTTT	52	-do-	
Nt_*GLA* FP	AGAACCCTGAACTGGAGAAACATT	54	-do-	50
Nt_*GLA* RP	GAACTGTCCCAAACTCCACCA	54		
Nt_*Osmotin* FP	CGACTAACCCTAGTGGAGGGAA	57	-do-	50
Nt_*Osmotin* RP	TTATTACATCCTCCGGGAACCC	55		
Nt_*Prb1b* FP	TGGTTGTGCTAGGGTTCGATG	54	-do-	50
Nt_*Prb1b* RP	TCAAGATCACCGTAGGGACGTT	55		
Nt_*L25* FP	CCCCTCACCACAGAGTCTGC	58	Tobacco endogenous internal controls for Real Time PCR	50
Nt_*L25* RP	AAGGGTGTTGTTGTCCTCAATCTT	54		

Nt_*EF-1 alpha* FP	TGAGATGCACCACGAAGCTC	54	-do-	50
Nt_*EF-1 alpha* RP	CCAACATTGTCACCAGGAAG TG	55		
Nt_*Ntubc2* FP	CTGGACAGCAGACTGACATC	54	-do-	50
Nt_*Ntubc2* RP	CAGGATAATTTGCTGTAACA GATTA	51		
*ACTIN*_RT FP	CAT TGC AGA TCG TAT GAG CAA	54	-do-	190
*ACTIN*_RT RP	GTG GAC AAT GGA AGG ACC A	54		
At_*ERF1*_RT FP	CCG ATC AAA TCC GTA AGC TC	52	Expression study of the transgene *ERF1* in *ERF1* tobacco transgenics using real time PCR	190
At_*ERF1*_RT RP	CTC TCA TCG AGA AAG CAG CT	54		

3.2.1.2 Overlap Primer Extension PCR

The genes *BIK1* and *AtEBP* were isolated from the gDNA of *Arabidopsis thaliana* using overlap primer extension PCR (Bryksin *et al*., 2010). In this method, primers were designed for each exon individually, with an overlap of at least ten bases of the next exon in the 5` of the forward primer and the reverse primer so as to facilitate the extension of the exons. The details of the designed primers are given in the table (Table..). The exons were amplified individually using the overlapping primers and were gel eluted and checked for the quantity and quality on 1% agarose gel. These exons were denatured at 99^0C for 4min and

immediately annealed at 4^0C and were then amplified to achieve the complete gene sequence.

In the case of *BIK1* gene isolation which contained five introns and six exons, the exons one, two and three were denatured, annealed and amplified using ExonI FP and ExonIII RP as set1. The *BIK1* set 1 fragment was cloned in InsT/A PCR cloning vector and was confirmed using M13 forward and reverse primers through sequencing. The amplified exons four, five and six were denatured, annealed and amplified using ExonIV FP and ExonVI RP as set2. The *BIK1* set 2 fragment was cloned in InsT/A PCR cloning vector and was confirmed using M13 forward and reverse primers through sequencing. Both the sets 1 and 2 were then denatured, annealed and amplified using ExonI FP and ExonVI RP to obtain the complete *BIK1* gene of 1188bp. The full length *BIK1* gene was cloned in InsT/A PCR cloning vector and was confirmed using M13 forward and reverse primers through sequencing. The confirmed *BIK1* gene was further used in cloning into the binary vector pCAMBIA 1300-ACS4-polyA-P to obtain the complete *BIK1* gene cassette.

The *AtEBP* gene contains one intron and two exons. The exonI and II were amplified individually using overlapping primers specific to each exon. The amplicons of each exon were denatured at 99^0C for 4min and annealed at 4^0C on ice. The annealed fragment was used to amplify the full length *AtEBP* gene of 747 bp using Exon I FP and Exon II RP. The full length *AtEBP* gene was cloned in InsT/A PCR cloning vector and was confirmed using M13 forward and reverse primers through sequencing. The confirmed *AtEBP* gene was further used in cloning into the binary vector pCAMBIA 1300-ACS7-polyA-PE to achieve the complete *AtEBP* gene cassette.

3.2.1.3 Plasmid DNA isolation

Plasmid DNA of required plasmid/vectors was isolated from 5 ml of overnight grown culture. The bacterial cells were spun down at 12000 g for 2 min. The bacterial pellet was

resuspended in 250 µl of STET (1N NaOH, 1M Tris, 0.5M EDTA, 0.5% Triton X-100) buffer. The bacterial cells were spun down at 12000 g for 2 min. The bacterial pellets were maintained in ice and resuspended in 100 µl of GET buffer (1M Glucose, 1M Tris, 0.5M ETDA) and vortexed to get an even suspesion. This was followed by addition of 200µl of (1% SDS, 0.2N NaOH of final concentration) and mixed gently. 150 µl of 3M potassium acetate (pH 5.2) was added, mixed by tapping and left on ice for 2 min and spun at 12000 g for 8 min. The supernatant was transferred to fresh tubes and the DNA was precipitated with two volumes of absolute ethanol and pelleted by centrifugation. The pellet was washed with 70% ethanol, air dried and dissolved in 50 µl $T_{10}E_1$ buffer. For sequencing purpose, the plasmid DNA from selected clones was isolated using QIAprep® Spin Miniprep Kit (Qiagen) following manufacturer's protocol.

3.2.1.4 PCR amplification

During the development of constructs, whenever the inserts were isolated by PCR, a preparative PCR (10 or 20 µl reaction volume) was carried out with primers specific to each of the components with the respective DNA. Once the expected size fragment was obtained a large volume PCR (60 or 80 µl reaction volume) was carried out with the same PCR conditions. The PCR reaction mixture consisted of 6-10 ng of DNA, 0.2 µM each of the forward and reverse primers, 100 µM of each dNTP, 1x concentration of *Taq* DNA polymerase buffer (10 mM Tris-HCl (pH 9.0), 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% gelatin), 1 unit of *Taq* DNA polymerase and milliQ water to a total volume of 60 µl. The product thus generated was used for cloning. The clones generated were confirmed by PCR and/or restriction digestion. Confirmation of the transgenic plants was done using PCR. Appropriate primers for confirming the transgene constructs were utilized in the PCR reaction. The general PCR program utilized for this study is given below. Only the annealing temperature (based on T_m of the primers) and/or the extension time varied (according to the length of the amplicons).

The cycling parameters adopted for PCR were:

Initial denaturation - 94^0C for 4 min

Denaturation - 92^0C for 30 sec

Annealing - 56-60^{0}C for 30 sec

Extension - 72^{0}C for 30 sec- 2.5min

Final extension - 72^{0}C for 7-10 min

3.2.1.5 Restriction analysis

Restriction digestion was performed to release inserts (from vectors) and to open vectors (linearize them) for cloning and also for confirmation of the developed clones (whether the expected product size is obtained on digestion). As a general rule, 1 µg DNA was used. The reaction mixture consisted of 5-10 units of enzyme, 1x final concentration of appropriate buffer, BSA to a final concentration of 100 µg/ml and sterile water. For cloning purpose, a large volume restriction digestion reaction was set up (60 or 80 µl) to obtain enough digested vector and insert concentrations for efficient ligation.

3.2.1.6 Quantification of DNA

Quantification of genomic DNA, plasmid DNA, *etc* was done by them on 0.8% agarose gels. Approximate DNA concentration was determined by visual comparison against a known amount of standard molecular weight marker (λ DNA/50 bp ladder/100 bp ladder/ λ DNA *Eco*RI + *Hin*dIII double digest).

3.2.1.7 Cloning

The general procedure followed for cloning is outlined below.

3.2.1.7-a. Preparation of vector and insert

The vectors as well as insert (to be cloned), were first isolated by setting up large volume PCR or restriction with appropriate enzymes (as mentioned earlier). The products were resolved on 0.8% agarose gel and the desired fragments were identified on the gel using standard marker (50 bp ladder or 100 bp ladder or λ DNA *Hin*dIII + *Eco*RI double digest). The agarose gel slabs containing the required fragments (cut vector as well as insert) were excised out from the gel using a sharp sterile scalpel blade. DNA was eluted from the excised gel slabs using Qiagen gel elution kit as per manufacturer's instructions. For each gel slab, three volumes of QG buffer was added to 1 volume of gel and incubated at 50˚C for 10 min (by vortexing intermittently). After bringing it to room temperature, one volume of

isopropanol was added (optional) and the whole mix was transferred to the supplied column. After centrifugation, the flow through was discarded and the column was washed twice with PE buffer. The DNA from the column was collected by adding 25-30 µl of elution buffer. The vector and insert DNA thus prepared were quantified by visual examination on 0.8% agarose gel.

3.2.1.7-b. Ligation

For all ligation reactions, the ratio of molar concentrations of insert to plasmid DNA used was 3:1. The amount of insert DNA needed for a known quantity of the vector DNA was calculated according to the following formula:

$$\text{Insert DNA (ng)} = \frac{(50/100 \text{ ng of vector DNA}) \times (\text{size of the insert in bp})}{\text{Size of the vector in bp}} \times 3$$

The ligation reactions consisted of 50 or 100 ng of vector DNA, insert DNA (as calculated from the formula mentioned above, 1x final concentration of the buffer, 40 units of T_4 DNA ligase and sterile water and the reaction mixture was incubated at 22^{o}C for 5 hrs.

3.2.1.7-c. Preparation of *E. coli* competent cells

The competent cells were prepared following the protocol as given by Ausubel *et al*. (1999). *E. coli* strain DH5α was grown at 37^{o}C in liquid LB medium (Himedia) overnight. An aliquot of the culture was transferred to 200 ml LB medium (Himedia) and grown to an OD_{590} of 0.375. The culture was distributed into four 50 ml pre-chilled sterile polypropylene tubes and left on ice for 5-10 minutes. The cells were pelleted by centrifugation at 1600 g for 7 min (4^{o}C). Each pellet was resuspended in 10 ml ice-cold $CaCl_2$ solution (15 % glycerol, 0.2 M $CaCl_2$, 0.1 M PIPES) and spun for 5 minutes at 1100 g (4^{o}C). The pellet was resuspended in 10 ml ice-cold $CaCl_2$ solution and left on ice for 30 minutes and spun at 1100g for 5 minutes (4^{o}C). The resultant pellet was completely resuspended in 2 ml of ice-cold $CaCl_2$ solution and 100 µl was aliquoted into pre-chilled sterile 1.75 ml eppendorf tubes and immediately frozen at -80^{o}C.

3.2.1.7-d. Transformation of *E. coli* competent cells

Heat shock method (Ausubel *et al*. 1999) was followed for the transformation of competent cells with the plasmid DNA or ligation mix. Frozen *E. coli* competent cells were thawed on ice, to which 1 ng of plasmid DNA (for control reaction) or 100 ng of ligation mix was added. The suspension was carefully mixed with pipette tip and incubated on ice for 15 min followed by heat shock at 42^{o}C for 2 min. Cells were incubated on ice for another 2 min, 1 ml LB medium was added and incubated at 37^{o}C for 1 h with shaking. Aliquots of the suspension were spread evenly on LA medium supplemented with appropriate antibiotic (ampicillin or kanamycin). The plates were incubated at 37^{o}C overnight (14-16 h). The following day single colonies were picked up and inoculated in 3 ml LB medium for plasmid isolation.

3.2.1.7-e. Confirmation of clones

The cloning at each step was confirmed by colony PCR, PCR with the isolated DNA and restriction analysis. The plasmid DNA of the new clones were isolated and used for this purpose. When PCR was used for confirmation of cloning, the procedure was same as mentioned above with the use of specific primers. During restriction analysis for confirmation of cloning, suitable restriction enzymes were used to confirm the presence of the insert as well as its orientation in the vector background. The procedure followed was as mentioned previously. Colony PCR was also done for preliminary confirmation of clones even before their plasmid DNA was isolated. For this, instead of plasmid DNA, the bacterial cells as such were used in the PCR reaction mixture. Also, the PCR reaction mixture with the bacterial cells was first heated at 95^{o}C for 5 min. The cycling parameters were the same as that for the respective gene fragments but the annealing temperature and extension time varied according to the primers used and the size of the amplicons expected.

3.2.1.8 Mobilization of the recombinant binary vectors into *Agrobacterium tumefaciens*

All the constructs developed (as mentioned above) were mobilized into *Agrobacterium* strain LBA4404 and further confirmed before they were used for plant transformation. The general procedures followed are explained below.

3.2.1.8-a. Preparation of LBA4404 chemically competent cells

Saturated culture of *Agrobacterium* strain LBA4404 was grown overnight in LB. 2 ml of this overnight grown culture was added to 50 ml of YEP medium without any antibiotics and allowed to grow upto an OD of 0.5 at A_{600}. The cells were pelleted down at 3000 rpm for 5 min at 4˚C in a sterile falcon tube. The cells were sequentially washed in 10 ml of 0.15 M sodium chloride and re-pelleted. The resultant pellet was resuspended in 1 ml of 20 mM ice-cold $CaCl_2$. 100 µl of aliquots were frozen and stored at -80˚C.

3.2.1.8-b. *Agrobacterium* Transformation by Electroporation

The double and triple gene cassettes developed in the binary vector (pCAMBIA1300) were mobilized into *Agrobacterium* strain LBA4404 by electroporation. Frozen *Agrobacterium* electro-competent cells were thawed on ice to which 1-2 µl binary plasmid DNA (50-100ng) was added. The suspension was carefully mixed with pipette tip and transferred to a pre-chilled 2mm Cuvette (Eppendorf). Electroporation was carried out at 2500V for nearly 5 msec. Immediately after electroporation, 1ml of growth medium (LB), without antibiotic, was added and the bacterial suspension was incubated at 28^{o}C for 1hr. The cells were pelleted by a brief spin and plated onto culture plates with appropriate antibiotic selection. The putative transformed colonies were picked up and cultured for plasmid isolation and confirmed for the mobilization of the binary plasmid into the bacterium.

3.2.1.8-c. Freeze-thaw transformation of *Agrobacterium tumefaciens*

The three gene cassettes of *BIK1, ERF1* and *AtEBP* developed in the binary vector (pCAMBIA1300) under the promoters ACS4, 5 and 7 respectively were mobilised into *Agrobacterium* strain LBA4404 by freeze-thaw method (Chen et al. 1994). The frozen *Agrobacterium* competent cells were thawed on ice and 1 µg of the recombinant binary plasmid DNA was added to competent cell mixture and mixed well. The entire mix was frozen in liquid nitrogen for 30 sec to 1 min and the frozen mix was thawed at 37^{0}C for 5 min. Immediately 1 ml of YEP was added. This culture was kept for shaking at 28˚C for 3-4 hrs. The cells were pelleted by giving a brief spin, the supernatant was decanted and the pellet was resuspended in the left out medium of ~100 µl and spread on YEP agar plates supplemented with kanamycin, streptomycin and rifampicin. After 2-3 days, the colonies were inoculated in 5-10 ml of YEP by adding appropriate antibiotics and plasmid DNA was isolated.

3.2.1.8-d. *Agrobacterium* plasmid DNA isolation

Plasmid DNA of LBA4404 strains harbouring binary recombinant plasmids were isolated from a starter culture of 10 ml each grown on rifampicin, streptomycin and kanamycin antibiotics. Fully-grown culture was incubated on ice for 5-10 min and the cells were pelleted at 3000 rpm for 15 min. The pellet was resuspended in 500 µl of GET (50 mM glucose, 25 mM Tris-Cl pH 5.8, 10 mM EDTA) and 250 µl lysozyme (@ 4 mg/ml) and incubated on ice for 10-15 min. 1 ml of NSW (0.2 N NaOH, 1% SDS) was added and incubated at room temperature for 10 min. To the same tube, 750 µl of 3 M potassium acetate (pH 5.2) was added and incubated on ice for 15 min. Centrifugation was done at 15000 rpm for 15 min at 4˚C. To the supernatant, equal volume of phenol: chloroform: isoamylalcohol (25:24:1) was added and mixed well and spun at 15000 rpm for 10min. The aqueous phase containing plasmid DNA was precipitated with double volume of ethanol and kept at -20˚C for 10 min. The pellet was washed with 70% ethanol, air-dried and dissolved in 50 µl of T_{10}.

3.2.1.8-e. Confirmation of constructs in *Agrobacterium*

3.2.1.8-e.1. 3-Ketoglycoside test for confirmation of *Agrobacterium* strain

This test was performed to check whether the strain was *Agrobacterium* or any other contaminant. *Agrobacterium* species grown on lactose make an enzyme called hexa pyranoside cytochrome C oxido-reductase that converts lactose to 3-keto-lactose, which in turn converts cupric oxide of Benedict's solution to cuprous oxide, a yellow colored precipitate. *Agrobacterium* strains (with recombinant DNA) streaked on 3-ketolactose medium (1% lactose, 0.1% yeast extract, 2% bacto-agar) were flooded with Benedicts reagent (17.3% sodium citrate, 10% sodium carbonate anhydrous, 1.74% copper sulphate). Yellow hallow of Cu_2O appears wherever 3-ketolactose is produced by *Agrobacterium*. By using this test all the *Agrobacterium* clones were confirmed.

3.2.1.8-e.2 Confirmation by restriction endonuclease digestion or PCR

The constructs were finally confirmed at *Agrobacterium* level also since recombinations is known to take place during transformation of *Agrobacterium*. Whenever the plasmid DNA was of good quality (as ascertained by viewing on agarose gels) as well as good quantity (at least 200 ng/µl), restriction digestion was used for confirmation. The reaction mixture consisted of 0.8 to 1.0 µg of plasmid DNA, 10-20 units of restriction enzyme, 1x final concentration of appropriate buffer, BSA to a final concentration of 100 µg/ml and milliQ water to make up the final volume. When plasmid DNA concentration was very low (less

than 200 ng/µl), PCR (as per the procedure mentioned in section above) was used for confirmation of the clones.

3.2.1.8-e.3 Confirmation by reverse mating

For further confirmation of orientation of cloning of the required gene cassette in binary vector, reverse mating (transfer of the binary plasmid back into *E. coli*) was done. Chemically competent *E. coli* cells of DH5α were transformed with ~50 ng of *Agrobacterium* plasmid DNA using the standard heat shock method described earlier. The plasmid DNA from the *E. coli* was subjected to appropriate restriction analysis or PCR.

3.2.2.0 Phylogenetic relationship between *B. ricini* and other *Botrytis* spp.

The phylogentic analysis of *B. ricini* was carried out as described by Staats el al., 2002. Three house- keeping gene fragment of *G3PDH*, *HSP60* and *RPB2* of B. ricini were compared with the gene sequences of other *Botrytis* spp available in GenBank.

3.2.2.1 Isolation/ amplification of gene fragments of *G3PDH, HSP60* and *RPB2*

The gene fragments of *G3PDH, HSP60* and *RPB2* were isolated from the mycelium of *B. ricini* through PCR using primers designed specific to the fragments as mentioned in Table 3.5. The amplified products were cloned in InsT/A vector and confirmed by PCR and restriction analysis. Five clones of each gene fragment were sequenced to obtain a proper consensus sequence.

3.2.2.2 Comparison of sequences using BLAST and Treebase

The consensus sequences of *B. ricini G3PDH, HSP60* and *RPB2* obtained through sequencing were compared for similarity with the gene fragments of other *Botrytis* spp using BLAST (Basic Local Alignment Search Tool). To deduce a phylogenetic relationship of *B. ricini* with other *Botrytis* species, Treebase analysis was carried out by Jan Van Kan at the Wageningen University, Laboratory of Phytopathology, Wageningen, Netherlands, who has a repository for all the gene sequences of the genus *Botrytis*.

3.2.3 Development of gene constructs

In the present investigation three genes *BIK1, ERF1, AtEBP* and the promoter *ACS4, ACS5* and *ACS7* of *Arabidopsis thaliana* were used. The terminator sequence polyA from pRT100

expression vector was used in each gene cassette. The promoters were validated for their expression in tobacco using GUS as the reporter gene.

3.2.3.1 Development of vectors to validate promoters ACS4, 5 and 7

The *Arabidopsis thaliana* promoters *ACS4, ACS5* and *ACS7* driving the elevated gene expression in the inflorescence were isolated from the gDNA by PCR and primers as mentioned in Table 3.5. The amplified promoter sequences were cloned in InsT/A vector, sequenced and further cloned in the binary vector pCAMBIA 1381z as *Eco*RI-*Hind*III fragment. These vectors were confirmed by PCR and restriction digestion as mentioned in sections 3.2.1.4 and 3.2.1.5 respectively. The confirmed clones were mobilized into *Agrobacterium* strain LBA4404 as mentioned in 3.2.1.8-c and used for tobacco transformations.

3.2.3.2 Isolation of *ERF1*

The *ERF1* is an intron-less gene and was isolated directly from the genomic DNA of *Arabidopsis thaliana* by PCR using specific primers as mentioned in (Table 3.5). The amplified gene product was cloned in InsT/A vector, sequenced and confirmed by PCR and restriction digestion. The *ERF1* gene was further cloned in the binary vector pCAMBIA 1300 as *BamH*I-*Sal*I fragment.

3.2.3.3 Isolation of *BIK1* and *AtEBP*

The *BIK1* gene contained six exons and the *AtEBP* gene with two exons. These genes were isolated from the genomic DNA of *Arabidopsis thaliana* by overlap extension PCR.

3.2.3.3.1 Isolation of exons

The primers specific to each exon of both *BIK1* (six exons) and *AtEBP* (two exons) were designed (Table 3.5) and was used for amplification by PCR as mentioned in 3.2.1.4. The six exons of *BIK1* gene were amplified, eluted cloned, sequenced, denatured and annealed in two sets of Exon I+II+III in set1 and Exon IV+V+VI in set2.

3.2.3.3.2 Amplification of subsets and obtaining full length gene

The subsets 1 and 2 of *BIK1* were cloned in InsT/A vector, sequenced and confirmed by PCR and restriction digestion. The confirmed sequences of set 1 and 2 were denatured, annealed

and amplified overlap extension PCR using the Exon I FP and Exon VI RP to obtain the full length *BIK1* gene coding region.

Similarly, the Exons I and II of *AtEBP* gene amplified products were cloned in InsT/A vector, sequenced and confirmed by PCR and restriction digestion. The confirmed sequences of Exons I and II were denatured, annealed and amplified by overlap extension PCR using the Exon I FP and Exon II RP to obtain the full length *AtEBP* gene coding region.

3.2.3.4 Isolation of polyA terminator

The polyA terminator sequence was isolated from pRT 100 vector by PCR using specific primers designed with flanking restriction enzyme sites i.e *Sal*I in the 5` end of forward primer and *Pst*I (P), *Pst*I/*Sac*I(PS) and *Pst*I/ *EcoR*I (PE) in the reverse primers (refer Table 3.5). These enzyme sites are incorpoprated to facilitate development of double and triple gene cassettes vectors. The amplified polyA fragment was cloned into InsT/A vector, sequenced and confirmed by PCR and restriction digestion.

3.2.3.5 Development of single gene cassettes

Single gene cassettes were developed by cloning the chosen promoter, gene and the terminator sequences. These component sequences were cloned in pCAMBIA 1300 binary vector and the confirmed clones were mobilized into *Agrobacterium* for plant transformation studies.

3.2.3.5.1 Development of AtACS4-*BIK1*-polyA

The *BIK1* single gene cassette was developed in pCAMBIA 1300 binary vector. First the polyA terminator was cloned as *Sal*I /*Pst*I fragment, followed by the promoter ACS4 as *Kpn*I/ *BamH*I fragment and the *BIK1* gene was cloned downstream to the promoter and upstream to the terminator as *BamH*I/*Sal*I fragment from their respective InsT/A clones to complete the assembling of the *BIK1* gene cassette. This vector is mentioned as pCAMBIA 1300-ACS4-*BIK1*-P and designated as “B”. The gene cassette was confirmed for the presence and orientation of all the components of the cassette by serial PCR, restriction digestion and sequencing. The confirmed clones were mobilized into *Agrobacterium* for plant transformation studies.

3.2.3.5.2 Development of AtACS5-*ERF1*-polyA

The *ERF1* single gene cassette was developed in pCAMBIA 1300 binary vector. First, the polyA terminator was cloned as *Sal*I*Pst*I fragment, followed by the promoter ACS5 as *Kpn*I-*BamH*I fragment and the *ERF1* gene was cloned downstream to the promoter and upstream to the terminator as *BamH*I-*Sal*I fragment from their respective InsT/A clones to complete the assembling of the *ERF1* gene cassette. This vector is mentioned as pCAMBIA 1300-ACS5-*ERF1*-PS and designated as "E". The gene cassette was confirmed for the presence and orientation of all the components of the cassette by serial PCR, restriction digestion and sequencing. The confirmed clones were mobilized into *Agrobacterium* for plant transformation studies.

3.2.3.5.3 Development of AtACS7-*AtEBP*-polyA

The *AtEBP* single gene cassette was developed in pCAMBIA 1300 binary vector. First the polyA terminator was cloned as *Sal*I-*Pst*I fragment, followed by the promoter ACS7 as *Kpn*I-*BamH*I fragment and the *AtEBP* gene was cloned downstream to the promoter and upstream to the terminator as *BamH*I-*Sal*I fragment from their respective InsT/A clones to complete the assembling of the *AtEBP* gene cassette. This vector is mentioned as pCAMBIA 1300-ACS7-*AtEBP*-PE and designated as "A". The gene cassette was confirmed for the presence and orientation of all the components of the cassette by serial PCR, restriction digestion and sequencing. The confirmed clones were mobilized into *Agrobacterium* for plant transformation studies.

3.2.3.6 Development of double gene cassette

The double gene cassette was developed by cloning ACS5-*ERF1*-PS gene cassette upstream of 1300-ACS4-*BIK1*-P gene cassette as a *Sac*I fragment in a head to head fashion. The double gene cassette was confirmed for the presence and orientation of all the components of the cassette by serial PCR, restriction digestion and sequencing. The double cassette vector is designated as "EB" and is mentioned as pCAMBIA 1300-EB. The confirmed clones were mobilized into *Agrobacterium* for plant transformation studies.

3.2.3.7 Development of triple gene cassette

The triple gene cassette vector was developed by cloning the ACS7-*AtEBP*-PE gene cassette upstream to the ACS5-*ERF1*-PS gene cassette in the double cassette vector as an *Eco*RI fragment in a tail to tail fashion. The double gene cassette was confirmed for the presence

and orientation of all the components of the cassette by serial PCR, restriction digestion and sequencing. The triple cassette vector is designated as "AEB" and is mentioned as pCAMBIA 1300-AEB. The confirmed clones were mobilized into *Agrobacterium* for plant transformation studies.

3.2.4 Tissue culture and Transformation

3.2.4.1 Transformation of Tobacco

3.2.4.1.1 Media preparation

Readymade mix of Murashige and Skoog (1962) medium supplemented with different hormones and 0.8% agar as gelling agent was used. The required media at various stages of explant growth in tissue culture work are as listed in the table 3.6.

3.2.4.1.2 Plant growth conditions

The culture plates with the medium and explants were incubated in growth room at temperature of 27±1 ºC under 16/8 hr photoperiod provided by cool fluorescent lights at an intensity of 30 μmol m^{-2} s^{-1}.

Table 3.6 Media used at various stages in tissue culture work

Medium	**Ingredients with hormones**	**pH**
Basal medium	MS powder + 1x $CaCl_2.2H_2O$	5.8
Half MS	Half the strength of MS	5.8
Shoot induction medium	BN (MS powder + 1x $CaCl_2$ + BAP 1 mg/l + NAA 0.1 mg/l	5.8
Shoot multiplication medium	MS + 1x $CaCl_2$ + BAP 0.5 mg/l	5.8
Shoot elongation medium	MS powder + 1x $CaCl_2$ + BAP 0.2 mg/l	5.8
Rooting medium	Half MS	5.8

Antibiotics and hormone stocks were filter sterilized and stored at -20˚C. All media with required hormones were autoclaved for 20 min at 121˚C, 15 lbs pressure and stored at 25˚C. Prior to use, required antibiotics were added to melted and cooled media.

3.2.4.1.3 Surface sterilization of seed for obtaining aseptic seedlings

Seed treatment is a must for obtaining healthy seedlings for further successful downstream tissue culture work. Good bold and healthy tobacco (*Nicotiana tabacum* cv.tabacum) seeds were selected and washed under running water for 2-3 hr after adding 2-3 drops of TritonX-100. Wetted seeds were soaked in 70% ethanol for 1 min. Later, seeds were surface sterilized with 0.1% $HgCl_2$ (mercuric chloride) for 1min followed by 5 min washing with sterile distilled water thrice with continuous shaking. The seeds were re-suspended in 0.5 ml of sterile water and inoculated on half strength MS media bottles at 8-10 seeds per bottle.

3.2.4.1.4 Inoculation of LBA4404 strain of *Agrobacterium*

The *Agrobacterium* strains harbouring single, double and triple gene cassette binary plasmids were inoculated into YEP/TB medium, with respective antibiotics and incubated at 28ºC for 12 hrs.

3.2.4.1.5 Co-cultivation

Standard leaf-disc transformation method for tobacco was followed. Sterile young tobacco leaves were cut out from aseptic tobacco seedlings (which are being routinely maintained under half strength MS medium in the laboratory). A 1:10 dilution of *Agrobacterium* culture (0.6 OD units) with liquid MS medium was taken into sterile petridish under laminar hood. The leaves, cut into small pieces, were placed in this mixture for 5 minutes. They were dried for a while on sterile blotting paper and placed on shoot induction medium. The plates were incubated in dark for 48 hrs.

3.2.4.1.6 Selection and shoot regeneration

The explants were then transferred to shoot induction medium with hygromycin (30 mg/l) and cefotaxime (250 mg/l) (selection I) and incubated for 15 days. The explants, which showed callusing were then sub-cultured on to plates with shoot elongation medium with hygromycin (30 mg/l) and cefotaxime (250 mg/l) for 15 days (selection II). Shoots obtained

were excised and sub-cultured to plates with shoot proliferation and elongation media with with hygromycin (30 mg/l) and cefotaxime (250 mg/l) for 15 days (selection III). At each level of selection, the dried and darkened explants (untransformed) were discarded. Simultaneously, some control (untransformed) explants sub-cultured to the first selection medium were also taken onto second and third cycle of selection even though the explants failed to produce callus in the first selection medium. Remaining control explants were maintained and sub-cultured on media similar in all aspects except that they did not have hygromycin and cefotaxime. The shoots obtained were maintained as controls for experiments aimed at molecular confirmation of the putative transgenics.

3.2.4.1.7 Rooting and maintenance

Well-established and healthy shoots were transferred and maintained in tubes with rooting medium and respective antibiotics. The time course for tobacco transformation and regeneration is shown in table 3.7.

3.2.4.2 Confirmation of the obtained transgenic tobacco plants

3.2.4.2.1 Isolation of tobacco genomic DNA

DNA was isolated from putative transgenic as well as control tobacco plants by following the modified CTAB procedure based on the protocol of Doyle and Doyle (1987). Fresh tobacco leaves (70-100 mg from tissue culture maintained plantlets) were harvested in to 1.5 ml microcentrifuge tubes, frozen in liquid nitrogen and ground to fine powder using pre-chilled micropestle. 400 µl of pre-warmed (60^{o}C) isolation buffer [2% (w/v) CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl (pH 8.0), 0.2% β-mercaptoethanol (added just before use)] was added and incubated at 60^{o}C for 30-60 minutes. The slurry was extracted twice with equal volume of chloroform-iso-amyl alcohol (24:1). The final aqueous phase was transferred to a fresh tube and DNA was precipitated with 0.6 volumes of ice-cold iso-propanol. The DNA pellet obtained after centrifugation was washed with 70% ethanol, air-dried and dissolved in 50 µl $T_{10}E_1$ buffer [10 mM Tris-HCl, 1 mM EDTA (pH 8.0)].

3.2.4.2.2 Quantification of DNA

The DNA samples were electrophoresed on a 0.8% agarose gel. The approximate DNA concentration was determined by visual comparison against a known amount of standard molecular weight marker (uncut λ DNA or λ DNA *Eco*RI + *Hin*dIII digest).

3.2.4.2.3 PCR analysis

Putative transgenics obtained were confirmed using PCR amplification of specific component sequences present in the constructs. The primers used and their sequence information is presented in the table 4. The PCR reaction mixture consisted of 20 ng of DNA, 0.2 µM each of the forward and reverse primers specific for the gene fragment, 1x final concentration of *Taq* DNA polymerase buffer [10 mM Tris-HCl (pH 9), 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% gelatin], 100 µM of each dNTP, 1 unit of *Taq* DNA polymerase, and sterile water (to make up the volume).

All PCR reactions were carried out with the following cycling parameters.

Step	Temperature (^oC)	Time	Cycles
Initial denaturation	94	4 min	1
Denaturation	92	30 sec	35
Annealing	*	45 sec	
Extension	72	*	
Final extension	72	7 min	1
Hold	4		1

* The annealing temperature and extension time were determined based on the primers used and the expected product size.

3.2.4.2.4 GUS assay

GUS histochemical assay was performed to confirm the putative transformants obtained using the promoter validation constructs. The protocol used for the GUS histochemical assay was as mentioned previously (Jefferson, 1987). X-GlcA (5-bromo-4-chloro-3-indolyl-_-D-glucuronic acid) is a substrate for β-D-glucuronidase (GUS) encoded by the *gusA* gene. The substrate is used as a qualitative histochemical marker of specific GUS expression in cells and tissues. X-GlcA is cleaved by GUS at the β1 glucuronic bond between glucuronic acid and the 5-bromo-4-chloro-3-indolyl part of X-GlcA via hydrolysis. This enzymatic cleavage of X-GlcA results in the precipitation of a water insoluble blue dichloro-dibromo-indigo precipitate. This reaction was used to confirm the presence of the transgene. X-GlcA solution was prepared by dissolving 5 mg of X-GlcA cyclohexylammonium salt in 50 µl dimethyl formamide (DMF) and 10 ml of autoclaved 0.05 M phosphate buffer solution at pH 7.0

(Sambrook and Russel, 2001). To this, 30 µl of Triton X 100 was added. Different tissues obtained from transgenic tobacco (for promoter study) like leaf, buds and flowers were immersed in GUS staining solution and incubated overnight in dark at 37 ºC. Before analysis, chlorophyll was bleached by extraction in ethanol. Untransformed explants (control) which were cultured under identical conditions served as control.

Table 3.7 Time course for transformation (tobacco) protocol

Steps	Description	Materials	Time (days)
1.	Inoculation of *Agrobacterium* culture	TB or YEP + rifampicin + kanamycin + streptomycin	Overnight
2.	Co-cultivation	O/N grown culture (A600nm=0.1) leaf explants on shoot induction medium in petri plates with no antibiotics	2
3.	Selection I for shoot induction	BN + hygromycin + cefotaxime	15
4.	Selection II for shoot proliferation	BA 0.5 mg/l + hygromycin + cefotaxime in petri plates	15
5.	Selection III for shoot elongation	BA0.2 mg/l + hygromycin + cefotaxime in petri plates	15
6.	Selection IV for rooting	½ MS + hygromycin + cefotaxime in bottles	15

3.2.4.2.5 Dot Blot analysis

The transgenic tobacco plants of the single gene constructs were tested for the stable integration of the transgene by dot blot analysis using the gDNA from the individual transgenics and untransformed control tobacco plants following the protocol by Hicks *et al*., 1995 for gDNA processing and spotting on Hybond-N+ positively charged nylon membrane from GE Healthcare. The hybridization of the gDNA with their specific probes was done using the direct labeling of DNA probes with alkaline phosphatase and chemiluminescent signal generation detection with CDP-*Star*^{TM} from Amersham. The gDNA sample of 1 µg was denatured using 2 M NaCl and 100 mM NaOH and boiling at 99^{0}C for 3 min. The denatured samples were then spotted on Hybond-N+ positively charged nylon membrane and

crosslinked in UV for 2 minutes. The membrane is rinsed in 2X SSC (3 M NaCl and 0.3 M Sodium citrate pH: 7.0) for 2 minutes and transferred to the hybridization buffer provided (supplemented with 0.5 M NaCl and 4% Blocking reagent) containing alkaline phosphatase labelled probe (600 ng) and incubated overnight for 10-12 hours at 55^0C. Post hybridization, the blots are washed stringently to remove excess probe using primary wash buffer (2M Urea, 0.1% SDS, 50 mM Sodium phosphate, 150 mM NaCl, 1mM $MgCl_2$, 0.2% Blocking reagent) twice for 30 minutes each at 55 ^{0}C and secondary wash buffer of 1: 20 dilution (1M Tris Base and 2 M NaCl) supplemented with 2 mM $MgCl_2$ twice for 10minutes each at room temperature. The signal generated by the hybridized probes was detected using CDP-*Star*™ chemiluminescent detection reagent that utilizes the probe bound alkaline phosphatase to calalyze the decomposition of a stabilized dioxetane substrate. The signal was then checked by exposing the dot blot to an X-ray film for one hour and developed subsequently to view the dots corresponding to the transgene in each spot on the dot blot membrane.

3.2.4.2.6 Pollen study:

3.2.4.2.6-a. Alexander Staining

It is a simplified method for differential staining of aborted and non-aborted pollen grains. The free anthers were collected before anthesis from stage 12 flower buds of the single gene transgenics and the untransformed control tobacco plants and fixed for two hours in Carony`s fixative (6 alcohol:3 Chloroform: 1 Acetic acid). 2-4 drops of Alexander stain (10 ml of 95% Alcohol, 1 ml Malachite green, 50 ml distilled water, 25 ml Glycerol, 5ml Acid Fuschin, 0.5 ml Orange G, 4 ml of Glacial Acetic Acid made up to 100 ml using distilled water) was applied to the anther before it got dried completely, on a glass slide. The slide on flame until near to boiling and incubate for 10-15 minutes at room temperature. Imaging of the heat fixed anther is done by placing a cover slip and applying even pressure on the anther. The cover slip was sealed on all sides using wax or nail polish and the pollen

grains were observed under the microscope to check for aborted and non-aborted nature. The untransformed pollen grains acted as control.

3.2.4.2.6-b. Pollen germination studies using Gibberellic acid

Pollen germination was studied in transgenic flowers with recessed anthers, no seed set and with flower drop. Free anthers were collected before anthesis from stage 12 flower buds. The pollen grains from these anthers were germinated in 50 µl of pollen germination medium (10% sucrose, 100 mg/L Boric acid and 125 µM Gibberellic acid) on a cavity slide and incubated in a humid chamber at room temperature for four hours. The pollen grains were checked for the formation of the pollen tubes under the microscope after incubation. The percentage of pollen germinated in each transgenic was calculated by the average of the pollen germinated at 10 different exposures under the microscope. The anthers germinated in distilled water acted as the negative control. The untransformed pollen grains acted as the positive control.

3.2.4.2.7 *Botrytis* infection assays

3.2.4.2.7-a. *In situ* infection studies on transgenic tobacco using *Botrytis cinerea*

Botrytis cinerea culture maintained on Potato Dextrose Agar (PDA) and marigold (*Calendula officinalis*) petals were used for the infection study both on excised leaf and inflorescence. The excised leaves of both the single gene transgenics and the untransformed control were subjected to *B. cinerea* infection. The mycelium of 6 day old grown *B. cinerea* spores on PDA was cut into small cubes and placed on the leaf surface of both transgenics and untransformed control and placed in a humid chamber, in dark at 22-24^{0}C for 10 days.

The infection by *B. cinerea* was measured based on the necrosis of the leaf tissue formed around the mycelium agar plug.

The excised inflorescence of both the single gene transgenics and the untransformed tobacco were placed in conical flasks containing water. A spore suspension of $1X10^6$ was sprayed on the inflorescence and maintained in a growth chamber with a photoperiod of 18 hrs of dark and 6hrs of light, a relative humidity of 80% humidity and 19^0C temperature for 1 week. The infection by *B.cinerea* was measured based on the necrosis of the flowers. The tobacco plants of both transgenic and untransformed control subjected to infection with water spray acted as the control in each case.

3.2.4.2.7-b. *In situ* infection studies on transgenic tobacco using *Alternaria alternata.*

The excised leaf explants of both the transgenics and untransformed control tobacco plants were subjected to infection using another necrotrophic fungi *Alternaria alternata* which causes death of infected tissue leading to loss of the whole plant. The mycelium of 6 day old grown *A.alternata* spores on maintained on PDA was cut into small cubes and placed on the leaf surface of both transgenics and untransformed control and placed in a humid chamber, in dark at 22-24^0C for 10 days. The infection by *A.alternata* was measured based on the necrosis of the leaf tissue around the inoculated mycelium agar plug.

3.2.4.3 Transformation of Castor

3.2.4.3.1 Meristem based transformation of castor

3.2.4.3.1-a. Media preparation

Readymade mix of Murashige and Skoog medium supplemented with different hormones and 0.8% agar as gelling agent was used. The required media at and the time course of various stages of explant growth in castor tissue culture work are as listed in the table 3.8

Table 3.8. Media and time course of various stages in castor transformation and plant regeneration.

Medium	**Ingredients with hormones and antibiotics**	**Time course (days)**
Embryo axes growth medium	MS powder + 1x $CaCl_2.2H_2O$ + BAP 0.1 mg/l	7
Co-cultivation medium	Full strength MS (MS powder + 1x $CaCl_2.2H_2O$)	5
Post co-cultivation medium	MS + 1x $CaCl_2$ + BAP 0.1 mg/l + cefotaxime 250 mg/l	10
Expansion and proliferation medium	MS + 1x $CaCl_2$ + TDZ 0.5 mg/l + cefotaxime 250 mg/l	10
Selection I medium	MS + 1x $CaCl_2$ + BAP 0.5mg/l + cefotaxime 250 mg/l + hygromycin 20 mg/l	15
Selection II medium	MS + 1x $CaCl_2$ + BAP 0.5mg/l + cefotaxime 250 mg/l + hygromycin 40 mg/l	15
Selection III medium	MS + 1x $CaCl_2$ + BAP 0.5mg/l + cefotaxime 250 mg/l + hygromycin 60 mg/l	15
Acclimatization (without selection) medium	MS + 1x $CaCl_2$ + BAP 0.5 mg/l + cefotaxime 250 mg/l	15
Shoot proliferation medium	MS + 1x $CaCl_2$ + BAP 0.5 mg/l	15
Shoot elongation medium	MS + 1x $CaCl_2$ + BAP 0.2 mg/l	15
Rooting medium	Half MS + NAA 2 mg/l	15

The pH of various media used in castor transformation and plant regeneration was maintained at 5.8. Antibiotics and hormone stocks were filter sterilized and stored at -20˚C. All media with required hormones were autoclaved for 20 min at 121˚C, 15 lbs pressure and stored at 25˚C. Prior to use, required antibiotics were added to melted and cooled media.

3.2.4.3.1-b. Explant preparation

Mature seeds were decoated and rinsed in running tap water for 30 min. The decoated seeds were surface sterilized with 0.1% (w/v) aqueous mercuric chloride solution for 12 min and subsequently rinsed 5 times with sterile distilled water. The seeds were blotted dry on sterile filter paper. The endosperm was carefully dissected to expose the embryos. Leaving the papery cotyledons on the endosperm, the embryo axes were excised and implanted on the culture medium, BA 0.1 mg/l.

3.2.4.3.1-c. Inoculation of LBA4404 strains of *Agrobacterium*

Agrobacterium strain LBA4404 harbouring double and triple gene constructs (pCAMBIA1300-EB and pCAMBIA1300-AEB) was inoculated into YEM with respective antibiotics and incubated at 28ºC for 12 hrs.

3.2.4.3.1-d. Transformation and plant regeneration

Embryo axes isolated from mature seeds were pre-cultured on medium supplemented with 0.1 mg/l BA for 7 days prior to infection by *Agrobacterium*. The seedlings were injured with two strokes of a sharp pointed blade (no.11) in the meristematic region, which is distinguishable by its characteristic swelling. The radicular regions and cotyledonary extensions were removed from the germinated embryo axes. The processed explants were immersed in bacterial suspension and subjected to vacuum infiltration for 30 min. Subsequently, the explants were blotted dry on sterile filter paper and co-cultivated for 5 days on full strength MS and incubated in dark. To enhance the penetration of the *Agrobacterium* containing the construct into the target tissues, explants were subjected to shaking (180 rpm) for 10 min during the bacterial incubation. Following co-cultivation, the explants were washed with 250 mg/l cefotaxime for 10 min, rinsed with sterile distilled water 3 times for 5 min each with constant stirring, and blotted dry on sterile filter paper. The procedure followed for castor transformation and plant regeneration is given below, schematically.

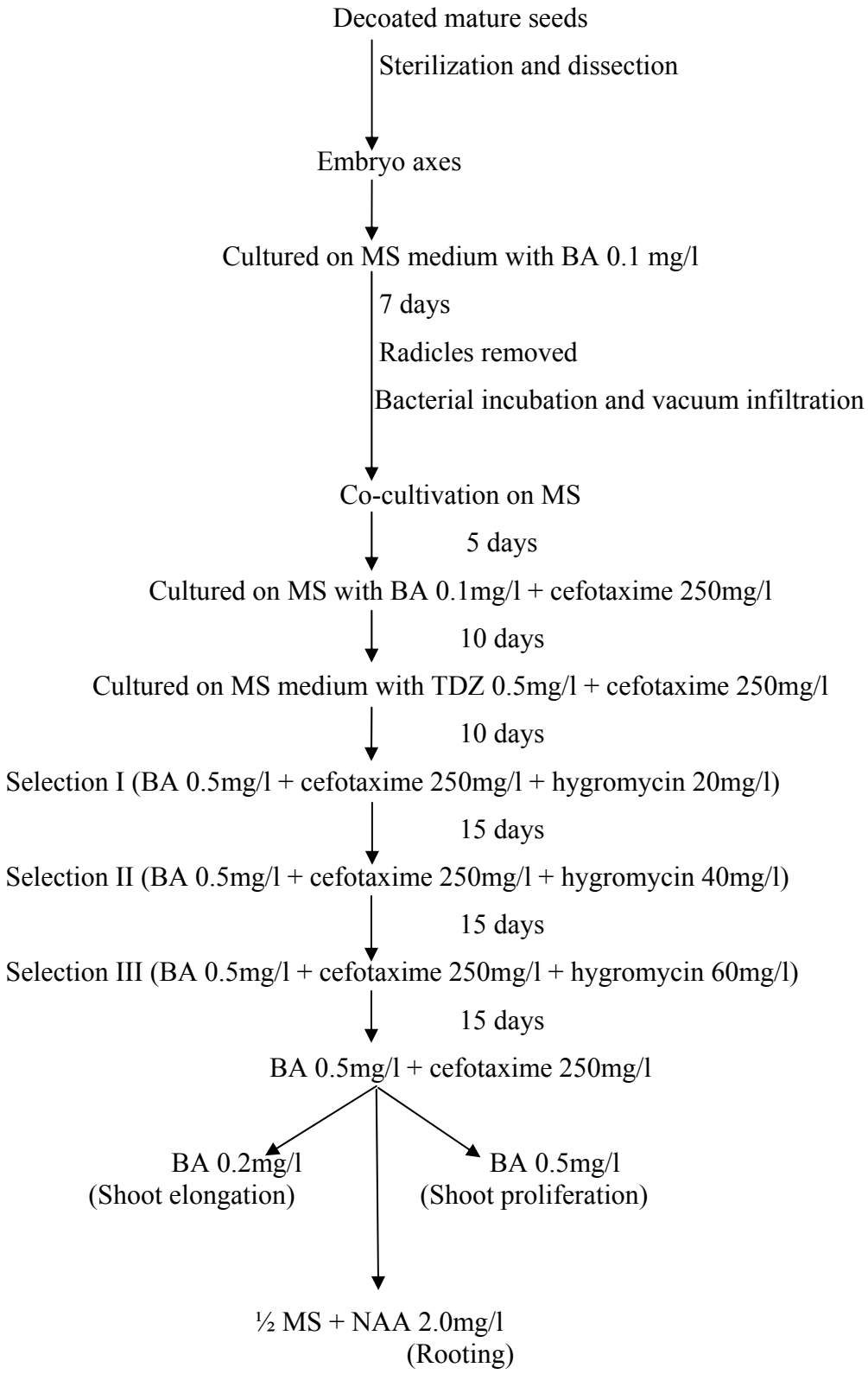


Fig. 3.7: Protocol for castor transformation and plant regeneration

3.2.4.3.2 *In planta* transformation:

Germinated seedlings of 6 day old castor of both genotypes DCS-9 (Jyoti) and 48-1 (Jwala) were used for the *in planta* transformation experiments. The seedlings were injured using a sterile needle at their shoot apical meristem and incubated along with the *Agrobacterium* strain EHA105 harbouring the triple gene cassette vector pCAMBIA 1300-AEB#1 for two hours with intermittent shaking at 22-24^{0}C. The seedlings were transferred on sterile tissue papers to drain the excess *Agrobacterium* culture and transplanted in vermiculite supplied with AB salts that served the required nutrition for the growth of the seedlings and incubated in dark at 24^{0}C for 3 days to elevate the *Agrobacterium* infection into the plant meristematic tissue. The seedlings were then transplanted to soil in the greenhouse for further growth until seed set. The seeds were then tested for the presence of the triple cassette T-DNA by germinating the seeds on MS medium containing hygromycin (60 mg/l). The germinated seedlings on the selection medium were allowed to grow further and then confirmed for the presence of the T-DNA using PCR specific to the selection marker and the triple gene cassette.

The present study was carried out at the Directorate of Oilseeds Research, Hyderabad with the aim of developing transgenic castor plants tolerant to necrotrophic fungus *Botrytis ricini*. The work was based on the hypothesis that stacking of multiple defense regulatory genes in castor and targeting the expression of these genes in the inflorescence (the main tissue affected during *Botrytis* infection) would increase the tolerance of the transgenic plant to a higher level when compared to trangenics obtained with the expression of single defense regulatory gene. Hence, a multi gene construct harboring three individual gene cassettes within a single T-DNA could provide cumulative tolerance against Botrytis infection. As the regeneration and transformation protocols available for castor are not repeatable and also very low in frequency, the vectors developed under this study were validated using tobacco as model system. The results obtained would be a prelude in obtaining castor transgenic plants with tolerance to *Botrytis ricini*. Based on this premise, the main objectives of this research study included

1. Phylogenetic analysis of *Botrytis ricini* to understand its relatedness with other *Botrytis* species.
2. Characterization of *Arabidopsis thaliana* promoters *ACS4*, 5 and 7 in tobacco using *gus* as the reporter gene.
3. Development of gene constructs using three inflorescence specific promoters *ACS4*, 5, 7 and three genes *ERF1, AtEBP* and *BIK1* from *Arabidopsis thaliana.*
4. To develop a multi gene cassette vector stacking the above three cassettes within a single T-DNA in the binary vector.
5. Transformation of model plant tobacco and target plant castor embryos using the developed constructs.
6. Validation of transgene expression and resistance of the obtained transgenic plants against *Botrytis.*

The results obtained during the present investigation are presented here under each of the following set of objectives:

4.1. Molecular phylogenetic analysis of *Botrytis ricini* – The Grey Mold of Castor

4.1.1. Isolation of house-keeping genes *G3PDH, HSP60* and *RPB2*

Three housekeeping genes *Glyceraldehyde-3-Phoshate dehydrogenase* (*G3PDH*) of 990bp, *Heat Shock Protein 60* (*HSP60*) of 1100bp and *Ribosome Protein Binding protein Subunit2* (*RPB2)*1000bp sequences were earlier reported to demonstrate the phylogenetic relationship of various *Botrytis* species. The three housekeeping gene sequences were acquired from NCBI-Nucleotide Database and gene specific primers were designed and used to isolate these gene fragments by PCR amplification using gDNA isolated from mycelium of *B. ricini* (Fig.4.0). The amplified gene fragments (Fig4.1) were gel eluted (Fig4.2) and cloned in T/A cloning vector (pTZ57R/T). Presence of gene fragments was confirmed by PCR using gene specific forward (FP) and (RP) (Fig4.3). The same was also confirmed by restriction digestion using *EcoRI* and *BamHI,* the restriction enzymes used for cloning the gene fragments was *Eco*RI and *Hind*III (Fig.4.4). The orientation of the inserted gene fragments in the cloning vector was confirmed by PCR using combination of M13 FP that binds on the vector backbone and gene specific RP and vice versa. The identified five positive clones of each gene were sequenced using the M13 forward and reverse primers flanking the multiple cloning site (MCS) of the InsT/A vector. The phylogenetic relationship of *Botrytis ricini* with all other existing *Botrytis* and *Sclerotineacae* members was done by TREEBASE analysis (Fig.4.6- 4.7). The sequences of *HSP60*, *RPB2* and *G3PDH* from *B. ricini* were submitted to NCBI GENBANK library and they were assigned Accession Nos.GQ860996.1, GQ860997.1 and GQ860998.1 respectively.

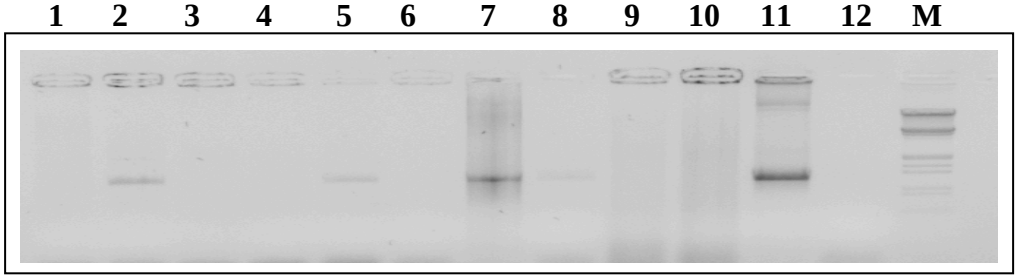


Fig.4.0. Quantification of *B. ricini* genomic DNA.

Lane no.1-12 : gDNA isolated from *Botrytis ricini* mycelium samples 1-12

Lane M : Lambda *HindIII-EcoRI* double digest.

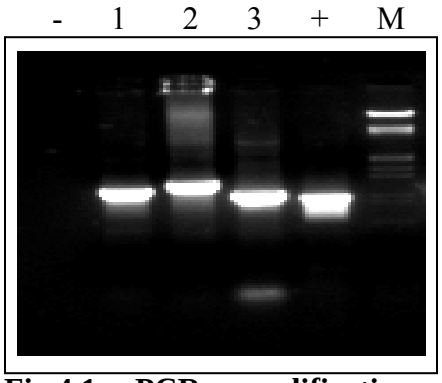


Fig.4.1. PCR amplification of *RPB2, HSP60* and *G3PDH* using *B.ricini* gDNA

Lane - : PCR negative

Lane 1: PCR of *RPB2*

Lane 2: PCR of *HSP60*

Lane 3: PCR of *G3PDH*

Lane +: PCR positive

Lane M: Lambda *HindIII-EcoRI* double digest.

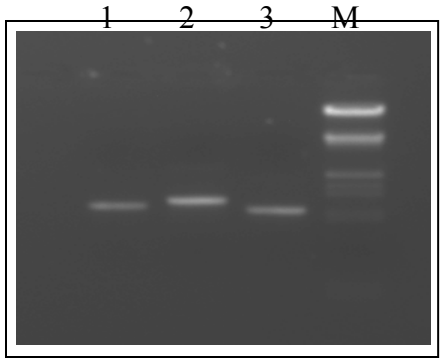


Fig.4.2. Quantification of the *RPB2, HSP60* and *G3PDH* elutes.

Lane 1: Elute of *RPB2*

Lane 2: Elute of *HSP60*

Lane 3: Elute of *G3PDH*

Lane M: Lambda *HindIII-EcoRI* double digest.

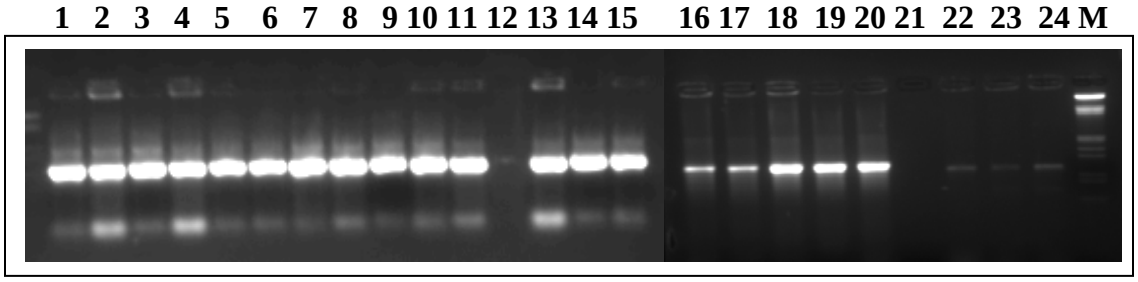


Fig.4.3.PCR of *G3PDH, RPB2* and *HSP60* – InsT/A plasmid DNA using gene specific forward and reverse primers.

Lane 1-15: PCR of *G3PDH* -InsT/A plasmid DNA from colonies #1-15 using *HSP60* FP/RP

Lane 16-20: PCR of *RPB2*-InsT/A plasmid DNA from colonies #1-5 using *RPB2* FP/RP

Lane 22-24: PCR of *HSP60*-InsT/A plasmid DNA from colonies #1-3 using *G3PDH* FP/RP

Lane M: Lambda *HindIII-EcoRI* double digest.

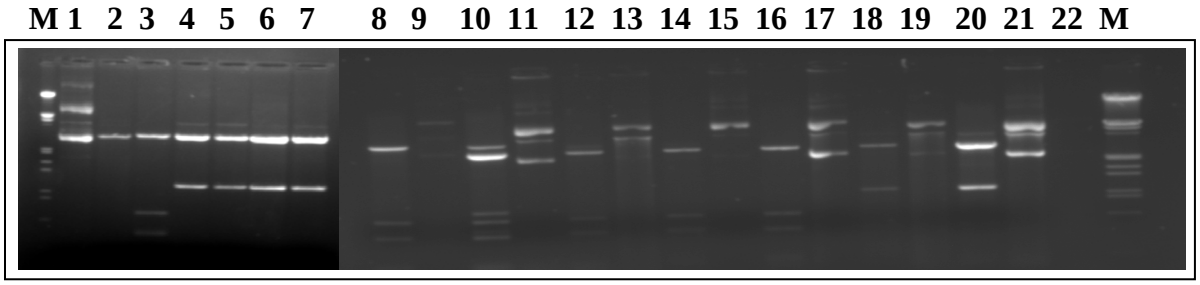


Fig.4.4. Restriction analysis of InsT/A plasmid DNA of *G3PDH, RPB2* and *HSP60* using *EcoRI- HindIII* double digestion.

Lane M: Lambda *HindIII-EcoRI* double digest.

Lane 1: Uncut InsT/A plasmid DNA of *G3PDH*

Lane 2-7: InsT/A plasmid DNA of *G3PDH* digested with *EcoRI-HindIII*

Lane 8,10,12,14,16: InsT/A plasmid DNA of *RPB2* digested with *EcoRI-HindIII*

Lane 9,11,13,15,17: Uncut InsT/A plasmid DNA of *RPB2*

Lane 18 and 20: InsT/A plasmid DNA of *HSP60* digested with *EcoRI-HindIII*

Lane 19 and 21: Uncut InsT/A plasmid DNA of *HSP60*

Phylogenetic analysis was carried out by using BLAST search tool with all sequences obtained for fragments of the three house-keeping genes isolated from *B. ricini.* The *in silico* analysis of these gene sequences of *B. ricini* indicated a 95% similarity with the existing *Botrytis* and *Sclerotinia* species. Hence, the 5% variation of *B. ricini* could be related to its unique nature of infecting the castor inflorescence causing grey mold. The query sequences and the summary of BLAST results obtained are provided in Appendix I**.** The dendrograms/phylogenetic trees obtained with three of the genes are shown in (Fig.4.5, 4.6 & 4.7).. These clearly indicated that *B. ricini* formed a distinct or separate clade and was between *Botrytis* and *Sclerotinium.*

Fig4.5. Phylogenetic tree obtained by comparing the sequence of *HSP60* gene fragment from *B. ricini* with other *Botrytis* species as well as *Sclerotinia sclerotiorum*

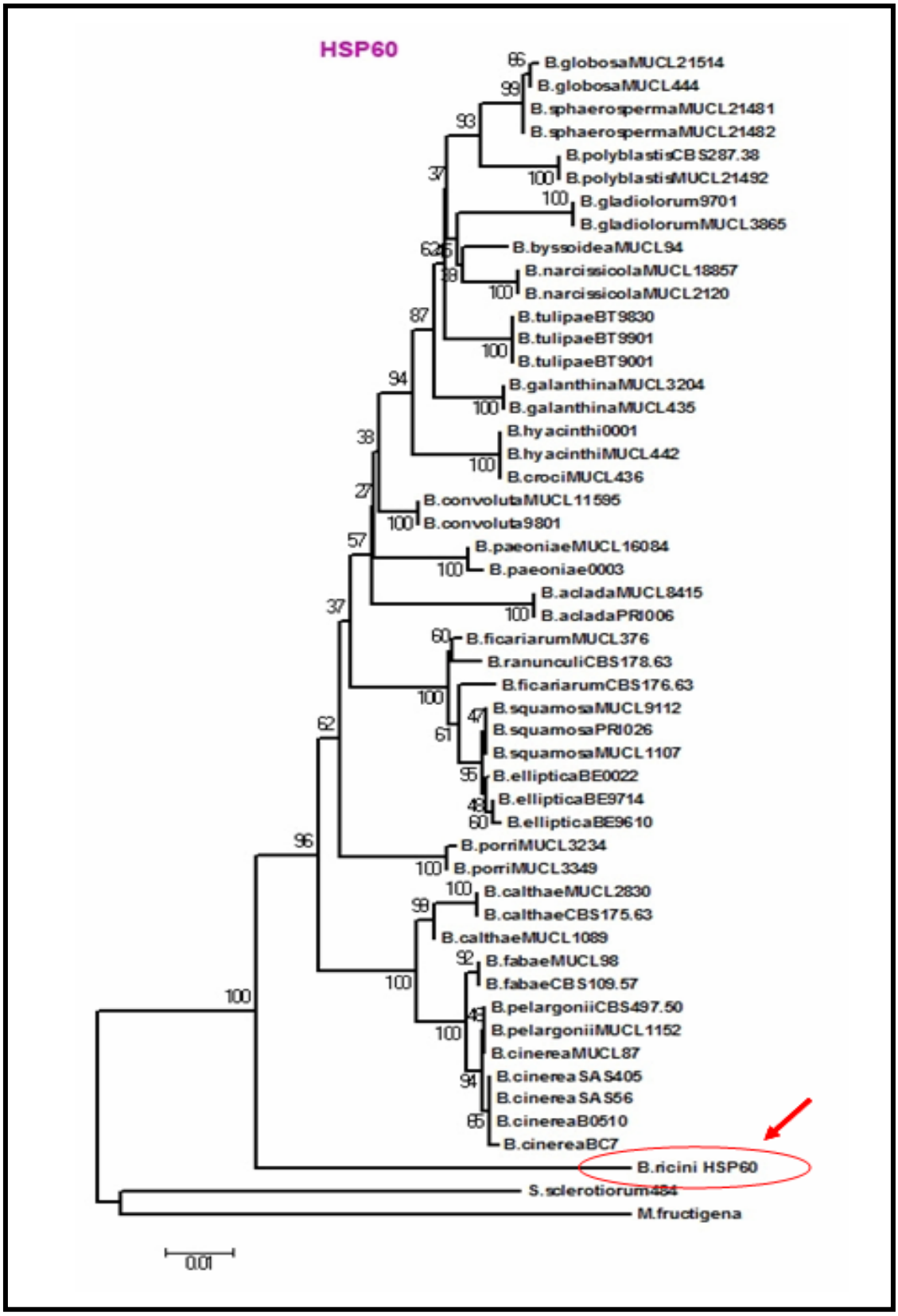


Fig4.6. Phylogenetic tree obtained by comparing the sequence of *G3PDH* gene fragment from *B. ricini* with other *Botrytis* species as well as *Sclerotinia sclerotiorum*

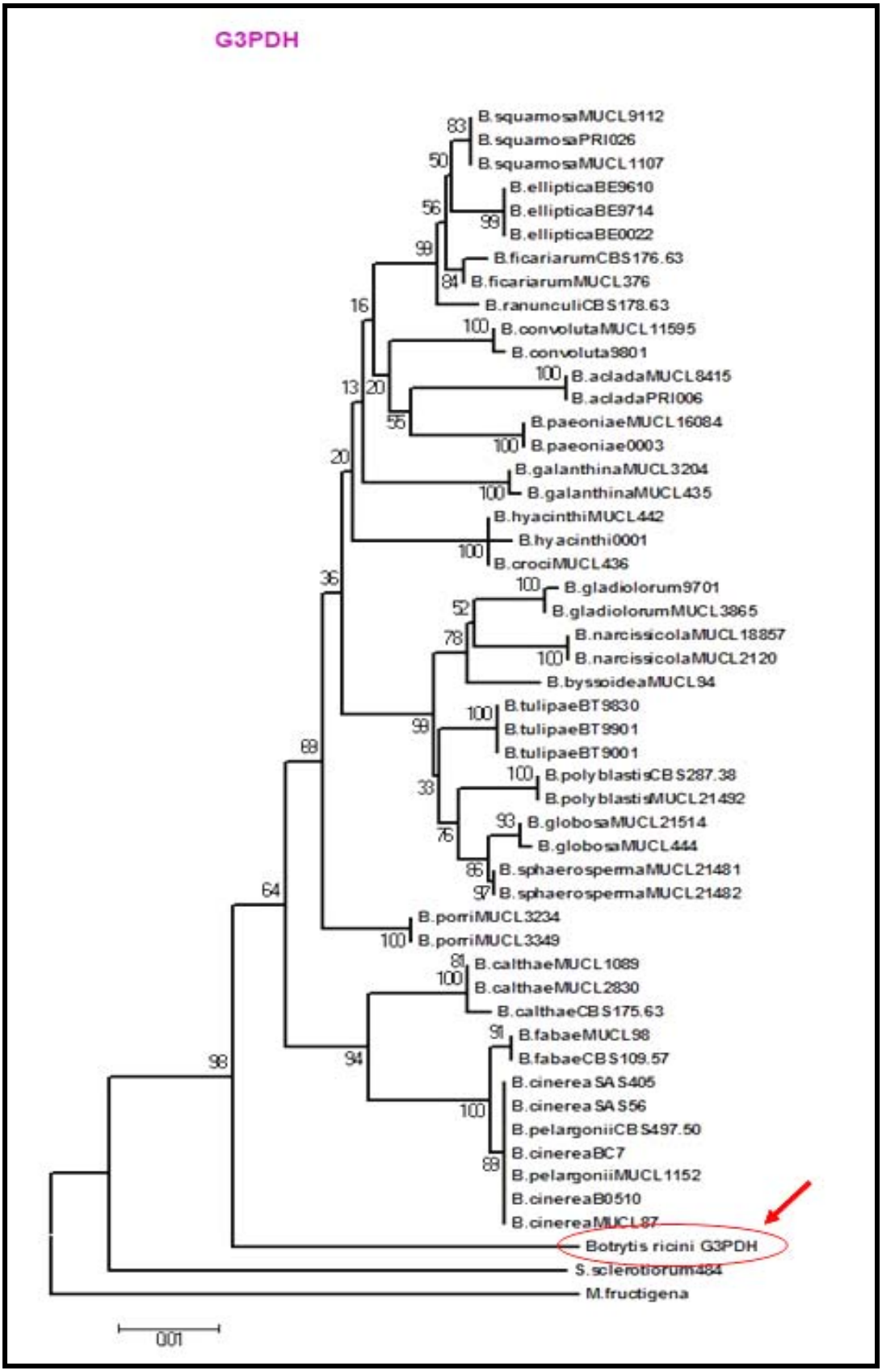
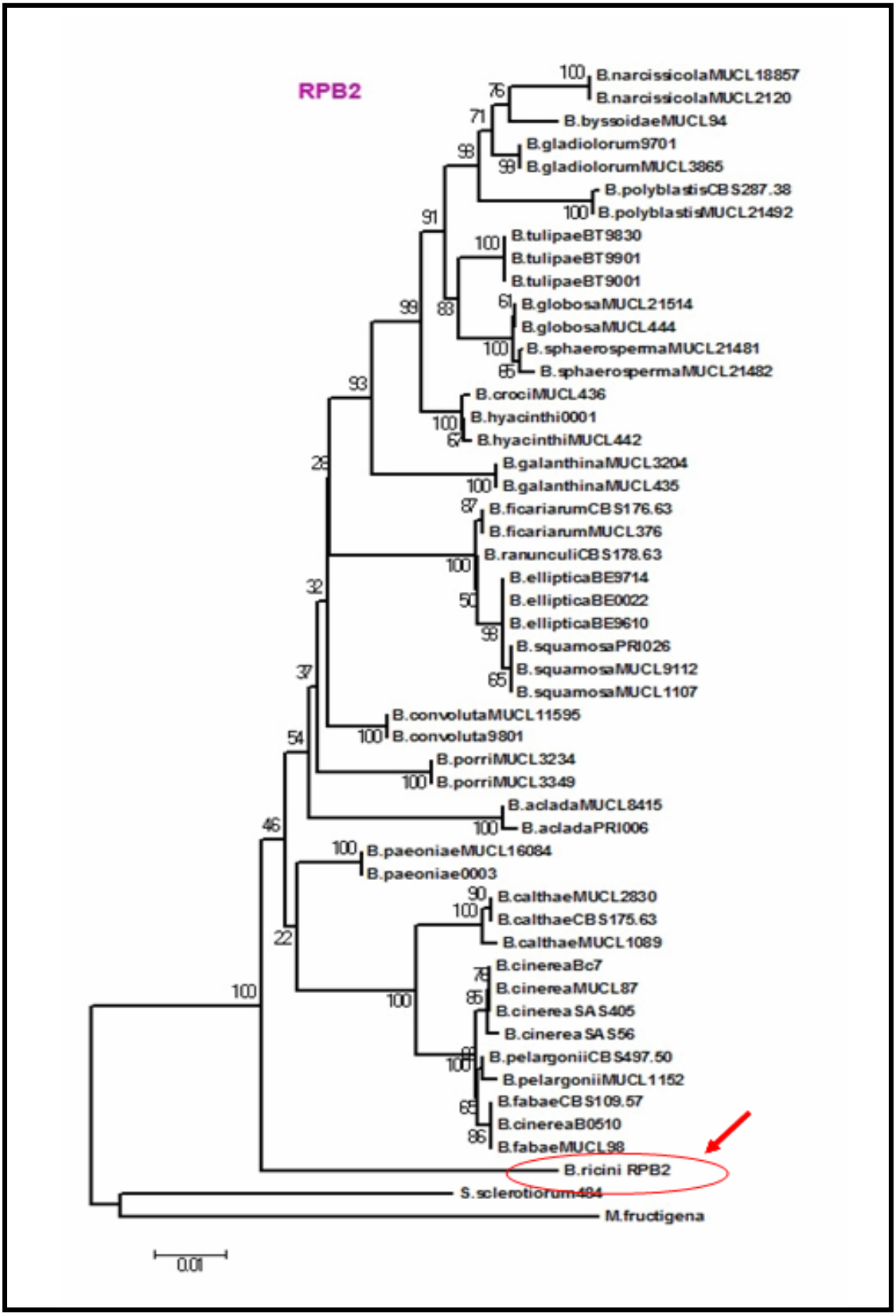


Fig4.7. Phylogenetic tree obtained by comparing the sequence of *RPB2* gene fragment from *B. ricini* with other *Botrytis* species as well as *Sclerotinia sclerotiorum*



4.2.0. Characterization of *Arabidopsis thaliana* promoters *ACS4,* 5 and 7 in tobacco using *gus (uidA)* as reporter gene

4.2.1. Isolation of *Arabidopsis thaliana* promoters *ACS4, 5* and *7*:

To achieve inflorescence elevated expression of the transgenes, promoters *ACS4* (1.2Kb), *ACS5* (1.22Kb) and *ACS7* (1.24Kb) were isolated from the gDNA of *Arabidopsis thaliana.* To isolate the promoters, PCR was performed using promoter specific primers designed (Appendix 1) based on the sequence information from NCBI GenBank nucleotide database.

The amplified products of *ACS4* (1.2 Kb), *ACS5* (1.22 Kb) and *ACS7* (1.24 Kb) were gel eluted and quantified for the estimation of its concentration by agarose gel electrophoresis. 50 ng of the eluted product was then ligated with the liberalized InsT/A vector and the ligated product was then transferred into *E.coli* DH5α cells. Ten colonies of each were subjected to colony PCR. The colonies which showed positive with the colony PCR (Fig.4.8.) were selected for further confirmation by restriction digestion, PCR and sequencing.

For the confirmation of the recombinant vector, plasmid DNA was subjected to restriction digestion using *EcoRI* and *Hind*III, at least three positive clones for each promoter was identified. The orientation of the promoters in the cloned vector was checked by PCR using promoter specific forward and vector specific reverse primer and *vice versa* (Fig.4.9). The presence and orientation of the promoters was also done by restriction digestion using *Kpn*I and *BamH*I enzymes for the release of 1.2kb fragments of the promoters (Fig.4.10 - 4.14.). Three recombinant clones for each promoter were sequenced using M13 forward and reverse primers and the sequence was analyzed using CHROMAS PRO software for the presence of insertions or deletions in the sequence of the three promoters. These promoter sequences were also checked for the presence of the *cis*-regulatory elements by *in silico* analysis of the sequences using PLACE (Plant *Cis*-acting Regulatory DNA Elements) database software, available online http://www.dna.affrc.go.jp/PLACE/html and PLANTCARE http://www.bioinformatics.psb.ugent.be/webtools/plantcare/html/ and PlantPAN- Plant Promoter Analysis Navigator http://www.plantpan.mbc.nctu.edu.tw/ for identifying combinatorial cis-regulatory elements with distance constraint in plant gene group. The list of these cis-regulatory elements with regard to *ACS4, ACS5* and *ACS7* promoter sequence are mentioned in Appendix I.

To investigate the presence of identity among the three promoter sequences with respect to the contigs in the sequence, a multiple sequence alignment of the three promoters was achieved using CLUSTALW software (http://www.ebi.ac.uk/Tools/msa/clustalw2/) (Apendix I). The promoter sequences were checked for the presence of any coding region in the sequence taken for the study by translating the DNA sequence to the probable protein sequence by ExPasy- TRANSLATE software (http://expasy.org/resources/translate/). All the six ORF`s were studied and the promoter sequences were then confirmed for the use as authentic promoter sequences for their expression in the heterologus system tobacco.

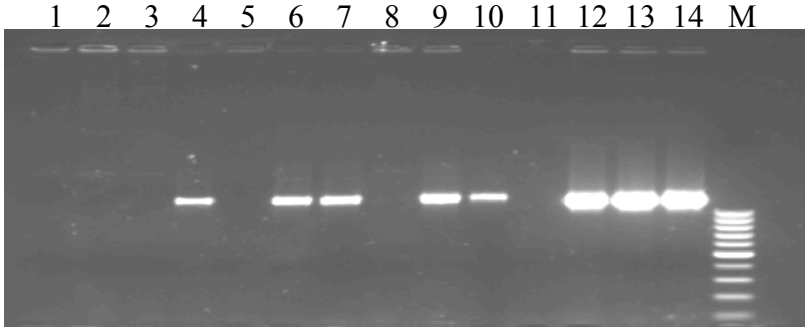


Fig.4.8.Colony PCR of ACS4, 5 and 7 using promoter specific primers
Lane 1-5: PCR of ACS5 colonies#1-5 using ACS5 FP/RP
Lane 6-10: PCR of ACS4 colonies#1-5 using ACS4 FP/RP
Lane 11-14: PCR of ACS7 colonies#1-4 using ACS7 FP/RP
Lane M: 100bp DNA ladder

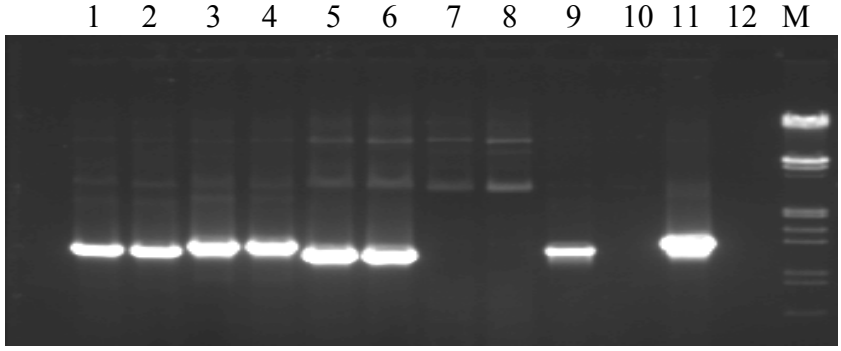


Fig.4.9. Orientation check of the promoter fragments by PCR
Lane 1-2: InsT/A-ACS 4#1 and 2 using ACS4 FP/RP
Lane 3-4: InsT/A-ACS 4#1 and 2 using ACS4 FP/ M13RP
Lane 5-6: InsT/A-ACS 7#2 and 3 using ACS7 FP/RP
Lane 7-8: InsT/A-ACS 7#2 and 3 using ACS7 FP/ M13RP
Lane 9: InsT/A-ACS 5#4 using ACS5 FP/RP
Lane 10: InsT/A-ACS 5#4 using ACS5 FP/ M13RP
Lane 11: InsT/A-ACS 5#4 using M13 FP/RP
Lane 12: Blank
Lane M: Lambda *Hind*III-*Eco*RI double digest

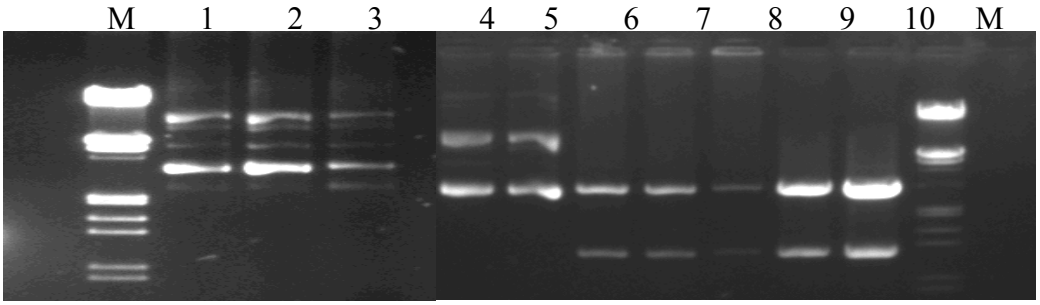


Fig.4.10. Restriction digestion of the InsT/A –ACS4, 5 and 7 plasmid DNA using *EcoR*I- *Hind*III restriction enzymes
Lane 1-2: Uncut InsT/A ACS 4 #1 and 2 plasmid DNA
Lane 3 : Uncut InsT/A ACS 5 #4 plasmid DNA
Lane 4-5: Uncut InsT/A ACS 7 #2 and 3 plasmid DNA
Lane 6-7: InsT/A ACS 4 #1 and 2 plasmid DNA digested with *EcoR*I-*Hind*III
Lane 8: InsT/A ACS 5 #4 plasmid DNA digested with *EcoR*I-*Hind*III
Lane 9-10: InsT/A ACS 7 #2 and 3 plasmid DNA digested with *EcoR*I-*Hind*III
Lane M: Lambda *Hind*III-*EcoR*I double digest

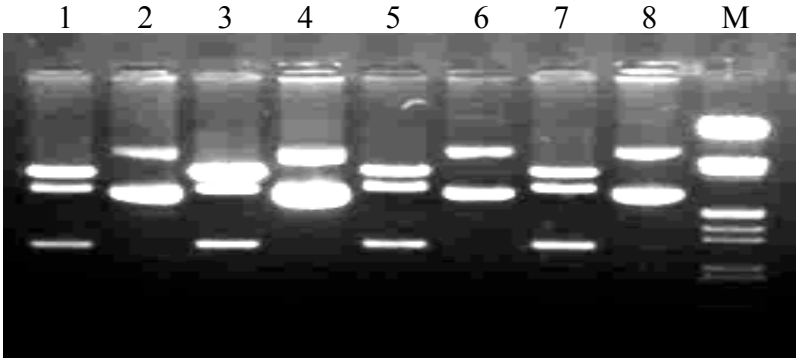


Fig.4.11. Restriction digestion of InsT/A-ACS4 #1-4 using *Kpn*I-*BamH*I
Lane 1,3,5,7: InsT/A-ACS4 #1-4 using *Kpn*I-*BamH*I
Lane 2,4,6,8: Uncut plasmid DNA of InsT/A-ACS4 #1-4
Lane M: Lambda *Hind*III-*EcoR*I double digest

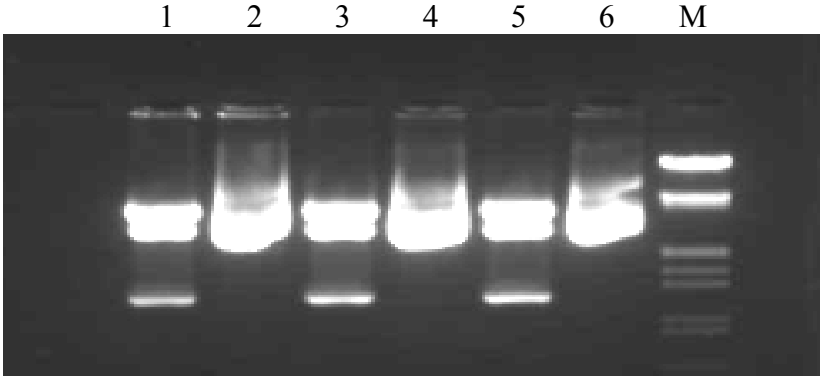


Fig.4.12. Restriction digestion of InsT/A-ACS5 #4,5,6 using *Kpn*I-*BamH*I
Lane 1,3,5: InsT/A-ACS5 #4,5,6 using *Kpn*I-*BamH*I
Lane 2,4,6: Uncut plasmid DNA of InsT/A-ACS5 #4,5,6
Lane M: Lambda *Hind*III-*EcoR*I double digest

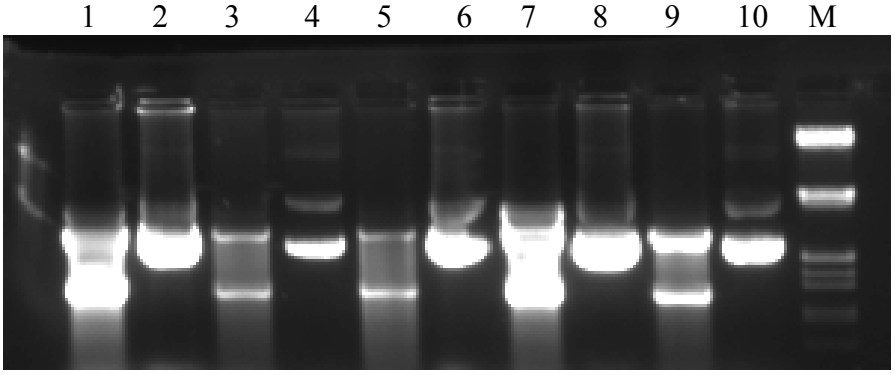


Fig.4.13. Restriction digestion of InsT/A-ACS7 #2,3,4,5,6 using *Kpn*I-*BamH*I
Lane 1,3,5,7,9: InsT/A-ACS7 #2,3,4,5,6 using *Kpn*I-*BamH*I
Lane 2,4,6,8,10: Uncut plasmid DNA of InsT/A-ACS7 # 2,3,4,5,6
Lane M: Lambda *Hind*III-*EcoR*I double digest

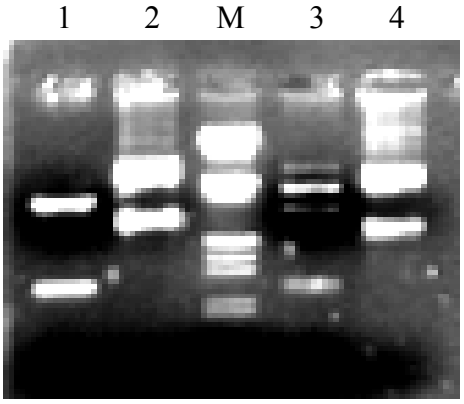


Fig.4.14. Restriction digestion of InsT/A-ACS4 #4 and 11 using *Xba*I
Lane 1,3: InsT/A –ACS4 # 4,11 digested with XbaI
Lane 2,4: InsT/A –ACS4 # 4,11 uncut plasmid DNA
Lane M: Lambda *Hind*III/*EcoR*I double digest DNA ladder

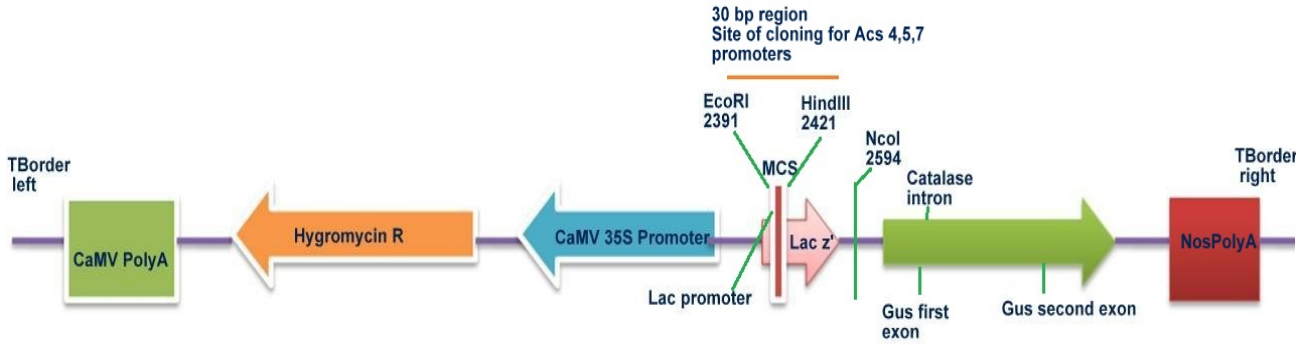
To develop individual gene cassettes, promoter sequences of *ACS4, ACS5* and *ACS7* were cloned in the binary vector pCAMBIA 1300. This was achieved by ligating the vector digested using *Kpn*I-*BamH*I with the promoter fragments excised from their respective InsT/A vectors which had been confirmed through sequencing using *Kpn*I-*BamH*I .

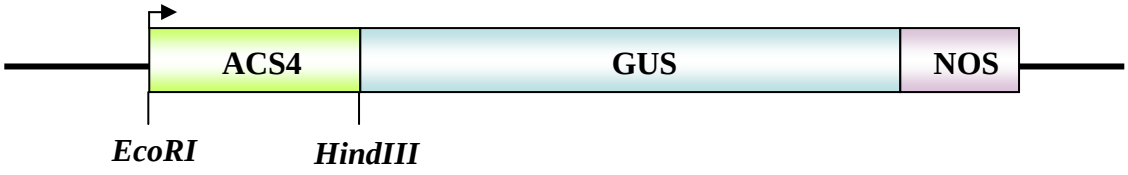
4.2.2. Development of constructs to validate the expression of *ACS4, 5* and *7* promoters using *gus* reporter

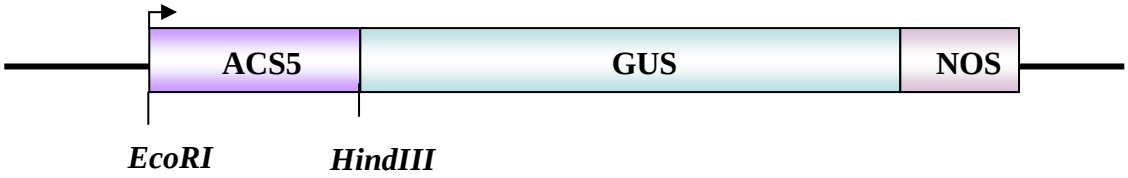
To validate the spatial and temporal expression of the *Arabidopsis thaliana* promoters *ACS4*, 5 and 7 in the heterologus system Tobacco, a *gus* reporter aided expression study was carried out. This was achieved by cloning the promoter fragments of *ACS4* (1.2 Kb), *ACS5* (1.22Kb) and *ACS7* (1.24Kb) upstream of *GUS* reporter gene in the binary vector pCAMBIA 1381z that has promoterless *gus* cassette. The schematic representation of cloning the promoter fragments in the binary vector is shown in Fig.4.15. The promoter fragments *ACS4, 5* and *7* were isolated as *EcoRI-HindIII* restriction fragments from the positive InsT/A promoter clones of *E.coli* which were confirmed through sequencing. These digested fragments of promoter were ligated with an *EcoRI-HindIII* digested pCAMBIA 1381z linearized vector (Fig.4.16.) to obtain the recombinant promoter vectors pCAMBIA 1381z–*ACS4-gus*-NOS, pCAMBIA 1381z–*ACS5-gus*-NOS and pCAMBIA 1381z–*ACS7-gus*-NOS expression cassettes (Fig.4.15) in T-DNA separately. The ligated DNA was then transferred into *E.coli* competent cells and the transformants were selected on a selection plate containing Kanamycin.

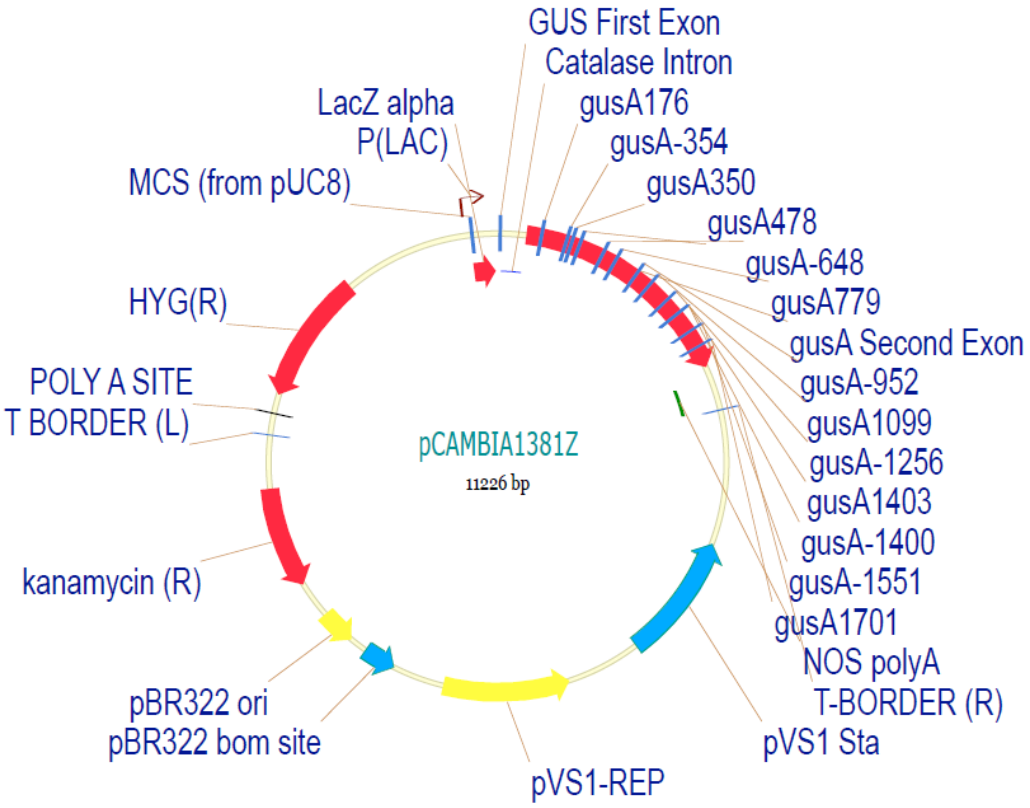
The positive clones of each promoter were identified by colony PCR of 18 transformants with respect to each promoter transformation using the promoter specific primers (Fig.4.18.). The presence of the promoters *ACS4* (7 clones), *ACS5* (10 clones) and *ACS7* (6 clones) in the binary vector pCAMBIA 1381z was confirmed by PCR (Fig.4.19) and restriction digestion using *Eco*RI/*Hind*III for the release of a 1.2 kb band (Fig.4.20-22). Three identified positive clones with regard to each promoter was checked for the orientation of the promoter with respect to the reporter by PCR using both the promoter (1.2 kb amlicon) and *gus* reporter (990bp amplicon) specific primers. The clones (one for each promoter) positive with PCR and restriction digestion analysis were mobilized in *Agrobacterium* strain LBA4404 for use in transformation of tobacco.

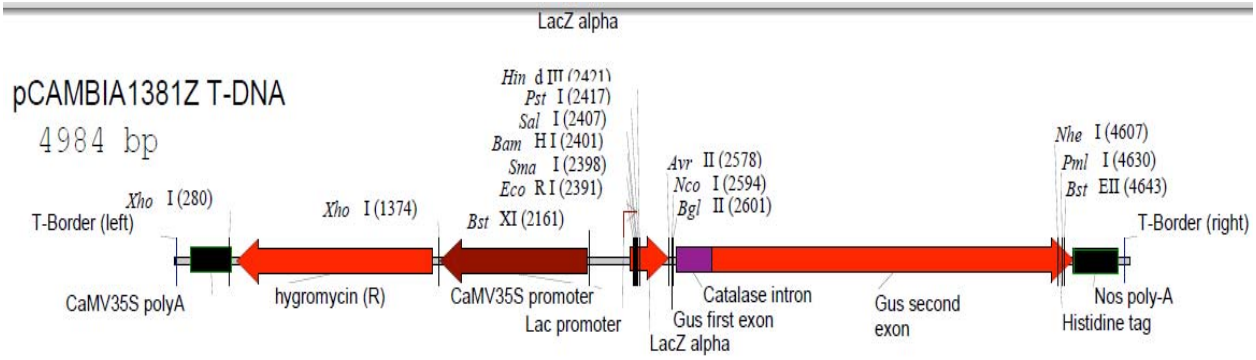
Fig. 4.15. Line diagram of the Promoter-GUS reporter constructs: pCAMBIA1381Z








GUS First Exon
Catalase Intron
LacZ alpha
P(LAC)
gusA176
gusA-354
MCS (from pUC8)
gusA350
gusA478
gusA-648
HYG(R)
gusA779
gusA Second Exon
POLY A SITE
T BORDER (L)
gusA-952
gusA1099
pCAMBIA1381Z
11226 bp
gusA-1256
gusA1403
gusA-1400
kanamycin (R)
gusA-1551
gusA1701
NOS polyA
T-BORDER (R)
pBR322 ori
pBR322 bom site
pVS1 Sta
pVS1-REP


LacZ alpha
pCAMBIA1381Z T-DNA
4984 bp
Hin d III (2421)
Pst I (2417)
Sal I (2407)
Bam H I (2401)
Sma I (2398)
Eco R I (2391)
Xho I (280)
T-Border (left)
Xho I (1374)
Bst XI (2161)
Avr II (2578)
Nco I (2594)
Bgl II (2601)
Nhe I (4607)
Pml I (4630)
Bst EII (4643)
T-Border (right)
CaMV35S polyA
hygromycin (R)
CaMV35S promoter
Lac promoter
Catalase intron
Gus first exon
LacZ alpha
Gus second exon
Nos poly-A
Histidine tag

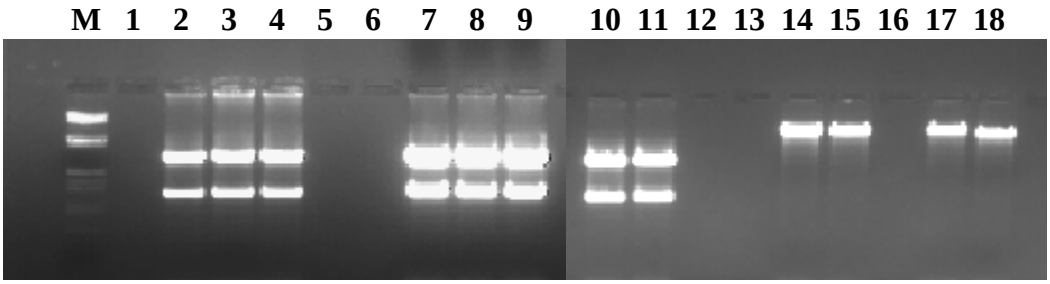


Fig.4.16. Restriction digestion of InsT/A-ACS 4, 5, 7 and pCAMBIA 1381z using *EcoR*I-*Hind*III restriction enzymes.

Lane M: Lambda *EcoR*I-*Hind*III double digest DNA marker

Lane 2,3 and 4: InsT/A-ACS4 restriction digestion using *EcoR*I-*Hind*III

Lane 7,8 and 9: InsT/A-ACS5 restriction digestion using *EcoR*I-*Hind*III

Lane 10 and 11: InsT/A-ACS7 restriction digestion using *EcoR*I-*Hind*III

Lane 14, 15,17 and 18: p CAMBIA 1381z restriction digestion using *EcoR*I-*Hind*III

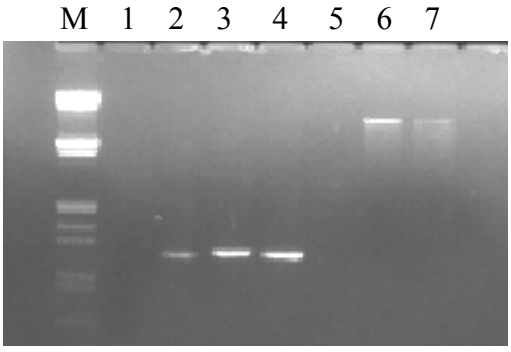


Fig.4.17.Quantification of the elutes of ACS4, 5, 7 and pCAMBIA 1381z *EcoR*I-*Hind*III linearized fragments.

Lane M: Lambda *EcoR*I-*Hind*III double digest DNA marker

Lane 2: ACS4 1.2 Kb promoter elute

Lane 3: ACS5 1.2 Kb promoter elute

Lane 4: ACS7 1.2 Kb promoter elute

Lane 6 and 7: pCAMBIA 1381z *EcoR*I-*Hind*III linearized fragments

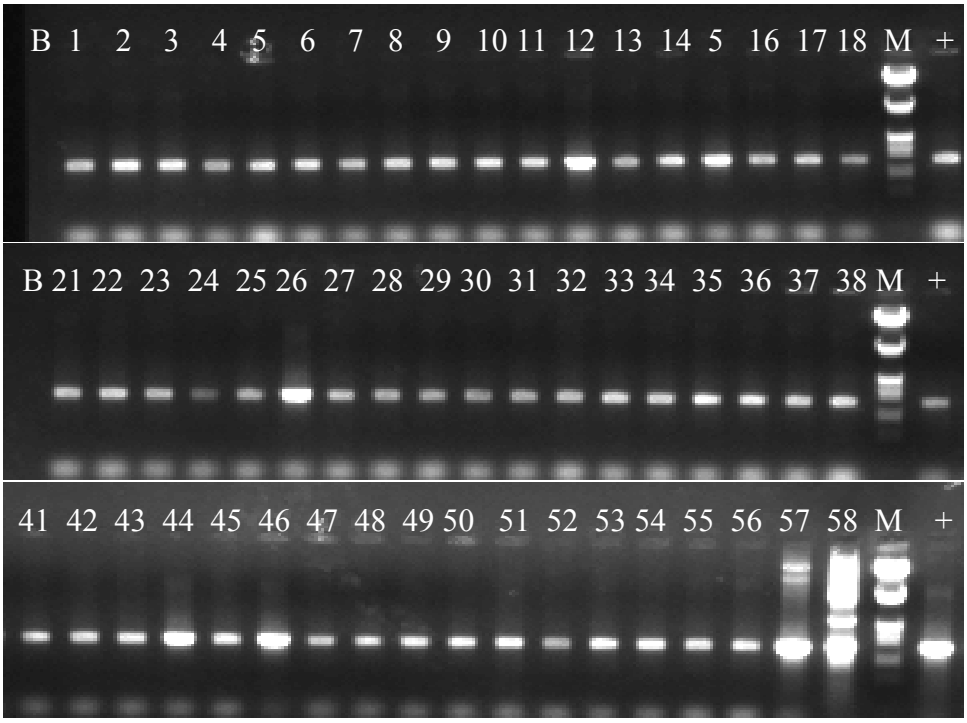


Fig.4.18. Colony PCR of pCAMBIA 1381z- ACS 4, 5 and 7 colonies using ACS4 FP/RP, ACS5 FP/RP and ACS7 FP/RP primers respectively.

Lane 1-18: pCAMBIA 1381z-ACS4 colonies PCR with ACS4 FP/RP

Lane 21-38: pCAMBIA 1381z-ACS5 colonies PCR with ACS5 FP/RP

Lane 41-58: pCAMBIA 1381z-ACS7 colonies PCR with ACS7 FP/RP

Lane B: Blank- No template control

Lane M: Lambda *Eco*RI-*Hind*III double digest DNA marker

Lane +: PCR positive

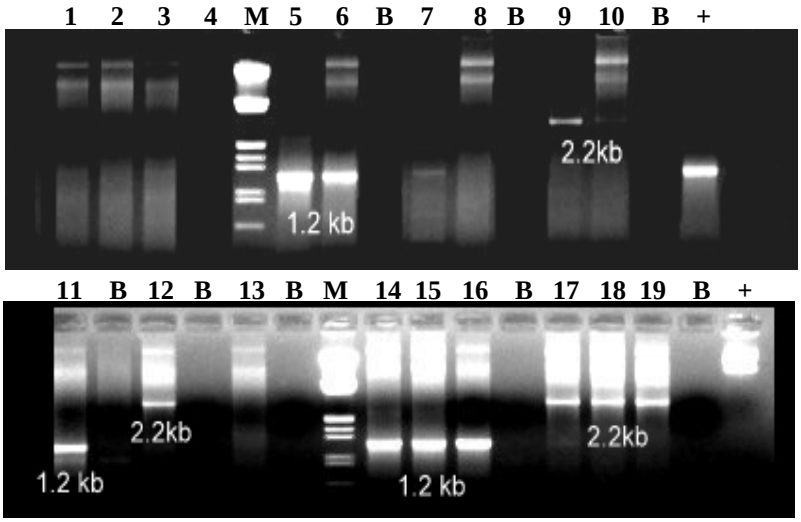


Fig.4.19. PCR confirmation of pCAMBIA-ACS4,5 and 7 plasmid DNA to check the orientation of the cloned promoter fragments with respect to the downstream GUS gene

Lane 1: pCAMBIA 1381z plasmid DNA PCR using ACS4 FP/RP

Lane 2: pCAMBIA 1381z plasmid DNA PCR using ACS5 FP/RP

Lane 3: pCAMBIA 1381z plasmid DNA PCR using ACS7 FP/RP

Lane 5,6: PCR of pCAMBIA 1381z –ACS7 #1,2 using ACS7 FP/RP

Lane 7,8: PCR of pCAMBIA 1381z –ACS7 #1,2 using ACS7 FP/GUS FP

Lane 9,10: PCR of pCAMBIA 1381z –ACS7 #1,2 using ACS7 FP/GUS RP

Lane 11: PCR of pCAMBIA 1381z –ACS4 #20 using ACS4 FP/RP

Lane 12: PCR of pCAMBIA 1381z –ACS4 #20using ACS4 FP/GUS RP

Lane 13: PCR of pCAMBIA 1381z –ACS4 #20 using ACS4 FP/GUS FP

Lane 14,15,16: PCR of pCAMBIA 1381z –ACS5 #6,22,45 using ACS5 FP/RP

Lane 17,18,19: PCR of pCAMBIA 1381z – ACS5 #6,22,45 using ACS5 FP/GUS RP

Lane B: PCR Blank

Lane +: PCR Positive

Lane M **:** Lambda *Eco*RI-*Hind*III double digest DNA marker

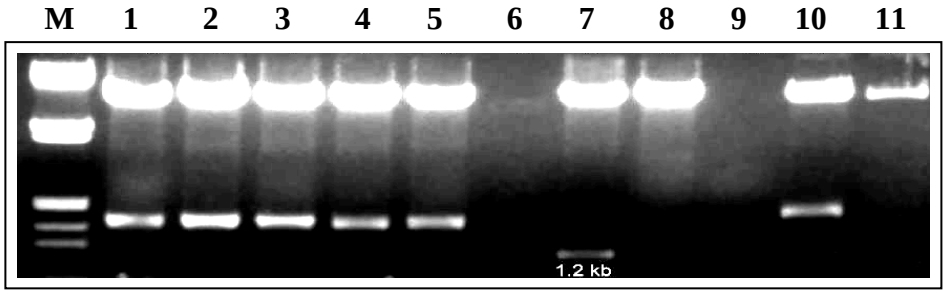


Fig.4.20. Restriction digestion confirmation of pCAMBIA 1381z-ACS4 plasmid DNA with *EcoR*I-*Hind*III

Lane M: Lambda *EcoR*I-*Hind*III double digest DNA marker

Lane 1,2,3,4,5,7and 8: Restriction digestion of pCAMBIA 1381z-ACS4 clone #3,9,10,12,16,20 and 27 respectively with *EcoR*I-*Hind*III

Lane 10: Restriction digestion of pCAMBIA 1381z-ACS4 clone# 20 with *EcoR*I

Lane 11: Restriction digestion of pCAMBIA 1381z-ACS4 clone# 20 with *Hind*III

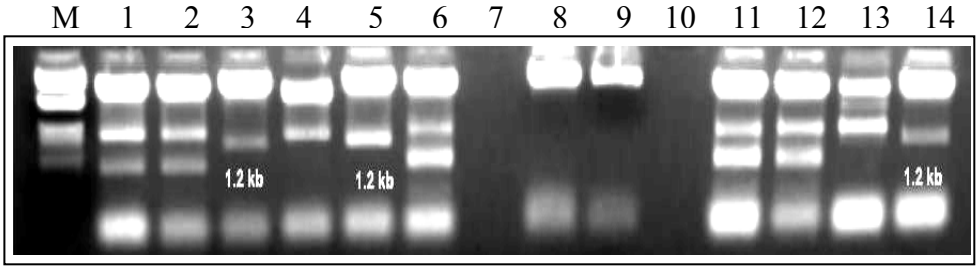


Fig.4.21. Restriction digestion confirmation of pCAMBIA 1381z-ACS5 plasmid DNA with *EcoR*I-*Hind*III

Lane M: Lambda *EcoR*I-*Hind*III double digest DNA marker

Lane 1,2,3,4,5,6,11,12,13 and 14: Restriction digestion of pCAMBIA 1381z-ACS5 clone #1,2,6,20,22,23,25,36,43 and 45 respectively with *EcoR*I-*Hind*III

Lane 8: Restriction digestion of pCAMBIA 1381z-ACS5 clone#6 with *EcoR*I

Lane 9: Restriction digestion of pCAMBIA 1381z-ACS4 clone#6 with *Hind*III

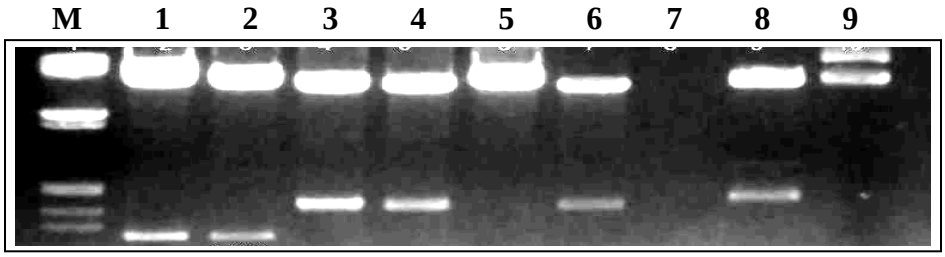


Fig.4.22. Restriction digestion confirmation of pCAMBIA 1381z-ACS7 plasmid DNA with *Eco*RI-*Hind*III

Lane M: Lambda *Eco*RI-*Hind*III double digest DNA marker

Lane 1,2,3,4,5 and 6: Restriction digestion of pCAMBIA 1381z-ACS7 clone #1,2,3,4,5 and 6 respectively with *Eco*RI-*Hind*III

Lane 8: Restriction digestion of pCAMBIA 1381z-ACS7 clone# 1 with *Eco*RI

Lane 9: Restriction digestion of pCAMBIA 1381z-ACS7 clone# 1 with *Hind*III

4.2.3. Mobilization of the promoter validation constructs into *Agrobacterium tumifaceans* strain LBA4404

The confirmed pCAMBIA 1381z–*ACS4-gus*-NOS, pCAMBIA 1381z–*ACS5-gus*-NOS and pCAMBIA 1381z–*ACS7-gus*-NOS and basal pCAMBIA 1381z (minus promoter) vectors were mobilized into *Agrobacterium* LBA4404. The *Agrobacterium* transformants were checked for the presence of the promoter vectors by PCR using plasmid DNA isolated from the *Agrobacterium* clones as the template and by restriction digestion using the plasmid DNA isolated from the *E.coli* colonies reverse transformed with the promoter vectors isolated from the positive clones of *Agrobacterium*. The purity of the *Agrobacterium* clones was checked by keto-lactose test for *Agrobacterium* before using the culture for transforming tobacco explants.

4.2.4. Tobacco transformation using promoter validation constructs

Tobacco leaf explants were transformed with *Agrobacterium* cultures harboring binary vectors pCAMBIA 1381z–*ACS4-gus*-NOS, pCAMBIA 1381z–*ACS5-gus*-NOS, pCAMBIA 1381z–*ACS7-gus*-NOS and pCAMBIA 1381z (minus promoter) vector as a control. Shoot regeneration was achieved on a medium containing MS + BAP 1mg/L + NAA 0.1mg/L with Hygromycin

30mg/L to selectively regenerate only the transfomants for 15 days. The regenerated shoots were proliferated further in a medium containing MS + BAP 0.5 mg/L + hygromycin 30mg/L for 15-30 days. The shoots were elongated in a medium containing MS + BAP 0.2 mg/L + Hygromycin 30mg/L for 15 days. The elongated shoots were transferred to a medium containing only MS salts (half strength) for rooting of the tobacco plants. Eight rooted plants with respect to each promoter and control were acclimatized in the green house (Fig.4.23.) and grown until flowering and completion of the reproductive cycle.

4.2.5. Molecular analysis of the promoter specific tobacco transgenic plants

The putative transgenic tobacco plants harboring the T-DNA with *ACS4-gus, ACS5-gus*, *ACS7-gus* and *gus* alone as the control were checked for the presence of the promoter and *gus* gene by PCR using the gDNA isolated from leaf as the template (Fig.4.24.). The control 1381z tobacco transgenic plants were also tested for the absence of the promoter fragments by PCR using the primers of all three promoters (Fig.4.25.). All the eight tobacco transgenic plants with respect to each promoter have showed positive with promoter specific PCRs indicating the presence of the promoter fragments in the binary vector. Five PCR positive plants were checked for the expression of *gus* gene by RT-PCR in different tissues like: leaf and flowers (Fig.4.26.). The expression was observed in both the tissues indicating the non-localized expression of the *Arabidopsis thaliana* promoters *ACS4, 5* and *7* in tobacco. However, RT-PCR being a PCR based technique could not clearly indicate any differences in the expression levels of the promoters in leaf and floral tissues analyzed. The expression of the *gus* reporter gene was not observed in the tobacco trangenics harbouring the empty vector pCAMBIA 1381z acting as a perfect control for the experiment. Expression pattern of house keeping gene actin was used as the endogenous control to ensure equal quantity of RNA taken for the RT-PCR experiments. To get the quantitative data on expression pattern histochemical analysis was carried out.

4.2.6. Histochemical staining of GUS expressing promoter validation tobacco transgenics

The histochemical staining of GUS expression was tested in different stages of development of leaves and inflorescence (Fig.4.28). The blue coloration was observed in all the stages of the flower development and was more evident in the later stages of floral development – more prominent in the anthers and stigma (Fig.4.27). The pollen grains also showed blue coloration (upto 30%) indicating the expression of the promoters. The GUS staining was also evident in the

capsules. This analysis indicated the elevated production of GUS in all the flower parts compared to leaves. Thus the selection of these promoters for developing gene constructs aimed at elevated expression in the inflorescence was justified and supported.

1381z control

1381z – *ACS4*

1381z – *ACS5*

1381z – *ACS7*

Fig 4.23. *ACS4, ACS5, ACS7* and 1381z (control) tobacco transgenic plants in the greenhouse

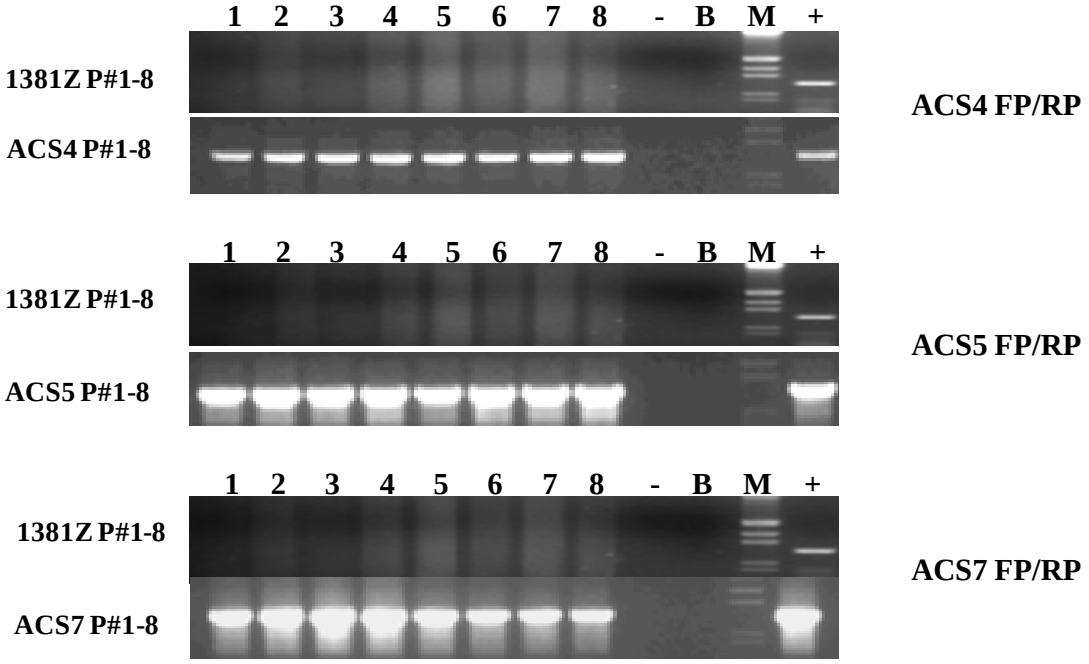


Fig.4.24. PCR confirmation of *ACS4, 5, 7* and 1381z (control) tobacco plants
Panel I: PCR of 1381z and *ACS4* p#1-8 using ACS4 FP/RP
Panel II: PCR of 1381z and *ACS5* p#1-8 using ACS5 FP/RP
Panel III: PCR of 1381z and *ACS7* p#1-8 using ACS7 FP/RP
Lane - : PCR negative
Lane B: PCR Blank
Lane M: Lanbda *Hind*III/*Eco*RI double digest
Lane + : PCR positive

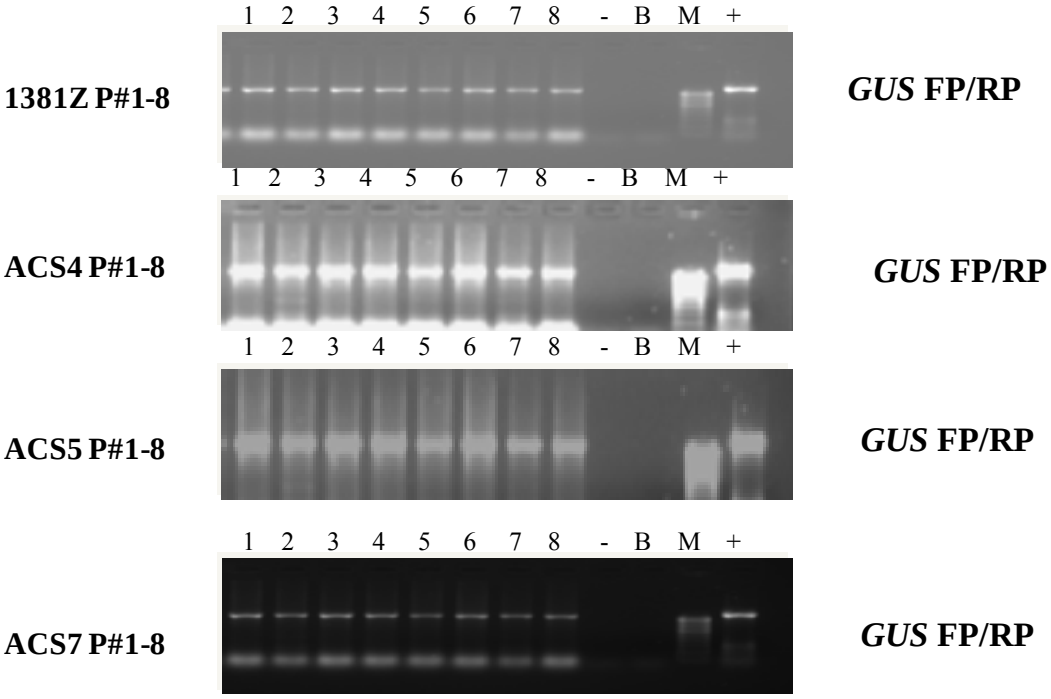


Fig.4.25. PCR confirmation of *ACS4,5,7* and 1381z control tobacco plants using GUS FP/RP
Panel I: PCR of 13181z (control) P#1-8 using GUS FP/RP
Panel II: PCR of *ACS4* P#1-8 using *gus* FP/RP
Panel III: PCR of *ACS5* P#1-8 using *gus* FP/RP
Panel IV: PCR of *ACS7* P#1-8 using *gus* FP/RP
Lane - : PCR negative
Lane B: PCR Blank
Lane M: Lanbda *Hind*III/*EcoR*I double digest
Lane + : PCR positive

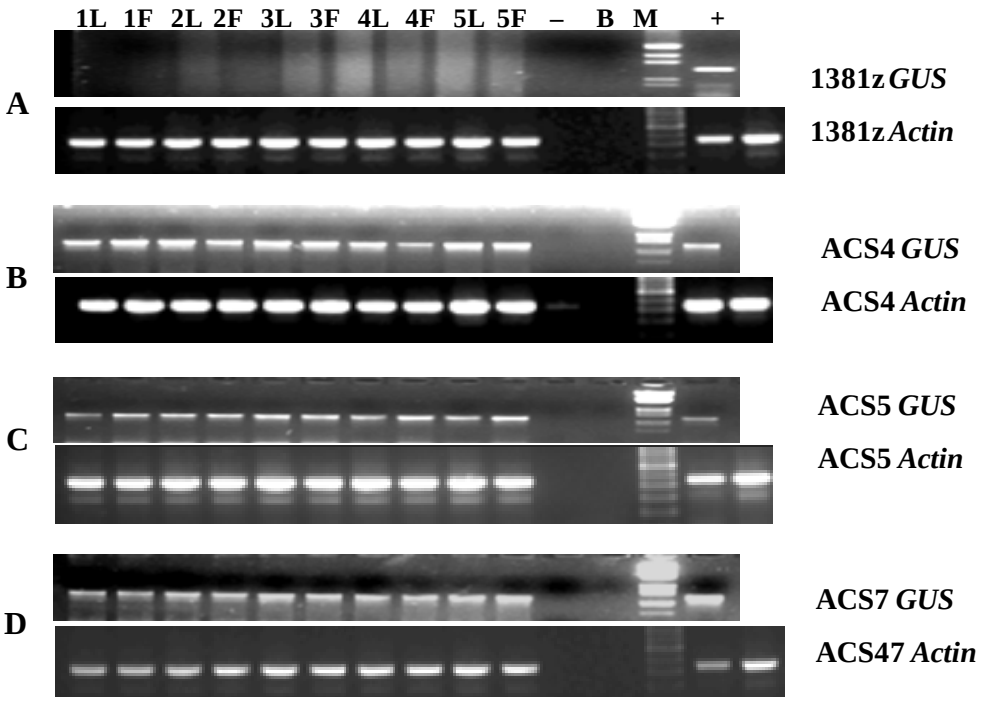


Fig.4.26. RT PCR analysis of promoter expressing tobacco plants using *GUS* and *Actin* FP/RP

Lane1,2,3,4,5L: RT PCR of leaf of 1381z control, ACS4, 5 and 7 promoter leaf cDNA

Lane 1,2,3,4,5F: RT PCR of leaf of 1381z control, ACS4, 5 and 7 promoter flower cDNA

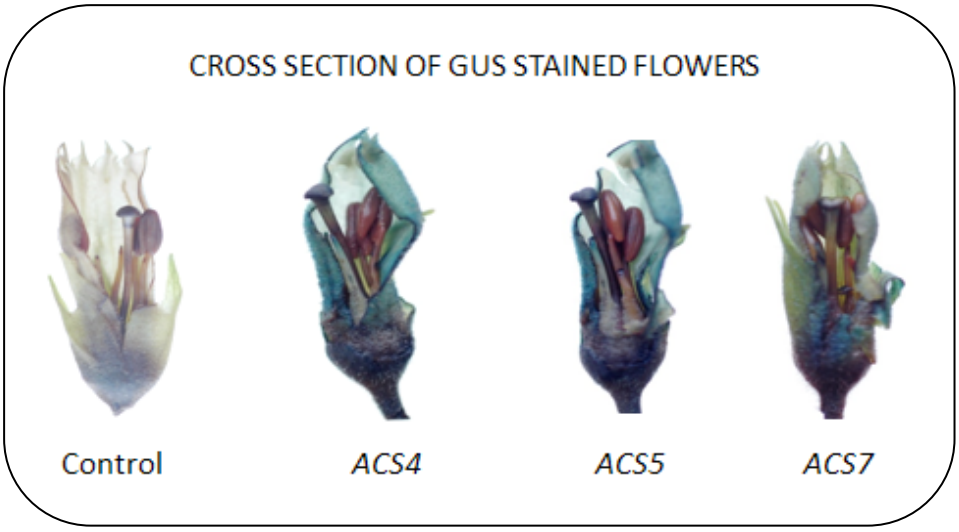


Fig.4.27. Cross section of the ACS4, 5, 7 promoter and untransformed tobacco flowers

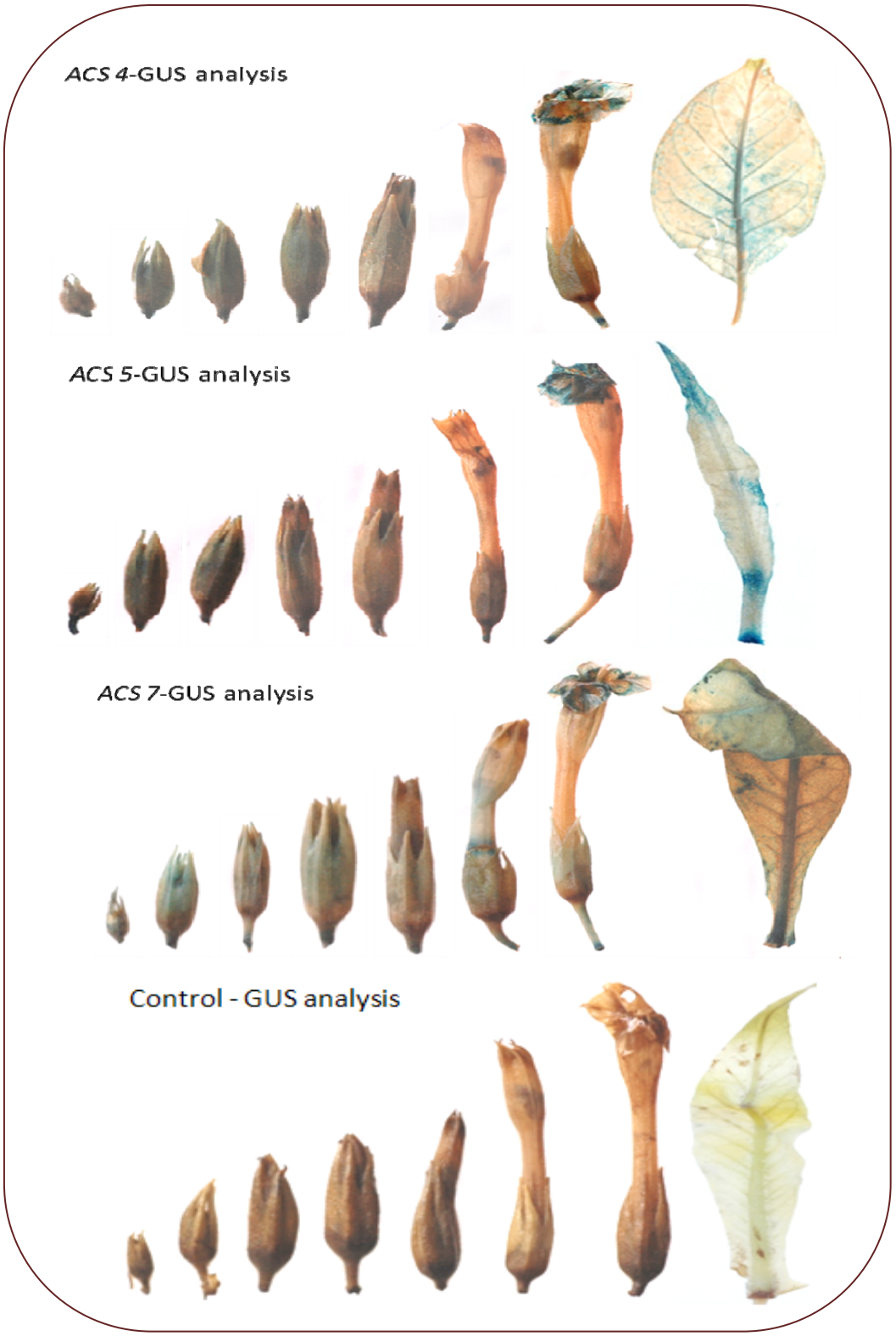
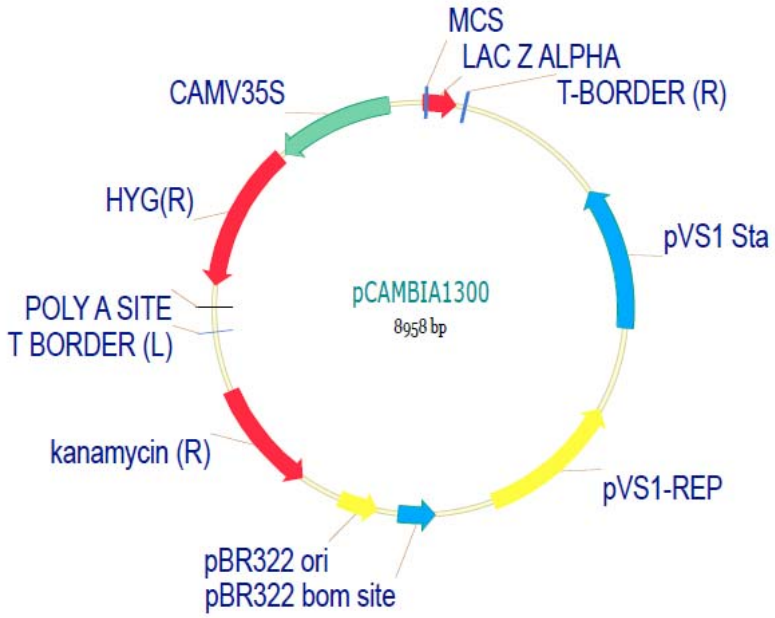
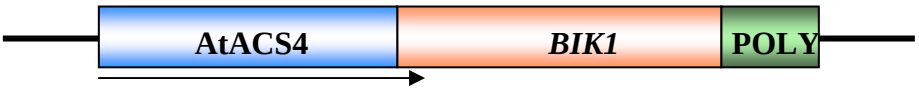


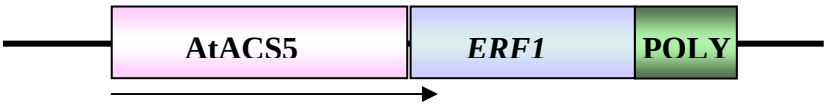
Fig 4.28. GUS histochemical staining of *AtACS 4,5,7* and control untransformed tobacco flowers of all stages of development and leaf.



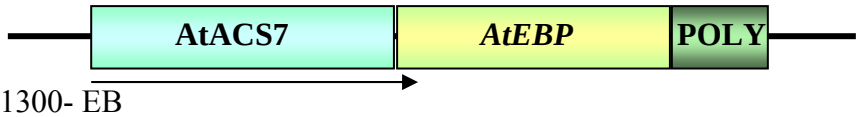
pCAMBIA 1300-ACS4-*BIK1*-P



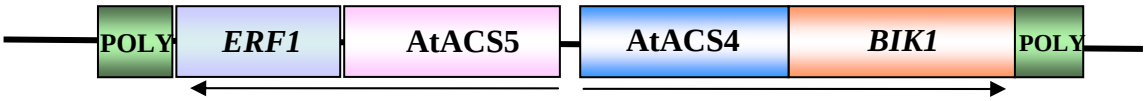
pCAMBIA 1300-ACS5-*ERF1*-PS



pCAMBIA 1300-ACS7-*AtEBP*-PE



pCAMBIA 1300- EB



pCAMBIA 1300- AEB

Fig.4.29. Schematic representation of the developed gene constructs
pCAMBIA 1300 binary vector :

4.3. Development of gene constructs using three inflorescence specific promoters *ACS4,* 5, 7 and three genes *BIK1, ERF1* and *AtEBP* from *Arabidopsis thaliana*

The three defense regulatory genes *BIK1, ERF1* and *AtEBP* were isolated from *Arabidopsis thaliana* using gene specific primers for *ERF1* and by rimer extension PCR for both *BIK1* and *AtEBP* genes. The primers for each of these genes were designed based on the sequences available at NCBI- GenBank database. These three genes were chosen based on the tolerance exhibited individually, against the necrotrophic fungi *Botrytis cinerea* in *Arabidopsis*. The genes *BIK1, ERF1* and *AtEBP* were used to develop individual gene cassettes using inflorescence specific promoters *ACS4*, 5 and 7 respectively. PolyA sequence from pRT 100 vector was used as the terminator in all the three cassettes developed. The schematic representation of development of individual gene cassettes is shown in Fig.4.30, 4.31 and 4.32.

The double gene cassette vector was developed by cloning the *ERF1* individual gene cassette (as a *Sac*I fragment) in the T-DNA of the *BIK1* gene cassette. The schematic representation of development of double gene cassette is shown in Fig.4.77. The promoter fragments of the two gene cassettes were placed in opposite orientation to each other so as to act as a bidirectional duplex promoter and also to avoid the read through effect of one cassette into another. The other two combinations of developing the double gene cassette i.e. *BIK1* with *AtEBP* and *ERF1* with *AtEBP* were not tried as the anti-apoptotic gene *AtEBP* expression is considered vital for the normal growth and development of the plant. Hence, its expression along with other genes might lead to detrimental effects in plants in which it is expressed ectopically. However, it was conjectured that these combinations could be achieved by bringing the two cassettes together in one plant by crossing the plants carrying individual plant cassettes.

A triple gene cassette vector harboring all the three individual cassettes within a single T-DNA was developed by cloning the *AtEBP* gene cassette upstream to the *ERF1* gene cassette in the double gene cassette vector as an *EcoRI* fragment. The schematic representation of development of triple gene cassette is shown below. The triple gene cassette vector was developed to stack the expression of all the three genes and study the cumulative expression of the three genes in a single plant with enhanced resistance to Botrytis infection.

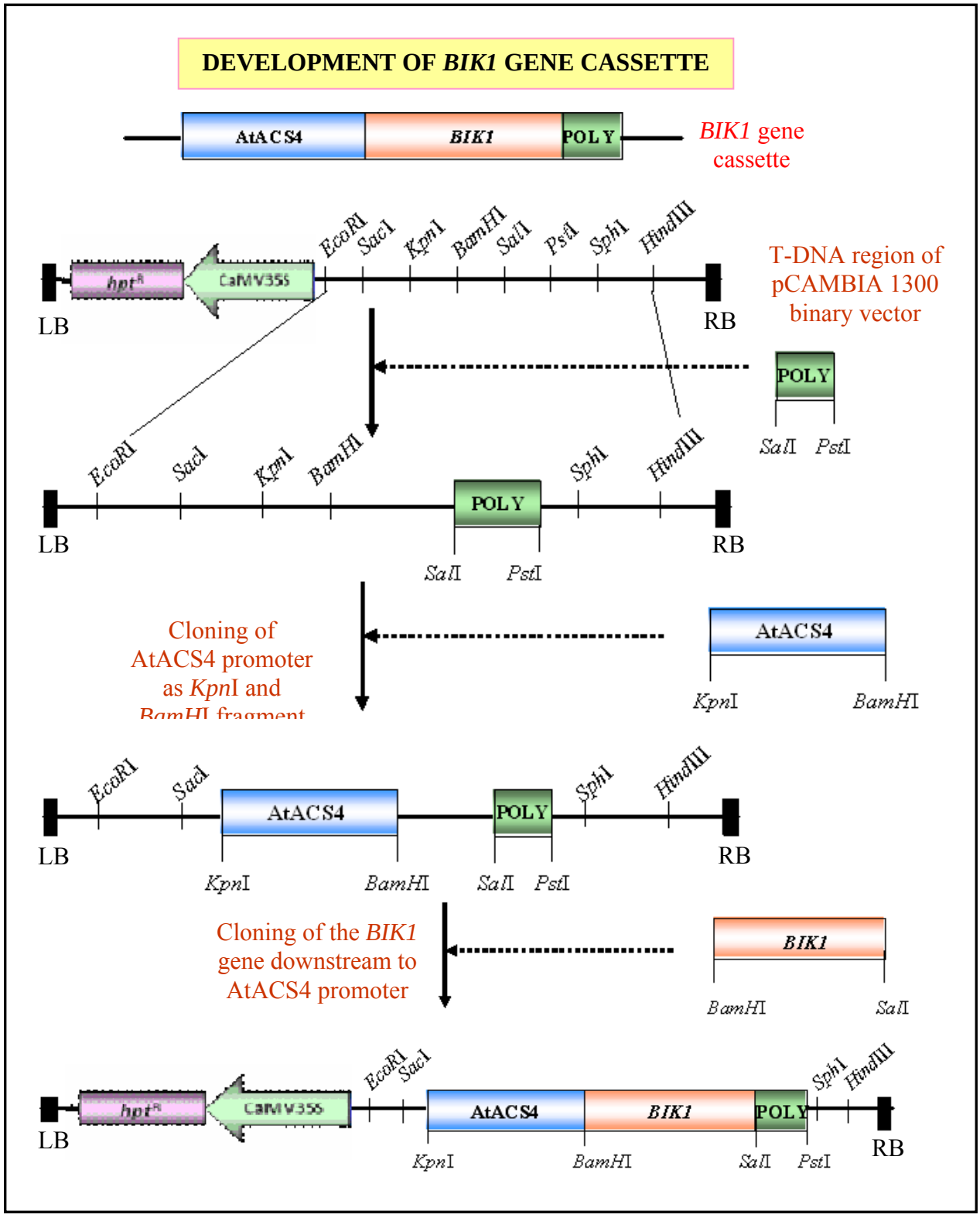


Fig.4.30.Schematic representation for the development of pCAMBIA 1300-*ACS4*-*BIK1*-P gene construct

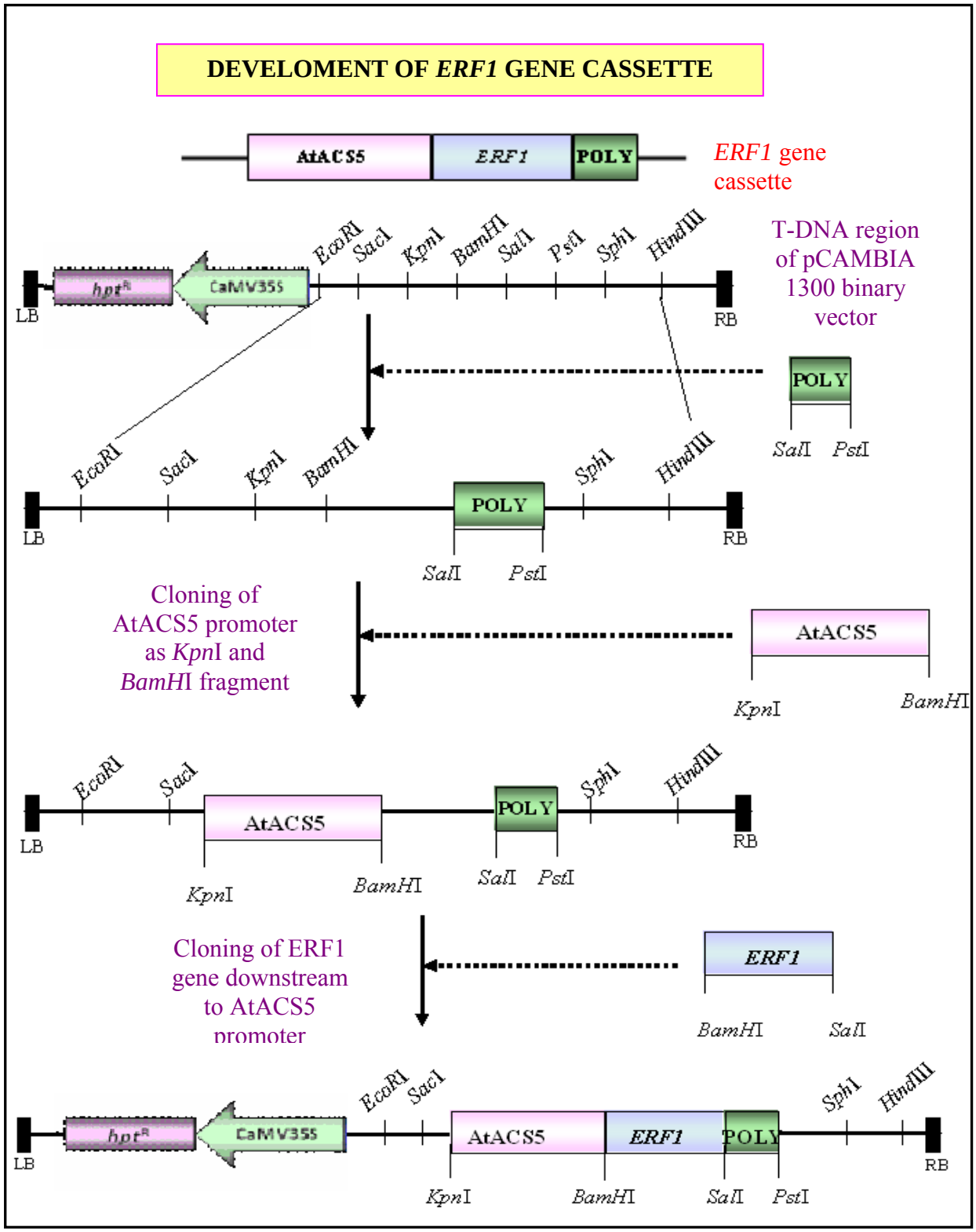


Fig.4.31. Schematic representation for the development of pCAMBIA 1300-*ACS5*-*ERF1*-PS gene construct

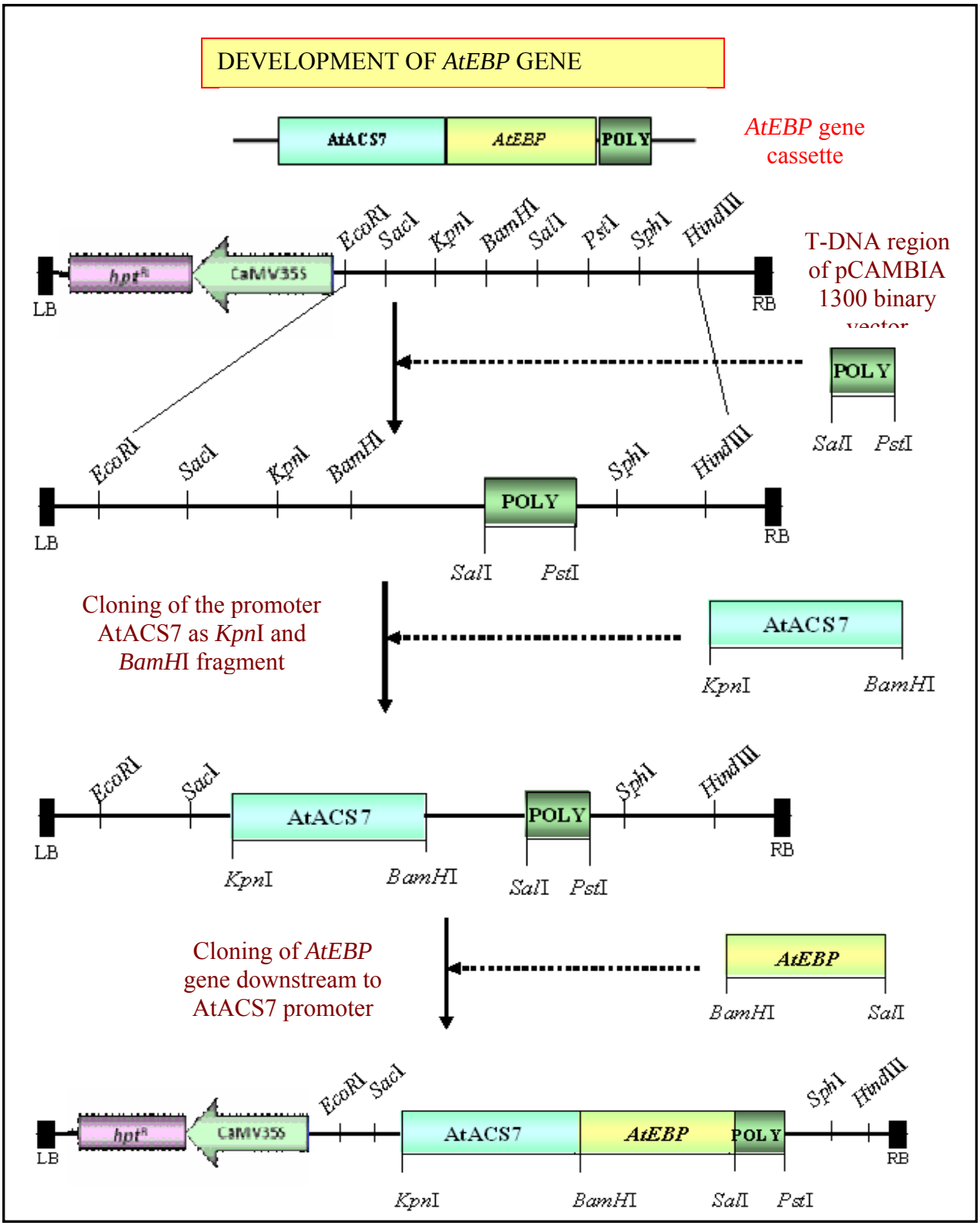


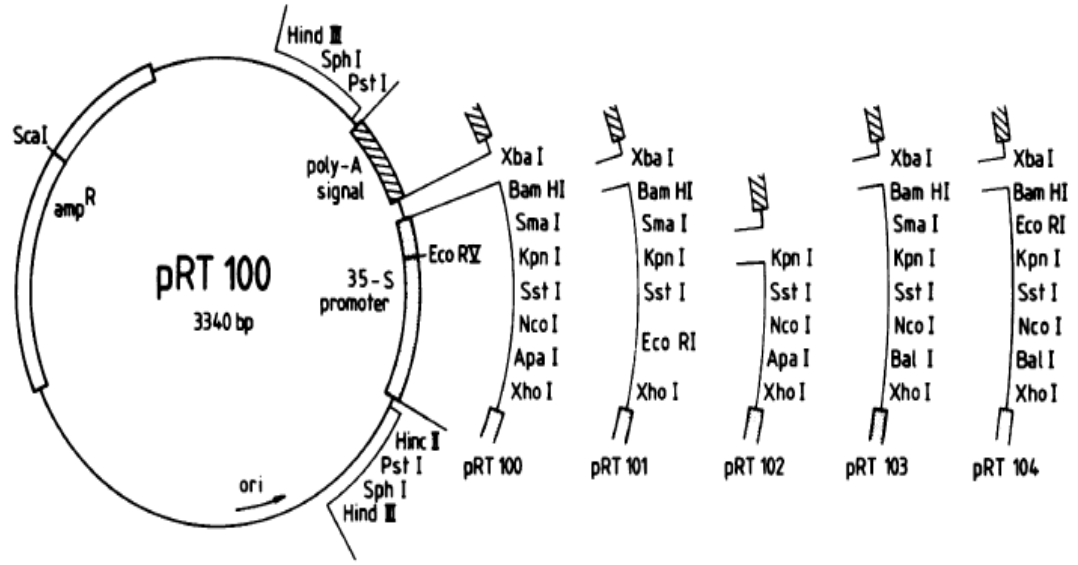
Fig.4.32. Schematic representation for the development of pCAMBIA 1300-*ACS7-AtEBP* PE gene construct

4.3.1. Isolation of *polyA* terminator from pRT100

The *polyA* terminator was isolated from the expression vector pRT100 and was cloned in InsTA vector earlier in the lab. The same *polyA* fragment was amplified using forward primer (tagged with *SalI*) and reverse primers tagged with three different restriction enzymes (*PstI, EcoRI-PstI* and *SacI-PstI*), independently, using InsT/A-*polyA* plasmid DNA as template. The three different combinations of reverse primers were used to facilitate the subcloning of the three gene cassettes into a single T-DNA in the binary vector pCAMBIA 1300, hence resulting in the formation of double cassette and triple cassette vector for stacking the action of two or three genes within a single plant tissue transformed with these vectors.

The amplified *polyA* fragments i.e., *SalI-polyA-PstI* (P), *SalI-polyA-EcoRI-PstI* (PE) and *SalI-polyA-SacI-PstI* (PS) were gel eluted and cloned in InsT/A vector to validate the *polyA* sequence by sequencing. Ten transformants of each polyA fragment was tested for the presence of their respective polyA fragments by colony PCR. Four clones showed amplification with respect to (P) and one each with PS and PE specific primers. The PCR positive clones of each case was further confirmed for the release of the 250 bp polyA fragment using different combinations of restriction enzymes. (Fig4.33. and 4.34.).

The InsT/A-polyA clones showing positive for the terminator fragment in each case were sequenced and the fragments were directionally cloned in the binary vector pCAMBIA 1300 as a *Pst*I/*Sal*I fragment to generate pCAMBIA 1300-P, pCAMBIA 1300-PE and pCAMBIA1300-PS binary vectors. One clone from combination showed positive on reconfirmation by restriction digestion using *Pst*I/*Sal*I for the release of the 250bp polyA fragment (Fig.4.35 and 4.36). These binary vectors harbouring the poly terminator fragments were used for cloning the promoters *ACS4, 5* and *7* upstream to assemble the gene cassette.




```
Hind3 SphI  PstI  HincII
aagcttgcatgcctgcaggtcAACATGGTGGAGCACGACACTCTCGTCTACTCCAAGAATATCAAAGATACAGTCTCAGAAGACCAGAGGGCTATTGAGACTTTTCAACAAAGGGTAATA 120
TCGGGAAACCTCCTCGGATTCCATTGCCCAGCTATCTGTCACTTCATCGAAAGGACAGTAGAAAAGGAAGATGGCTTCTACAAATGCCATCATTGCGATAAAGGAAAGGCTATCGTTCAA 240
                                                                                                    EcoRV
GAATGCCTCTACCGACAGTGGTCCCAAAGATGGACCCCCACCCACGAGGAACATCGTGGAAAAAGAAGACGTTCCAACCACGTCTTCAAAGCAAGTGGATTGATGTGATATCTCCACTGA 360
                                                                  XhoI ApaINcoI    SstI  KpnISmaI BamHI
CGTAAGGGATGACGCACAATCCCACTATCCTTCGCAAGACCCTTCCTCTATATAAGGAAGTTCATTTCATTTGGAGAGGACctcgaggcccatgggcgagctcggtacccggggatcct 480
XbaI
ctagagtcGCGAAATCACCAGTCTCTCTCTACAAATCTATCTCTCTCTATTTTCTCCAGAATAATGTGTGAGTAGTTCCCAGATAAGGGAATTAGGGTTCTTATAGGGTTTCGCTCATGT 600
                                                                                  PstI  SphI  Hind3
GTTGAGCATATAAGAAACCCTTAGTATGTATTTGTATTTGTAAAATACTTCTATCAATAAATTTCTAATTCCTAAAACCAAAATCCAGTgacctgcaggcatgcaagctt 711
```

1 2 3 4 5 6 7 M 8 9 10 11 12 13 14 15 16 17 18 19 M

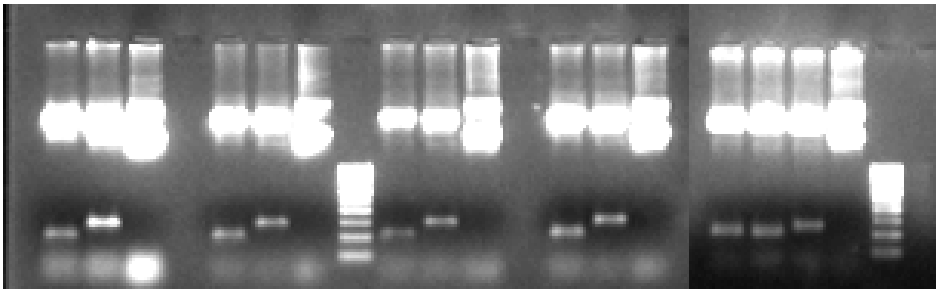


Fig.4.33. Restriction digestion confirmation of InsT/A-PolyA-P,PE and PS plasmid DNA

Lane 1,5,8,12: InsT/A PolyA –P clones #1-4 digested with *Sal*I/*Pst*I
Lane 2,6,9,13: InsT/A PolyA –P clones #1-4 digested with *Hind*III/*Eco*RI
Lane 3,7,10,14: InsT/A PolyA –P clones #1-4 uncut plasmid DNA
Lane 16: InsT/A PolyA –PE clone #1 digested with *Sal*I/*Pst*I
Lane 17: InsT/A PolyA –PE clone #1 digested with *Sal*I/*Eco*RI
Lane 18: InsT/A PolyA –PE clone #1 digested with *Hind*III/*Eco*RI
Lane 19: InsT/A PolyA –PE clone #1 uncut plasmid DNA
Lane M: 100bp DNA ladder

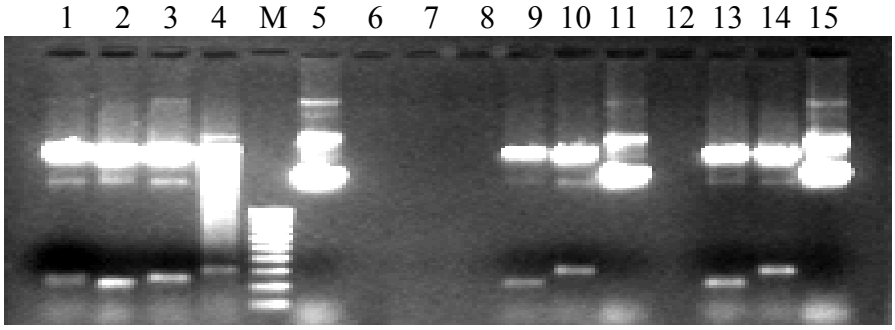


Fig.4.34. Restriction digestion confirmation of InsT/A-PolyA-P,PE and PS plasmid DNA with *Sal*I,*Sac*I,*EcoR*I,*Hind*III/*EcoR*I

Lane 1: InsT/A PolyA –PS clone #1 digested with *Sal*I/*Sac*I
Lane 2: InsT/A PolyA –PE clone #1 digested with *Sal*I/*Pst*I
Lane 3: InsT/A PolyA –PE clone #1 digested with *Sac*I
Lane 4: InsT/A PolyA –PE clone #1 digested with *Hind*III/*EcoR*I
Lane 5: InsT/A PolyA –PE clone #1 uncut plasmid DNA
Lane 9,13: InsT/A PolyA –P clones #5,6 digested with *Sal*I/*Pst*I
Lane 10,14: InsT/A PolyA –P clones #5,6 digested with *Hind*III/*EcoR*I
Lane 11,15: InsT/A PolyA –P clone #5,6 uncut plasmid DNA
Lane 6,7,8 : empty wells
Lane M: 100bp DNA ladder

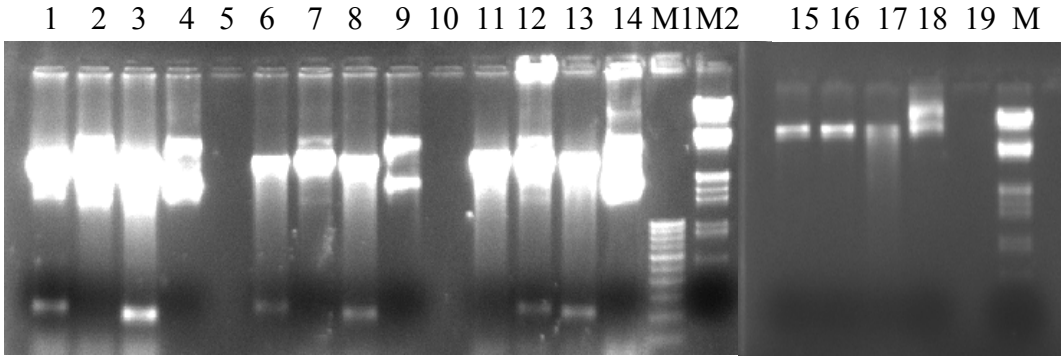


Fig.4.35. Restriction digestion confirmation of pCAMBIA 1300-PolyA-P,PE and PS plasmid DNA
Lane 1,6,11: pCAMBIA 1300-PolyA-P,PE and PS#1 digested with *Pst*I
Lane 2,7,12: pCAMBIA 1300-PolyA-P,PE and PS#1 digested with *Sal*I
Lane 3,8,13: pCAMBIA 1300-PolyA-P,PE and PS#1 digested with *Pst*I/*Sal*I
Lane 4,9,14: pCAMBIA 1300-PolyA-P,PE and PS#1 uncut plasmid DNA
Lane 15: pCAMBIA 1300 #1 digested with *Pst*I
Lane 16: pCAMBIA 1300 #1 digested with *Sal*I
Lane 17: pCAMBIA 1300 #1 digested with *Pst*I/*Sal*I
Lane 18: pCAMBIA 1300 #1 uncut plasmid DNA
Lane M1: 100bp DNA ladder
Lane M2: Lambda *Hind*III/*EcoR*I double digest DNA ladder

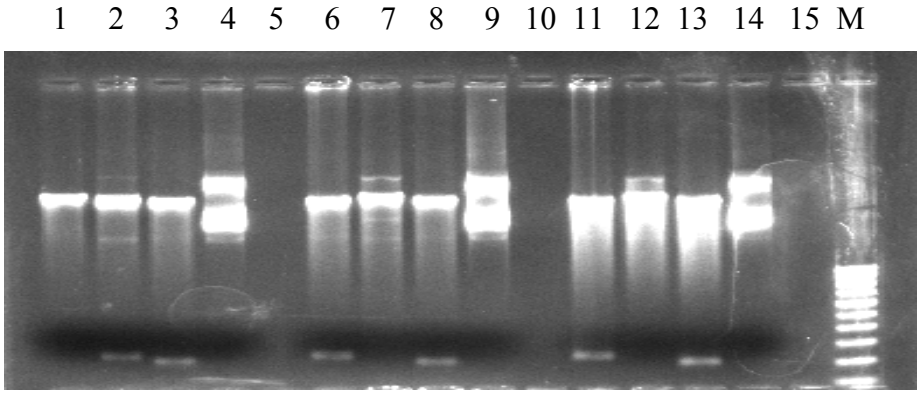


Fig.4.35-a. Restriction digestion confirmation of pCAMBIA 1300-PolyA-P plasmid DNA
Lane 1,6,11: pCAMBIA 1300-PolyA-P # 2,3,4 digested with *Pst*I
Lane 2,7,12: pCAMBIA 1300-PolyA-P #2,3,4 digested with *Sal*I
Lane 3,8,13: pCAMBIA 1300-PolyA-P #2,3,4 digested with *Pst*I/*Sal*I
Lane 4,9,14: pCAMBIA 1300-PolyA-P #2,3,4 uncut plasmid DNA
Lane M: 100bp DNA ladder

4.3.2. Cloning of the promoter fragments of *ACS4, 5* and *7* in pCAMBIA 1300-polyA binary vectors

To develop the individual gene cassettes for the present study, promoter sequences of *ACS4, ACS5* and *ACS7* were cloned in the binary vector pCAMBIA 1300-P, PS and PE respectively to generate pCAMBIA-1300-*ACS4*-P, pCAMBIA-1300-*ACS4*-PE and pCAMBIA-1300-*ACS4*-PS vectors. This was achieved by ligating the eluted fragments of the binary vectors pCAMBIA 1300-P, PS and PE digested using *KpnI-BamHI* with the promoter fragments excised after digesting their respective InsT/A vectors (which had been confirmed through sequencing) with *KpnI-BamHI* (Fig.4.36, 4.38 and 4.39). Ten transformants with respect to each promoter was checked using colony PCR using primers specific to each promoter (Fig.4.37). One clone of each promoter set was further checked for the release of the 1.2 kb promoter fragments by restriction digestion using *KpnI* and *BamHI* (Fig.4.40).

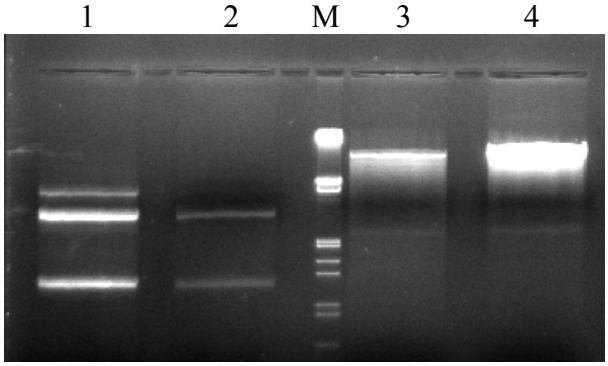


Fig.4.36. Restriction digestion of InsT/A ACS4, pCAMBIA 1300-PolyA –P and PS with *Kpn*I/*BamH*I

Lane 1: InsT/A-ACS4 plasmid DNA digested with *Kpn*I/*BamH*I
Lane 2: InsT/A-ACS5 plasmid DNA digested with *Kpn*I/*BamH*I
Lane 3: pCAMBIA 1300-PolyA –P plasmid DNA digested with *Kpn*I/*BamH*I
Lane 4: pCAMBIA 1300-PolyA –PS plasmid DNA digested with *Kpn*I/*BamH*I
Lane M: Lambda *Hind*III/*EcoR*I double digest DNA ladder

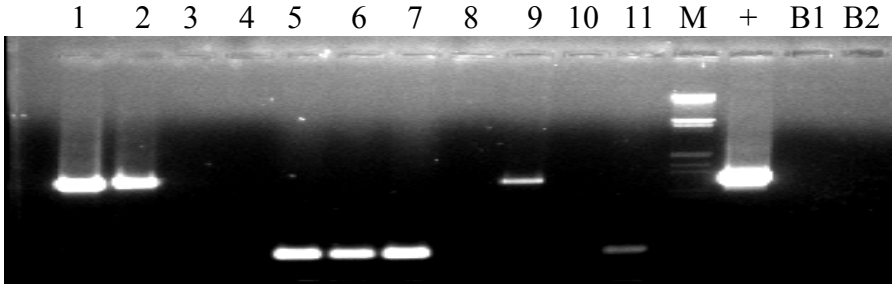


Fig.4.37. PCR of pCAMBIA 1300-ACS4-PolyA-P # 1-5 plasmid DNA with ACS4 FP/RP and PolyA FP/RP

Lane 1,2,3,4,9: PCR of pCAMBIA 1300-ACS4-PolyA-P # 1-5 using ACS4 FP/RP
Lane 5,6,7,8,11: PCR of pCAMBIA 1300-ACS4- PolyA-P # 1-5 using PolyA FP/RP
Lane + : PCR positive of ACS4 promoter fragment
Lane B1: PCR negative for ACS4 FP/RP
Lane B2: PCR negative for PolyA FP/RP
Lane M: Lambda *Hind*III/*EcoR*I double digest DNA ladder

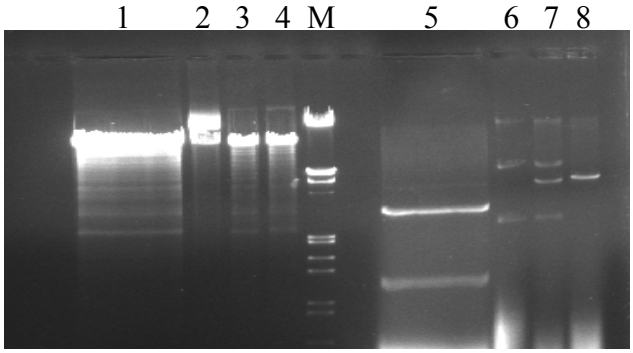


Fig.4.38. Restriction digestion of InsT/A ACS5, pCAMBIA 1300-PolyA PS with *Kpn*I/*BamH*I

Lane 1: InsT/A-ACS5 plasmid DNA digested with *Kpn*I/*BamH*I
Lane 2: InsT/A-ACS5 uncut plasmid DNA
Lane 3: InsT/A-ACS5 plasmid DNA digested with *Kpn*I
Lane 4: InsT/A-ACS5 plasmid DNA digested with *BamH*I
Lane 5: pCAMBIA 1300-PolA-PS digested with *Kpn*I/*BamH*I
Lane 6: pCAMBIA 1300-PolA-PS uncut plasmid DNA
Lane 7: pCAMBIA 1300-PolA-PS digested with *Kpn*I
Lane 8: pCAMBIA 1300-PolA-PS digested with *BamH*I
Lane M: Lambda *Hind*III/*EcoR*I double digest DNA ladder

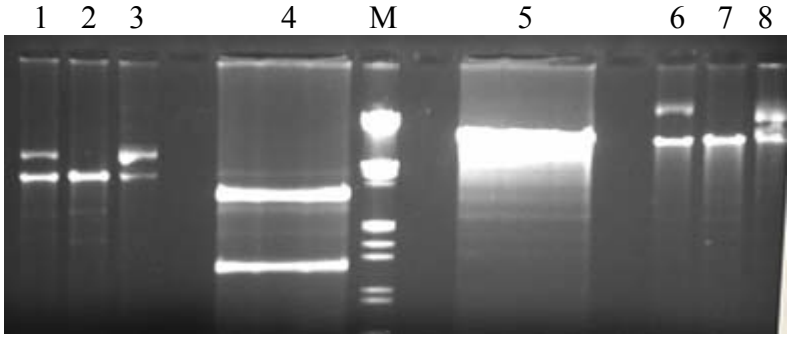


Fig.4.39. Restriction digestion of InsT/A –ACS7 and pCAMBIA 1300-PolyA-PE with *Kpn*I/*BamH*I

Lane 1: InsT/A –ACS7 plasmid DNA digested with *Kpn*I
Lane 2: InsT/A –ACS7 plasmid DNA digested with *BamH*I
Lane 3: InsT/A –ACS7 uncut plasmid DNA
Lane 4: InsT/A –ACS7 plasmid DNA digested with *Kpn*I/ *BamH*I
Lane 5: pCAMBIA 1300-PolyA-PE plasmid DNA digested with *Kpn*I
Lane 6: plasmid DNA digested with *BamH*I
Lane 3: pCAMBIA 1300-PolyA-PE uncut plasmid DNA

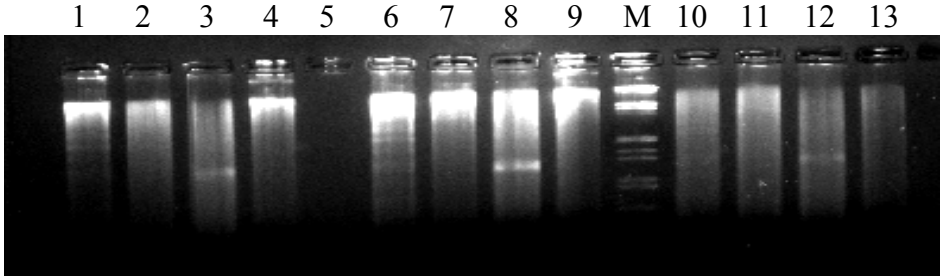


Fig.4.40. Restriction digestion confirmation of the pCAMBIA 1300-ACS4-PolyA-P #1,2 and 5 plasmid DNA
Lane 1: pCAMBIA 1300-ACS4-PolyA-P #1 using *Kpn*I
Lane 2: pCAMBIA 1300-ACS4-PolyA-P #1 using *BamH*I
Lane 3: pCAMBIA 1300-ACS4-PolyA-P #1 using *Kpn*I/*BamH*I
Lane 4: pCAMBIA 1300-ACS4-PolyA-P #1 uncut plasmid DNA
Lane 6: pCAMBIA 1300-ACS5-PolyA-PS #1 using *Kpn*I
Lane 7: pCAMBIA 1300-ACS5-PolyA-PS #1 using *BamH*I
Lane 8: pCAMBIA 1300-ACS5-PolyA-P S#1 using *Kpn*I/*BamH*I
Lane 9: pCAMBIA 1300-ACS5-PolyA-PS #1 uncut plasmid DNA
Lane 10: pCAMBIA 1300-ACS7-PolyA-PE #1 using *Kpn*I
Lane 11: pCAMBIA 1300-ACS7-PolyA-PE #1 using *BamH*I
Lane 12: pCAMBIA 1300-ACS7-PolyA-PE #1 using *Kpn*I/*BamH*I
Lane 13: pCAMBIA 1300-ACS7-PolyA-PE # 1 uncut plasmid DNA
Lane M: Lambda *Hind*III/*EcoR*I double digest DNA ladder

4.3.3 Isolation of the selected genes

4.33-a. Isolation of *Botrytis Induced Kinase I* gene from *Arabidopsis thaliana*

The *BIK1* gene of *Arabidopsis thaliana* is a kinase gene of 1188bp (Ac. no. At2G39660), with six exons and five introns. The isolation of this gene from *Arabidopsis* involved the amplification of six exons from the gDNA separately by PCR amplification (Fig.4.42) using primers specific to each exon. The six exons were then spliced in two sets to form the complete *BIK1* gene coding sequence by primer extension PCR.

The *BIK1* Exon I (49bp) was first amplified using Exon I specific AMV *BIK1* ExonI forward and *BIK1* ExonI reverse primers (Fig.4.41,43 and 44). The amplified *BIK1* Exon I fragment was then gel eluted, quantified and cloned into InsT/A vector through ligation at 22^{o}C, for 16hrs. The ligated DNA was then transferred into *E.coli*. For the confirmation of the recombinant vector carrying the *BIK1*-ExonI, plasmid DNA was isolated from the recombinant bacterial suspension

by alkaline lysis method. The plasmid DNA concentration and quality was checked by gel analysis. The plasmids were checked for the presence of *BIK1*-ExonI by PCR using exon specific forward and reverse primers (Fig.4.44). The size of the cloned *BIK1*-ExonI gene was confirmed by restriction digestion using *EcoRI* and *HindIII*. The orientation of the *BIK1*gene in the cloned vector was checked by PCR using *BIK1* gene specific forward and vector specific reverse primer and *vice versa*. The orientation of the Exon I was also confirmed by restriction digestion.

Secondly, Exon II (308bp) and III (136 bp) were amplified (Fig.4.45) and the two fragments were denatured at 95^{o}/4min and immediately annealed at 4^{o}C. The annealed product was used as a template to amplify both the exons together (444bp). The Exons II+III fragment was cloned in InsT/A vector and sequenced. The confirmed sequences of both ExonI and II+III were then amplified using PR Polymerase, denatured and annealed to obtain Exon I+II+III fragment (548bp) which was then cloned in pJET cloning vector and two positive clones in each case were sequenced to check for any mutations in the annealed product. *BIK1*gene Exon IV (143bp), V (124 bp) and VI (428 bp) fragments were amplified separately using primers specific to these Exons and gDNA of *Arabidopsis* as the template (Fig.4.46). Exon IV amplified fragment was cloned in InsT/A vector and confirmed through sequencing the positive clones. The fragments of Exon V and VI were denatured, annealed, amplified and cloned in InsT/A vector and was confirmed by sequencing. The confirmed sequences of both ExonIV and V+VI (552 bp) were then amplified using PR Polymerase then denatured and annealed to obtain Exon IV+V+VI fragment (695 bp) which was then cloned in pJET cloning vector and two positive clones in each case were sequenced to check for any mutations in the annealed product.

The Exon I+II+III (548 bp) and IV+V+VI (695 bp) were amplified from their respective pJET plasmid vectors using Exon I FP/ Exon III RP and Exon IV FP/ Exon VI RP respectively (Fig.4.47) using KOD polymerase. The amplified products of I+II+III (548 bp) and IV+V+VI (695 bp) were denatured, annealed, amplified using *BIK1* ExonI FP and Exon VI RP (Fig.4.48) and cloned in InsT/A vector. Fourteen colonies were subjected to colony PCR to check for the presence of the full length *BIK1* gene fragment and all of them were positive with an amplicon of 1188bp (Fig.4.49). Ten positive clones were also confirmed by restriction digestion using

*BamH*I/*Sal*I out of which only four clones (#2,8,9 and10) released the expected 1188bp *BIK1* gene fragment (Fig.4.50). The positive clones were confirmed through sequencing using M13 FP/RP and Exon III FP and Exon IV RP to span the entire *BIK1* gene (AMV+1188bp) fragment. The sequence was analyzed using CHROMAS PRO software to check for the presence of insertions or deletions in the gene sequence.

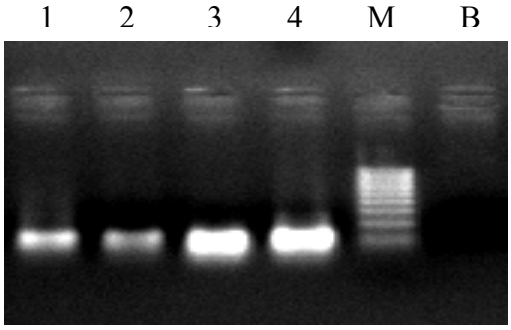


Fig 4.41. PCR amplification of *BIK1* –ExonI using *BIK1*-ExonI specific FP/RP
Lane 1-4: PCR of *BIK1* –ExonI using *BIK1*-ExonI specific FP/RP
Lane B: PCR Blank
Lane M: 100bp DNA ladder

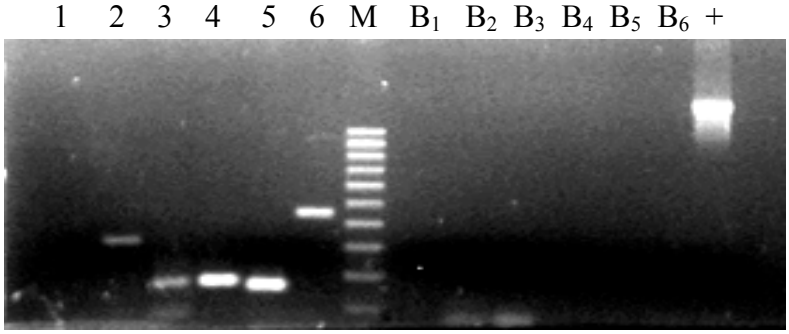


Fig.4.42.PCR amplification of Exon II-VI using Exon specific FP/RP and *Arabidopsis* gDNA as template
Lane 1**:** PCR of *BIK1* ExonI using ExonI FP/RP (faint band)
Lane 1: PCR of *BIK1* ExonII using ExonII FP/RP
Lane 2: PCR of *BIK1* ExonIII using ExonIII FP/RP
Lane 3: PCR of *BIK1* ExonIV using Exon IV FP/RP
Lane 4: PCR of *BIK1* ExonV using ExonV FP/RP
Lane 5: PCR of *BIK1* ExonVI using ExonVI FP/RP
Lane 6-10: PCR Blank of Exon II-VI primers
Lane 11: PCR positive
Lane M: 100bp DNA ladder

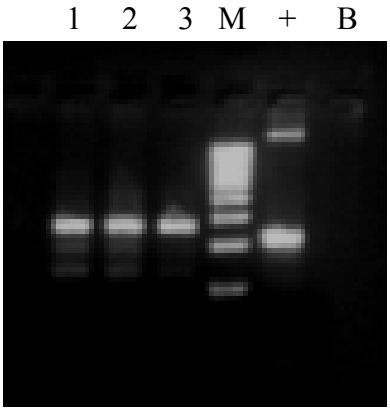


Fig4.43. PCR of InsT/A-*BIK1* Exon I clones#1-3 using ExonI FP/RP
Lane 1-3: PCR of InsT/A-*BIK1* Exon I clones#1-3 using ExonI FP/RP
Lane +: PCR Positive
Lane B: PCR Blank
Lane M: 100bp DNA ladder

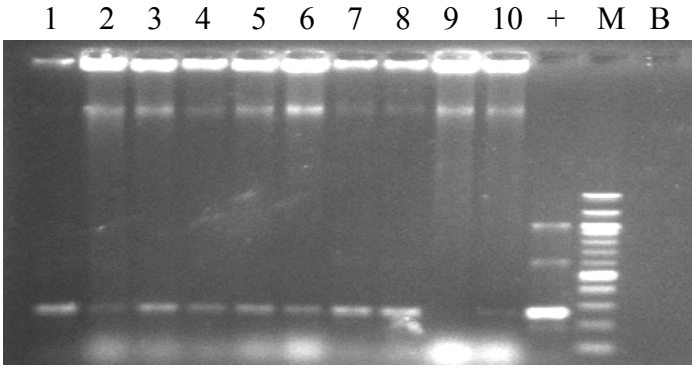


Fig.4.44.Colony PCR of *BIK1* ExonI colonies #1-10 using Exon I FP/RP
Lane 1-10: PCR of *BIK1* ExonI colonies #1-10 using Exon I FP/RP
Lane +: PCR ositive
Lane B: PCR Blank
Lane M: 100bp DNA ladder

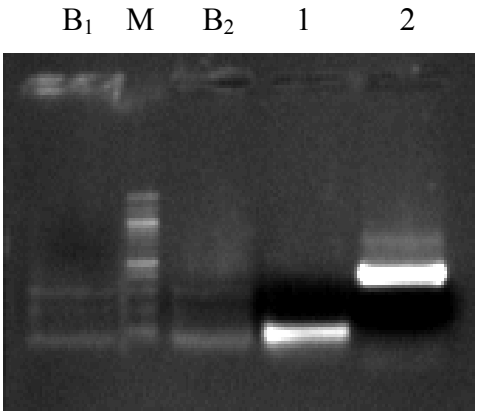


Fig.4.45.Large volume PCR of *BIK1* ExonI and Exon(II+III)
Lane 1: PCR of *BIK1* Exon I using ExonI elute as the template and ExonI FP/RP for amplification
Lane 2: PCR of *BIK1* Exon II+III using Exon II+III elutes as the template and ExonII FP and Exon III RP for amplification
Lane B_1: PCR Blank of Exon I FP/RP
Lane B_2: PCR Blank of Exon II FP/ Exon III RP
Lane M: 100bp DNA ladder

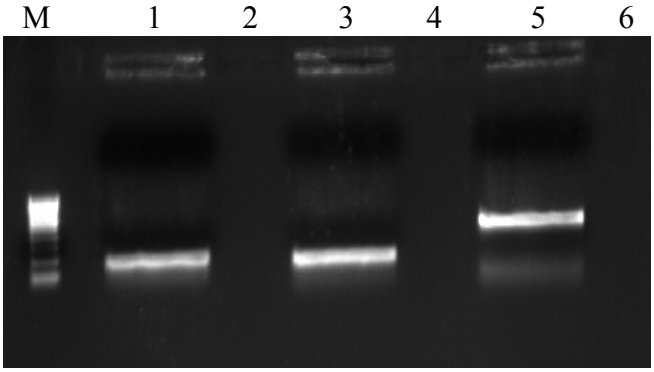


Fig.4.46.Large volume PCR of *BIK1* Exon IV, V, VI
Lane 1: PCR of *BIK1* Exon IV using Exon IV FP/RP
Lane 2: PCR Blank of Exon IV FP/RP
Lane 3: PCR of *BIK1* Exon V using Exon V FP/RP
Lane 4: PCR Blank of Exon V FP/RP
Lane 5: PCR of *BIK1* Exon VI using Exon VI FP/RP
Lane 6: PCR Blank of Exon VI FP/RP
Lane M: 100bp DNA ladder

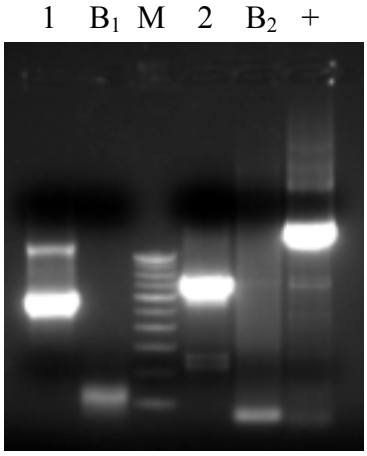


Fig .4.47.Primer extension PCR of *BIK1* Exon I+II+III and Exon IV+V+VI for cloning into pJET cloning vector
Lane 1: PCR of *BIK1* Exon I+II+III using Exon I FP/ Exon III RP and Exon I+II+III annealed elutes as template
Lane 2: PCR of *BIK1* Exon IV+V+VI using Exon IV FP/ Exon VI RP and Exons IV+V+VI annealed elutes as template
Lane +: PCR positive
Lane B_1: PCR Blank of Exon I FP/ Exon III RP
Lane B_2: PCR Blank of Exon IV FP/ Exon VI RP
Lane M: 100b DNA ladder

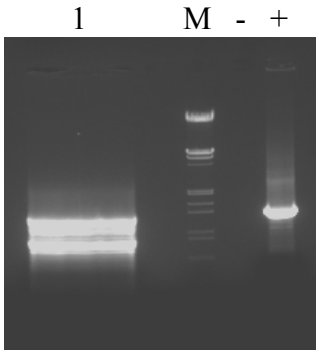


Fig.4.48. Large volume PCR of *BIK1* gene (Exon I+II+III+IV+V+VI) using ExonI FP/ Exon VI RP for cloning into pJET 1.2 cloning vector
Lane 1: PCR of *BIK1* gene (Exon I+II+III+IV+V+VI) using ExonI FP/ Exon VI RP
Lane -: PCR negative
Lane +: PCR positive
Lane M: Lambda *Hind*III/*Eco*RI double digest DNA ladder

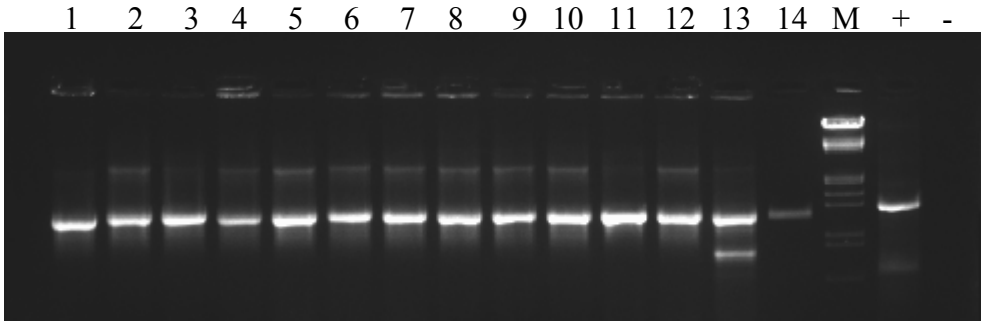


Fig.4.49. Colony PCR of *BIK1* Clones#1-14 using *BIK1* Exon I FP and Exon VI RP
Lane 1-14: PCR of clones #1-14 using *BIK1* Exon I FP and Exon VI RP
Lane +: PCR positive
Lane -: PCR negative
Lane M: Lambda *Hind*III/*Eco*RI double digest DNA ladder

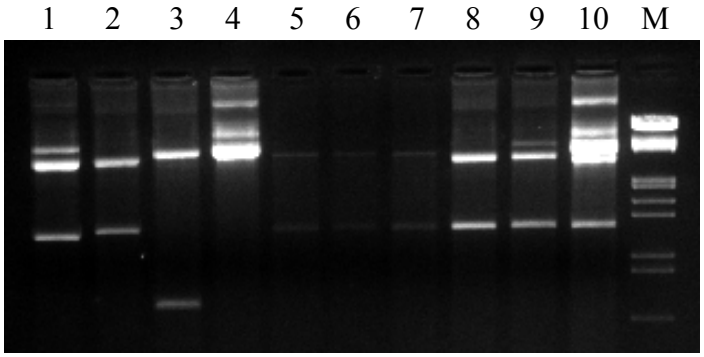


Fig.4.50. Restriction digestion of pJET-*BIK1* #1-10 plasmid DNA using *BamH*I/*Sal*I
Lane 1-10: pJET-*BIK1* #1-10 plasmid DNA digested using *BamH*I/*Sal*I
Lane M: Lambda *Hind*III/*Eco*RI double digest DNA ladder

4.33-b. Isolation of *Ethylene Responsive Factor I (ERF1)* gene from *Arabidopsis thaliana:*

The *Ethylene Responsive Factor I* is a defense regulatory gene of 650bp in *Arabidopsis* (Ac. no. At3G23240). This gene has no introns in the coding region and hence was isolated directly from the gDNA of *Arabidopsis* using PCR. The isolation of *ERF1* gene was achieved by PCR amplification of the gDNA using the gene specific forward and reverse primers. The amplified *ERF1* gene was then gel eluted, quantified and ligated into InsT/A vector. *E.coli* was transformed with the ligation mix and the transformants were selected. The lines were confirmed using PCRs and restriction analysis carried with different restriction digestion (Fig.4.51 and 4.52).

Five clones showing positive for the *ERF1* gene were sequenced using M13 forward and reverse primers and the sequence was analyzed using CHROMAS PRO software to check for the presence of insertions or deletions in the gene sequence. The *ERF1* gene was cloned downstream of *ACS5* promoter as a *BamH*I-*Sal*I fragment in pCAMBIA 1300-*ACS5*-PolyA-PS binary vector.

4.33-c. Isolation of *Ethylene Responsive Element Binding Protein (AtEBP)* gene from *Arabidopsis thaliana*

The *AtEBP* gene is a 747 bp codong region (Ac. No. At3G16700). The *AtEBP* gene contains two exons (160bp and 587bp) with one interveining intron. The isolation of the *AtEBP* gene was achieved by amplifying the two exons separately by PCR using primers specific to both the exons using gDNA as the template. The obtained amplicons of each exon I and II was then denatured and annealed to achieve the complete coding fragment of *AtEBP* gene by primer extension PCR (Fig.4.53-4.55). The amplified *AtEBP* gene was then gel eluted, quantified and cloned into InsT/A vector. *E.coli* transformants were identified on a selection plate and plasmid DNA was isolated from the clones. The plasmids were checked for the presence of *AtEBP* gene by PCR using gene specific forward and reverse primers. The size of the cloned *AtEBP* gene was confirmed by restriction digestion using *EcoRI* and *HindIII* (Fig.4.56 and 57).The orientation of the *AtEBP* gene in the cloned vector was checked by PCR using *AtEBP* gene specific forward and vector specific reverse primer and vice versa. The orientation of the *AtEBP* gene was also checked by restriction digestion using different combinations of enzymes.

Five clones positive for the *AtEBP* gene were sequenced using M13 forward and reverse primers and the sequence was analyzed using software CHROMAS PRO for the presence of insertions or deletions in the gene sequence. The *AtEBP* gene was cloned downstream of *ACS7* promoter as a *BamH*I-*Sal*I fragment in pCAMBIA 1300-*ACS7*-PolyA-PE binary vector.

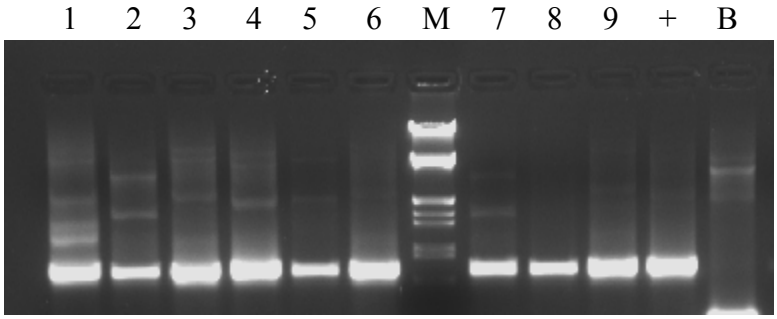


Fig.4.51. PCR amplification of *ERF1* clones #1-9 using *ERF1* FP/RP
Lane 1-9: *ERF1* clone #1-9 PCR using *ERF1* gene FP/RP
Lane +: PCR positive
Lane B: PCR blank
Lane M: Lambda *Hind*III/*Eco*RI double digest DNA ladder

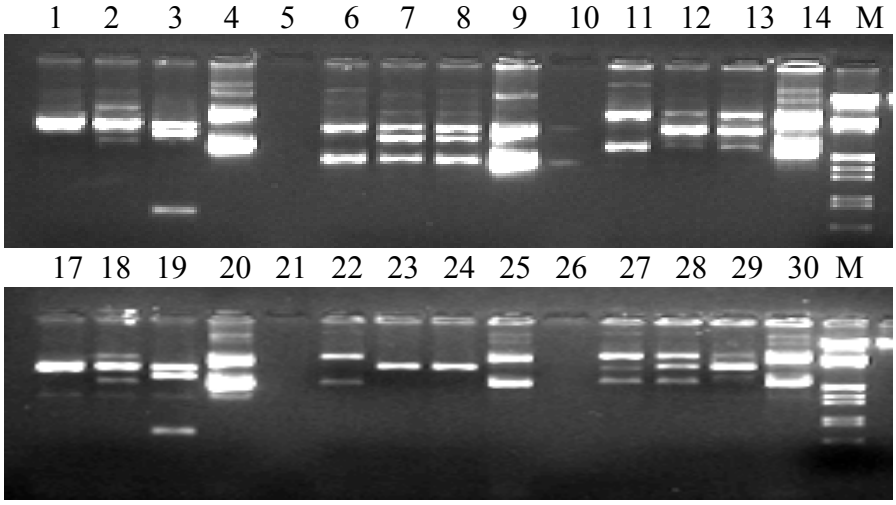


Fig.4.52.Restriction digestion confirmation of InsT/A *ERF1* plasmid of # 1-6
Lane 1,6,11,17,22,27: InsT/A *ERF1* plasmid of # 1-6 with *Bgl*II
Lane 2,7,12,18,23,28: InsT/A *ERF1* plasmid of # 1-6 with *Sal*I
Lane 3,8,13,19,24,29: InsT/A *ERF1* plasmid of # 1-6 with *Bgl*II/*Sal*I
Lane 4,9,14,20,25,30: InsT/A *ERF1* plasmid of # 1-6 with uncut plasmid DNA
Lane M: Lambda *Hind*III/*Eco*RI double digest DNA ladder

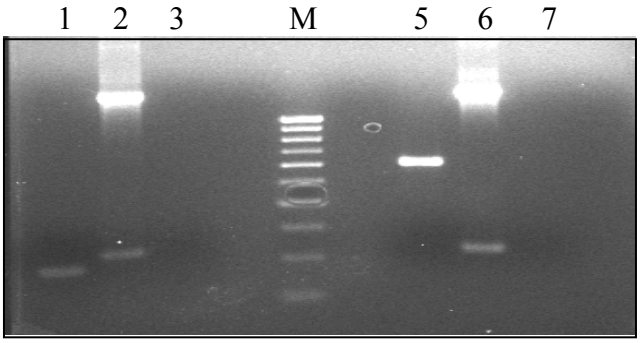


Fig.4.53. PCR amplification of ExonI and II of *AtEBP* gene
Lane 1: PCR of *AtEBP* ExonI (159 bp) using AMV-*AtEBP* Exon I FP and RP using *Arabidopsis* gDNA as a template
Lane 5: PCR of *AtEBP* ExonII (586bp) using *AtEBP* Exon II-FP and RP using *Arabidopsis* gDNA as a template
Lane 2,6: PCR positive
Lane 3,7: PCR negative using *AtEBP* ExonI and II specific primers
Lane M: 100bp DNA ladder

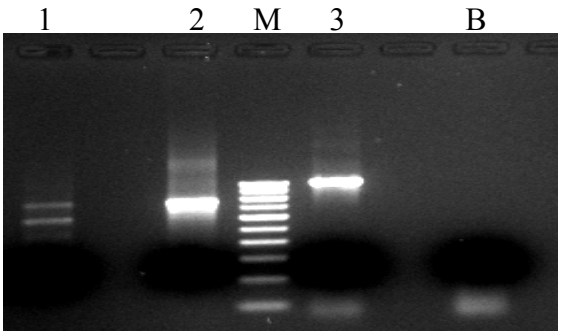


Fig.4.54. Primer extension PCR of *AtEBP* ExonI+ExonII annealed fragments using AMV-*AtEBP* ExonI-FP/ *AtEBP* ExonII-RP
Lane 1: PCR of *AtEBP* ExonI + II annealed fragments without primers
Lane 2: PCR of *AtEBP* ExonI + II annealed fragments with AMV-*AtEBP* ExonI-FP/ *AtEBP* ExonII-RP
Lane 3: PCR of *Arabidopsis* gDNA using AMV-*AtEBP* ExonI-FP/ *AtEBP* ExonII-RP
Lane B: PCR Blank of AMV-*AtEBP* ExonI-FP/ *AtEBP* ExonII-RP
Lane M: 100bp DNA ladder

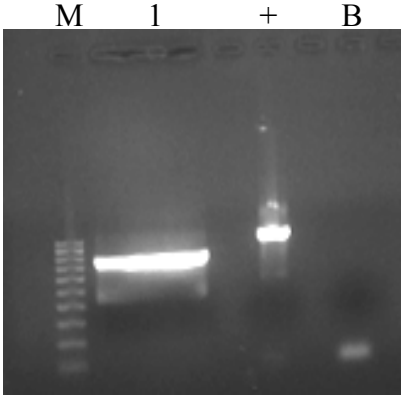


Fig.4.54a. Large volume PCR of *AtEBP* gene for elution and cloning in InsT/A vector
Lane 1: PCR of *AtEBP* ExonI + II annealed fragments with AMV-*AtEBP* ExonI-FP/ *AtEBP* ExonII-RP
Lane +: PCR positive
Lane -: PCR Blank
Lane M:100bp DNA ladder

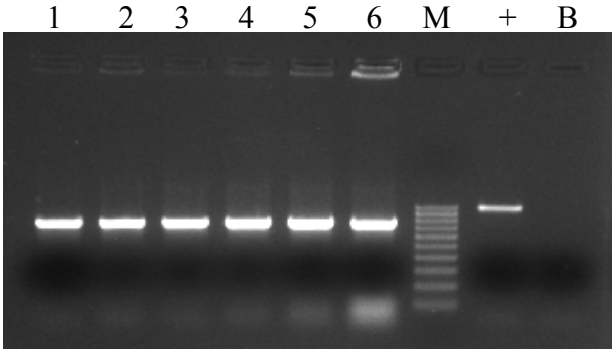


Fig.4.55. PCR check of InsT/A-*AtEBP* clones #1-6 plasmid DNA with *AtEBP* FP/RP
Lane 1-6: PCR of InsT/A-*AtEBP* clones #1-6 plasmid DNA with *AtEBP* FP/RP
Lane +: PCR positive
Lane B: PCR Blank
Lane M: 100bp DNA ladder

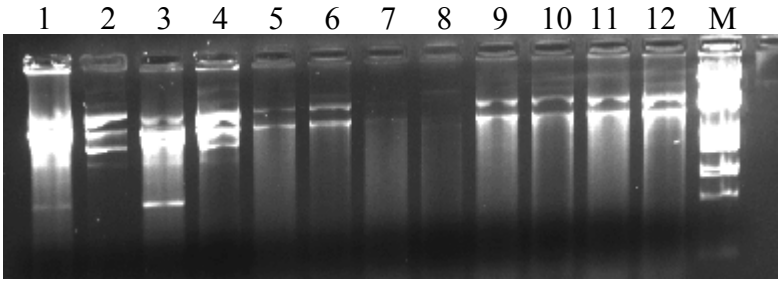


Fig.4.56. Restriction digestion of InsT/A-*AtEBP* #1-6 plasmid DNA using *HindIII/EcoRI*
Lane 1,3,5,7,9,11: InsT/A-*AtEBP* #1-6 plasmid DNA digested with H*indIII/EcoRI*
Lane 2,4,6,8,10,12: InsT/A-*AtEBP* #1-6 uncut plasmid DNA
Lane M: Lambda *Hind*III/*Eco*RI double digest DNA ladder

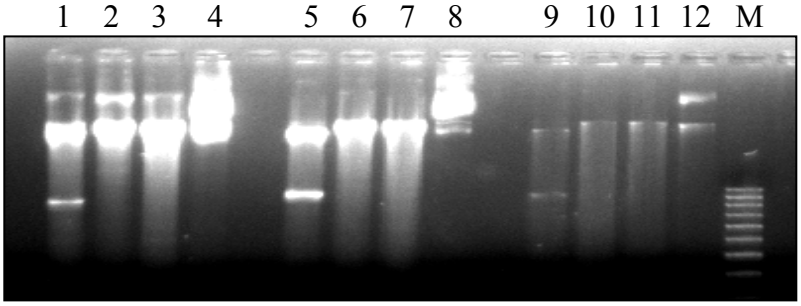


Fig.4.57. Restriction digestion of InsT/A-*AtEBP* #1-3 with *BamH*I/*Sal*I
Lane 1,5,9: InsT/A-*AtEBP* #1-3 plasmid DNA digested with *BamH*I/*Sal*I
Lane 2,6,10: InsT/A-*AtEBP* #1-3 plasmid DNA digested with *BamH*I
Lane 3,7,11: InsT/A-*AtEBP* #1-3 plasmid DNA digested with *Sal*I
Lane 4,8,12: InsT/A-*AtEBP* #1-3 uncut plasmid DNA
Lane M: 100bp DNA ladder

4.3.4. Development of the gene cassettes *ACS4*-*BIK1*-polyA, *ACS7*-*AtEBP*-polyA and *ACS5*-*ERF1*-polyA

The promoters *ACS4, 5* and *7*, genes *BIK1, ERF1* and *AtEBP*, terminators PolyA-P, PolyA-PS and PolyA-PE were assembled together by cloning into the multiple cloning site (MCS) of pCAMBIA 1300 binary vector.

First, the PolyA terminator fragments isolated from InsT/A-PolyA vector through PCR using primers specific for PolyA fragment including flanking restriction enzymes at their 3` end with *Pst*I (P) for use in PolyA-RP of *BIK*1 cassette, *Pst*I-*Sac*I (PS) for use in the PolyA-RP of *ERF1* gene cassette and *Pst*I-*EcoR*I (PE) for use in the PolyA-RP of *AtEBP* gene cassette and were confirmed through sequencing. These fragments were cloned in the MCS of pCAMBIA 1300 as a *Sal*I-*Pst*I fragment to form pCAMBIA 1300-PolyA-P, pCAMBIA 1300- PolyA-PS, pCAMBIA 1300- PolyA-PE respectively. The pCAMBIA 1300-PolyA vectors were confirmed both by PCR and restriction digestion.

Second, the promoter sequences *ACS4, 5* and *7* were isolated through PCR from the gDNA of *Arabidopsis thaliana* using forward primer flanked with *Kpn*I restriction enzyme site at the 3` end and reverse primer flanked with *BamH*I restriction enzyme site at its 3` end. The isolated promoter sequences were cloned as *Kpn*I-*BamH*I fragment in the MCS of pCAMBIA 1300-PolyA-P, pCAMBIA1300-PolyA-PS and pCAMBIA 1300- PolyA-PE respectively to form pCAMBIA-*ACS4*-PolyA-P, pCAMBIA-*ACS5*-PolyA-PS and pCAMBIA-*ACS7*-PolyA-PE. These vectors were confirmed both by PCR and restriction digestion.

Thirdly, the gene sequences of *BIK1, ERF1* and *AtEBP* which were isolated from *Arabidopsis thaliana* and cloned in the InsT/A vector were flanked with *BamH*I restriction site and AMV enhancer sequence in their 5`end and *Sal*I at their 3` end using PCR. The genes AMV-*BIK1,* AMV-*ERF1* and AMV-*AtEBP* were cloned downstream to the promoters *ACS4*, *ACS5* and *ACS7* respectively as *BamH*I-*Sal*I fragment in the developed vectors pCAMBIA-*ACS4*-PolyA-P, pCAMBIA-*ACS5*-PolyA-PS and pCAMBIA-*ACS7*-PolyA-PE thereby leading to the formation of three individual gene cassettes ***ACS4*-*BIK1*-polyA-P, *ACS4*-*ERF1*-polyA-PS and *ACS4*-**

***AtEBP*-polyA-PE.** These developed individual gene cassettes were confirmed both by PCR and restriction digestion (Fig.4.58-67).

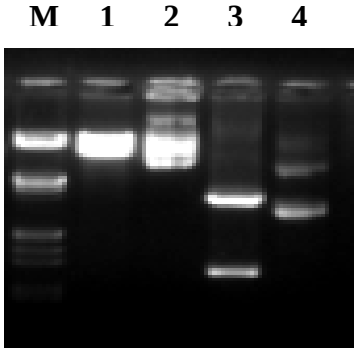


Fig.4.58. Restriction digestion of ACS4-PolyA-P#1 and pJET-*BIK1*#2 using *BamH*I/*Sal*I
Lane 1: ACS4-PolyA-P#1 digested with *BamH*I/*Sal*I
Lane 2: ACS4-PolyA-P#1 uncut plasmid DNA
Lane 3: pJET-*BIK1*#2 digested with *BamH*I/*Sal*I
Lane 4: pJET-*BIK1*#2 uncut plasmid DNA
Lane M: Lambda *Hind*III/*EcoR*I double digest DNA ladder

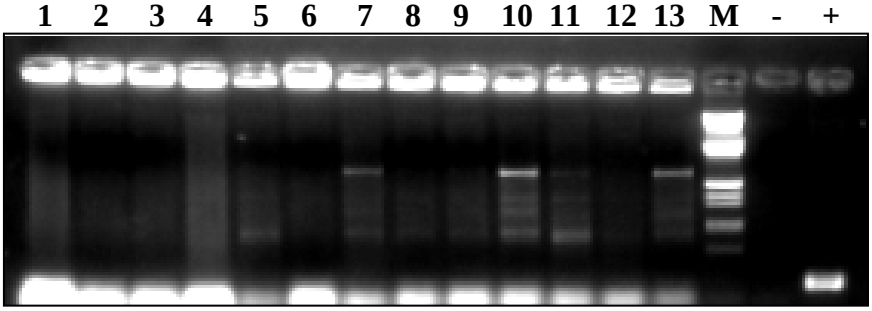


Fig.4.59. Colony PCR of ACS4-*BIK1*-PolyA-P #1-13 using ACS4 FP/ *BIK1*-ExonVI RP
Lane 1-13: PCR of ACS4-*BIK1*-PolyA-P #1-13 using ACS4 FP/ *BIK1*-ExonVI RP
Lane -: PCR negative
Lana +: PCR ppositive
Lane M: Lambda *Hind*III/*EcoR*I double digest DNA ladder

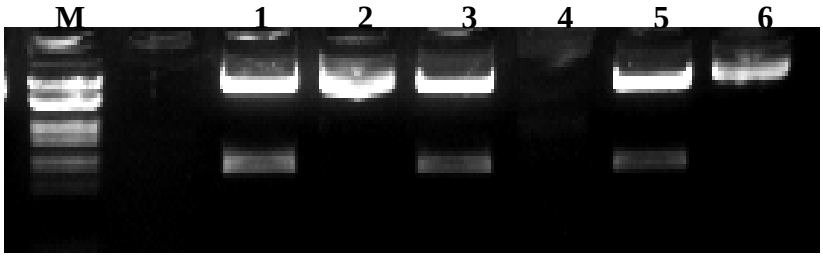


Fig.4.60. Restriction digestion of ACS4-*BIK1*-PolyA-P #7,10,13 using *BamH*I/*Sal*I

Lane 1,3,5: Restriction digestion of ACS4-*BIK1*-PolyA-P #7,10,13 using *BamH*I/*Sal*I

Lane 2,4,6: ACS4-*BIK1*-PolyA-P #7,10,13 uncut plasmid DNA

Lane M: Lambda *Hind*III/*EcoR*I double digest DNA ladder

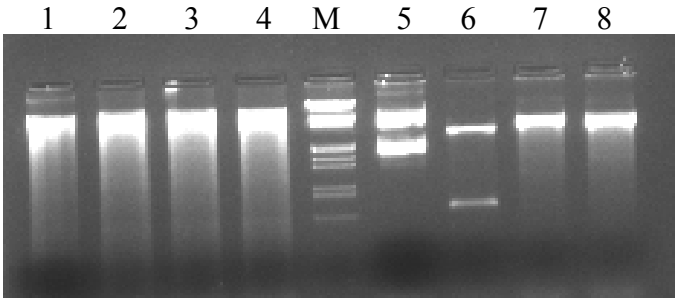


Fig.4.61. Restriction digestion pCAM 1300-ACS5-PS#1 and InsT/A-*ERF1*#1 for ligation

Lane 1: pCAM 1300-ACS5-PS#1 uncut plasmid DNA

Lane 2: pCAM 1300-ACS5-PS#1 digested with *BamH*I/*Sal*I

Lane 3: pCAM 1300-ACS5-PS#1 digested with *BamH*I

Lane 4: pCAM 1300-ACS5-PS#1 digested with *Sal*I

Lane 5: InsT/A-*ERF1*#1 uncut plasmid DNA

Lane 6: InsT/A-*ERF1*#1 digested with *BamH*I/*Sal*I

Lane 7: InsT/A-*ERF1*#1 digested with *BamH*I

Lane 8: InsT/A-*ERF1*#1 digested with *Sal*I

Lane M: Lambda *Hind*III/*EcoR*I double digest DNA ladder

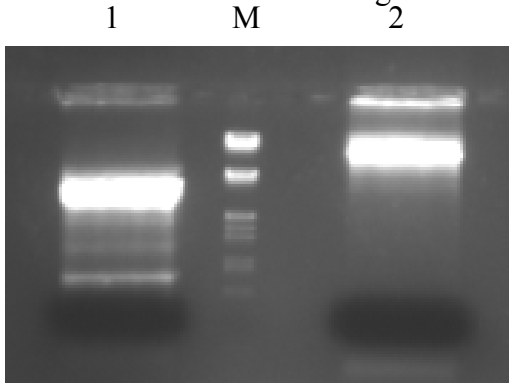


Fig.4.62. Restriction digestion pCAM 1300-ACS5-PS#1 and InsT/A-*ERF1*#1 for ligation

Lane 1: InsT/A-*ERF1*#1 digested with *BamH*I/*Sal*I

Lane 2: pCAM 1300-ACS5-PS#1 digested with *BamH*I/*Sal*I

Lane M: Lambda *Hind*III/*EcoR*I double digest DNA ladder

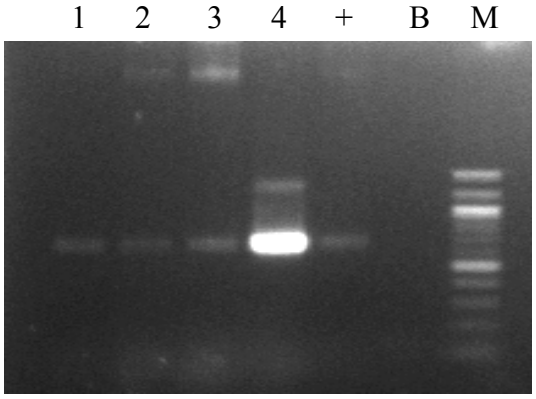


Fig.4.63.Colony PCR of CAMBIA 1300-ACS5-AMV-*ERF1*-PS colony# 1-4 using *ERF1* FP/RP
Lane 1-4 : PCR of CAMBIA 1300-ACS5-AMV-*ERF1*-PS colony# 1-4 using *ERF1* FP/RP
Lane +: PCR positive
Lane B: PCR Blank
Lane M: 100bp DNA ladder

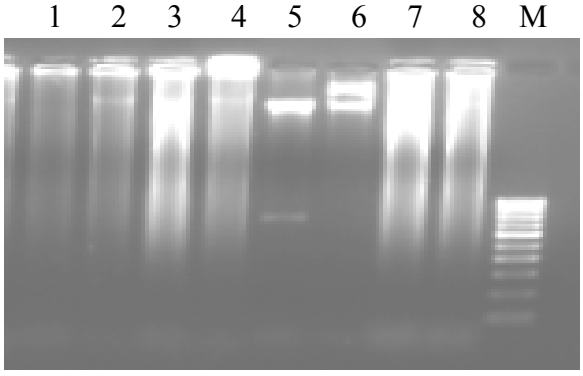


Fig. 4.64. Restriction digestion of pCAMBIA 1300-ACS5-AMV-*ERF1*-PS colony# 1-4 using *Bgl*II//*Sal*I
Lane 1,3,5,7: pCAMBIA 1300-ACS5-AMV-*ERF1*-PS colony# 1-4 using *Bgl*II//*Sal*I
Lane 2,4,6,8: pCAMBIA 1300-ACS5-AMV-*ERF1*-PS colony# 1-4 uncut plasmid DNA
Lane M: 100b DNA ladder

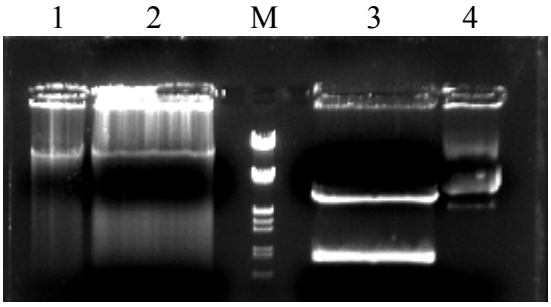


Fig.4.65. Large volume restriction digestion of pCAMBIA 1300-ACS7-PolyA-PE and InsT/A-*AtEBP* using *BamH*I/*Sal*I
Lane 1: pCAMBIA 1300-ACS7-PE uncut plasmid DNA
Lane 2: pCAMBIA 1300-ACS7-PE digested with *BamH*I/*Sal*I
Lane 3: InsT/A-*AtEBP* digested with *BamH*I/*Sal*I
Lane 4: InsT/A-*AtEBP* uncut plasmid DNA
Lane M: Lambda *Hind*III/*Eco*RI double digest DNA ladder.

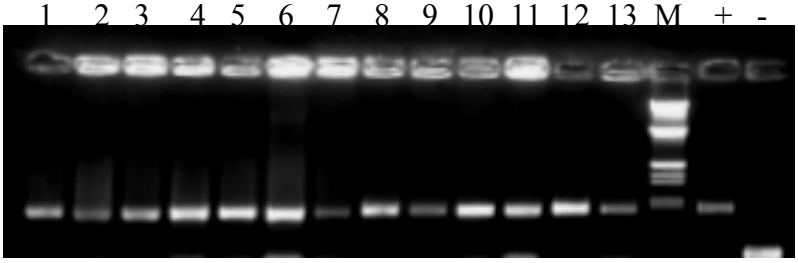


Fig.4.66. PCR confirmation of ACS7-AMV-*AtEBP*-PolyA-PE#1-13 using *AtEBP* FP/RP
Lane 1-13: PCR confirmation of **ACS7-AMV-*AtEBP*-PolyA-PE#1-13 using *AtEBP* FP/RP**
Lane +: PCR positive
Lane -: PCR negative
Lane M: Lambda *Hind*III/*Eco*RI double digest DNA ladder.

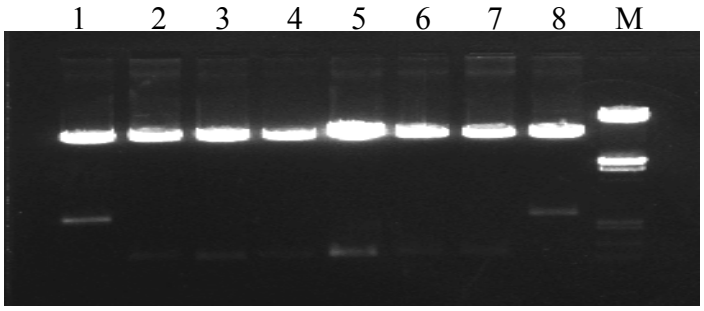


Fig.4.67. Restriction digestion of ACS7-AMV-*AtEBP*-PolyA-PE #1-8 with *EcoR*I
Lane 1-8: ACS7-AMV-*AtEBP*-PE #1-8 digested with *Eco*RI
Lane M: Lambda *Hind*III/*Eco*RI double digest DNA ladder.

4.3.5. *Agrobacterium* transformation with the developed individual gene cassettes

The confirmed individual gene cassette vectors pCAMBIA 1300- ***ACS4-BIK1*-polyA-P, *ACS5-ERF1*-polyA-PS** and ***ACS7-AtEBP*-polyA-PE** were mobilized into *Agrobacterium tumefaciens* strain LBA4404 by freeze-thaw method of transformation (Dityatkin., *et al*. (1972). The *Agrobacterium* transformants were selected on a YEP agar medium containing Rifampicin (25mg/L), Streptomycin (25mg/L) and Kanamycin (50mg/L). The *Agrobacterium* tranformants were checked for the presence of all three gene cassettes *ACS4-BIK1*-polyA-P, *ACS5-ERF1*-polyA-PS and *ACS7-AtEBP*-polyA-PE in the T-DNA of the binary vector pCAMBIA 1300 by colony PCR (Fig4.68, 69 and 4.72-75).

To further confirm the individual gene cassettes by restriction digestions, plasmid DNA were isolated from PCR positive clones of *Agrobacterium* and were retransferred into *E.coli*. The *E.coli* transformants obtained in the kanamycin selection plates were screened for the presence of the individual gene cassettes by colony PCR. PCR positive Plasmid DNA was isolated from *E.coli* clones and subjected to restriction digestions with different combinations of restriction enzymes to confirm the presence of the individual gene cassettes (Fig.4.70, 71 and4.76).

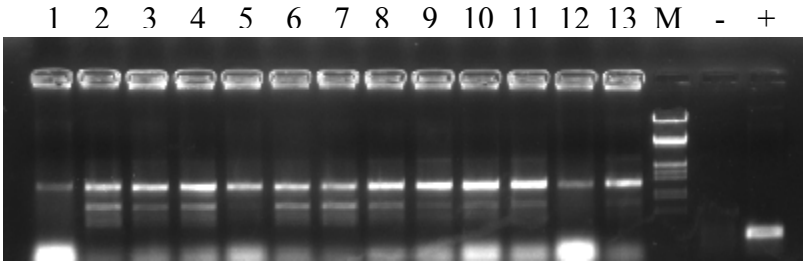


Fig 4.68.Colony PCR of LBA4404 colonies of pCAMBIA 1300- ACS4-*BIK1*-polyA-P using *BIK1* FP/RP
Lane 1-13: PCR of LBA4404 colonies of pCAMBIA 1300- ACS4-*BIK1*-polyA-P using BIK1 FP/RP
Lane -: PCR negative
Lane +: PCR positive
Lane M: Lambda *Hind*III/*Eco*RI double digest DNA ladder.

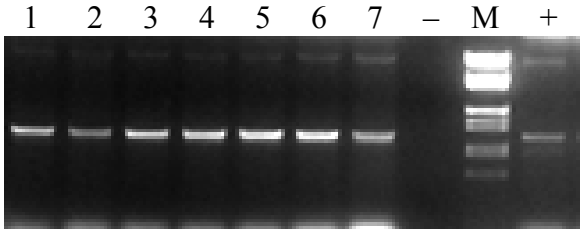


Fig.4.69. PCR check of LBA4404- pCAMBIA 1300- ACS4-*BIK1*-polyA-P #1-7 using ACS4 FP/RP
Lane 1-7: PCR check of LBA4404- pCAMBIA 1300- ACS4-*BIK1*-polyA-P #1-7 using ACS4 FP/RP
Lane -: PCR negative
Lane +: PCR positive
Lane M: Lambda *Hind*III/*Eco*RI double digest DNA ladder.

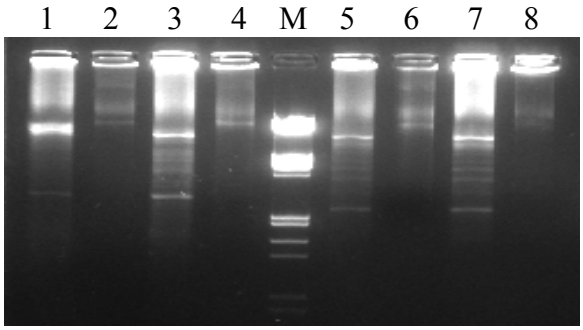


Fig 4.70. Restriction digestion of LBA4404- pCAMBIA 1300- ACS4-*BIK1*-polyA-P #1,2 and LBA4404- pCAMBIA- ACS5-*ERF1*-polyA-PS # 1,2 using *Kpn*I/*Sal*I
Lane 1,3: LBA4404- pCAMBIA 1300- ACS4-*BIK1*-polyA-P #1,2 digested with *Kpn*I/*Sal*I
Lane 2,4: LBA4404- pCAMBIA 1300- ACS4-*BIK1*-polyA-P #1,2 uncut plasmid DNA
Lane 5,7: LBA4404- pCAMBIA- ACS5-*ERF1*-polyA-PS # 1,2 digested using *Kpn*I/*Sal*I
Lane 6,8: LBA4404- pCAMBIA- ACS5-*ERF1*-polyA-PS # 1,2 uncut plasmid DNA
Lane M: Lambda *Hind*III/*Eco*RI double digest DNA ladder.

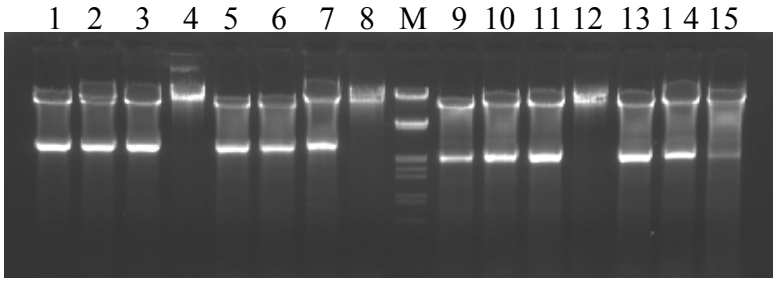


Fig 4.71. Restriction digestion of reverse mated clones of LBA4404- pCAMBIA 1300-ACS4-*BIK1*-polyA-P #1,2 and LBA4404- pCAMBIA- ACS5-*ERF1*-polyA-PS # 1,2 using *Kpn*I/*Sal*I

Lane 1,2,3: Reverse mated clones #1,2,3 transformed with LBA4404- pCAMBIA 1300-ACS4-*BIK1*-polyA-P #1 digested with *Kpn*I/*Sal*I

Lane 5,6,7: Reverse mated clones #1,2,3 transformed with LBA4404- pCAMBIA 1300-ACS4-*BIK1*-polyA-P #2 digested with *Kpn*I/*Sal*I

Lane 9,10,11: Reverse mated clones #1,2,3 ransformed with LBA4404- pCAMBIA-ACS5-*ERF1*-polyA-PS #1 digested with *Kpn*I/*Sal*I

Lane 13,14,15: Reverse mated clones #1,2,3 ransformed with LBA4404- pCAMBIA-ACS5-*ERF1*-polyA-PS #2 digested with *Kpn*I/*Sal*I

Lane 4,8,12: Uncut plasmid DNA

Lane M: Lambda *Hind*III/*Eco*RI double digest DNA ladder.

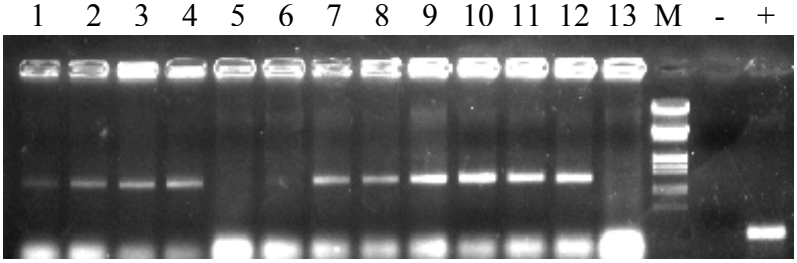


Fig.4.72. Colony PCR of LBA4404 colonies of pCAMBIA- ACS5-*ERF1*-polyA-PS using *ERF1* FP/RP

Lane 1-13: PCR of LBA4404 colonies of pCAMBIA- ACS5-*ERF1*-polyA-PS using *ERF1* FP/RP

Lane -: PCR negative

Lane +: PCR positive

Lane M: Lambda *Hind*III/*Eco*RI double digest DNA ladder.

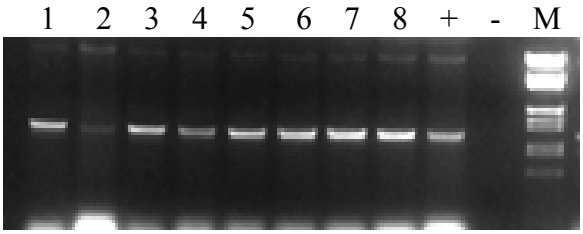


Fig.4.73. PCR check of LBA4404- pCAMBIA 1300- ACS5-*ERF1*-polyA-PS #1-8 using ACS5 FP/RP
Lane 1-8: PCR check of LBA4404- pCAMBIA 1300- ACS5-*ERF1*-polyA-PS #1-8 using ACS5 FP/RP
Lane -: PCR negative
Lane +: PCR positive
Lane M: Lambda *Hind*III/*Eco*RI double digest DNA ladder.

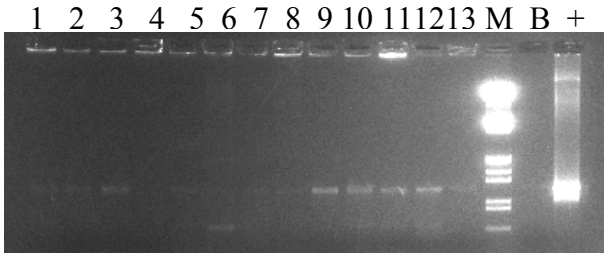


Fig.4.74. Colony PCR of LBA4404 colonies of pCAMBIA- ACS7-*AtEBP*-polyA-PE using *ACS7* FP/RP
Lane 1-13: PCR of LBA4404 colonies of pCAMBIA- ACS7-*AtEBP*-polyA-PE using ACS7 FP/RP
Lane B: PCR blank
Lane +: PCR positive
Lane M: Lambda *Hind*III/*Eco*RI double digest DNA ladder

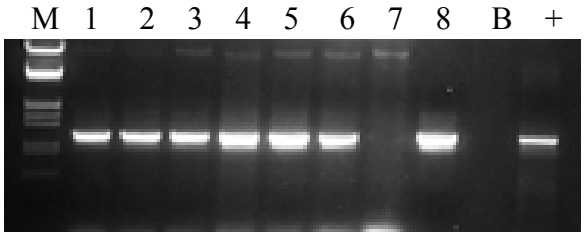


Fig.4.75. PCR check of LBA4404- pCAMBIA 1300- ACS7-*AtEBP*-polyA-PE #1-8 using ACS7 FP/RP

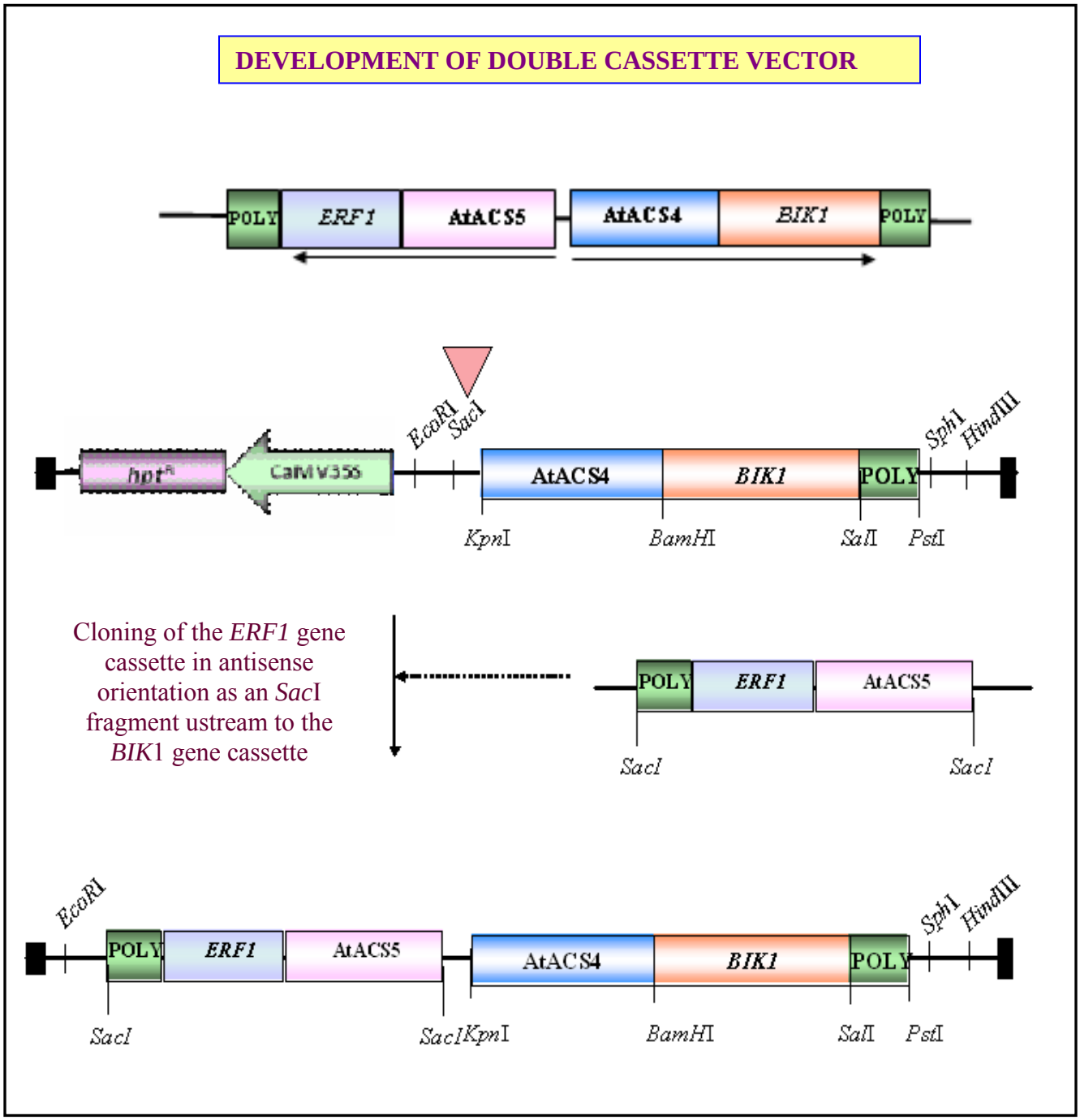
Lane 1-8: PCR check of LBA4404- pCAMBIA 1300- ACS7-*AtEBP*-polyA-PE #1-8 using ACS7 FP/RP

Lane B: PCR blank

Lane +: PCR positive

Lane M: Lambda *Hind*III/*Eco*RI double digest DNA ladder.

4.76. Schematic rereseentation of development of double gene cassette vector



4.4. Development of multiple cassette vector stacking the above three cassettes into one vector.

4.4.1. Development of Double Cassette *ACS5-ERF1*-PolyA-PS +*ACS4-BIK1*-PolyA-P vector in pCAMBIA 1300

The double cassette vector *ACS5-ERF1*-PolyA-PS +*ACS4-BIK1*-PolyA-P (referred as EB) was developed using *ERF1* and *BIK1* individual gene cassettes. The schematic representation of developing the double gene cassette vector is shown in Fig.4.77. The *AtEBP* gene cassette was not taken into consideration due to the anti apoptotic nature of the gene and unpredictable developmental defects in the plants over-expressing the gene. The *ACS5-ERF1*-PolyA-PS gene cassette of (2.2Kb) was cloned as a *SacI* fragment upstream to *ACS4-BIK1*-PolyA-P gene cassette in a divergent orientation in the binary vector pCAMBIA 1300-*ACS4-BIK1*-PolyA-P.

The ligated plasmid was transferred into *E.coli* by heat shock method and the transformants were selected on a selection plate containing Kanamycin (50mg/L). The positive colonies were identified by colony PCR (Fig.4.77-78) using primers specific for both *ERF1* and *BIK1* genes. The double cassette binary plasmid DNA was isolated from the positive clones of *E.coli* and was confirmed for the presence and divergent (cassettes in opposite) orientation of *ACS5-ERF1*-PolyA-PS cassette with respect to the *ACS4-BIK1*-PolyA-P gene cassette in the same T-DNA of the binary vector by PCR and restriction digestion (Fig.4.79-80). Plasmid DNA isolated from *E.coli* clones EB# 1, 3 and 6 were subjected to restriction digestions with different combinations of restriction enzymes to confirm the presence and orientation of the individual gene cassettes within the T-DNA (Fig.4.81). EB#3 was identified to harbor the two individual gene cassettes in divergent orientation.

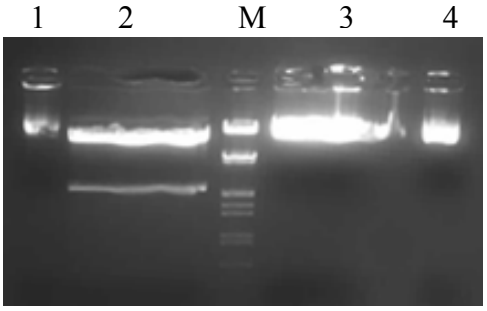


Fig.4.77.Large volume restriction digestion of ACS5-*ERF1*-PolyA-PS and ACS4-*BIK1*-PolyA-P with *EcoR*I

Lane 1: Uncut plasmid DNA of ACS5-*ERF1*-PolyA-PS
Lane 2: ACS5-*ERF1*-PolyA-PS digested with *EcoR*I
Lane 3: Uncut plasmid DNA of ACS4-*BIK1*-PolyA-P
Lane 4: ACS4-*BIK1*-PolyA-P digested with *EcoR*I
Lane M: Lambda *Hind*III/*EcoR*I double digest DNA ladder.

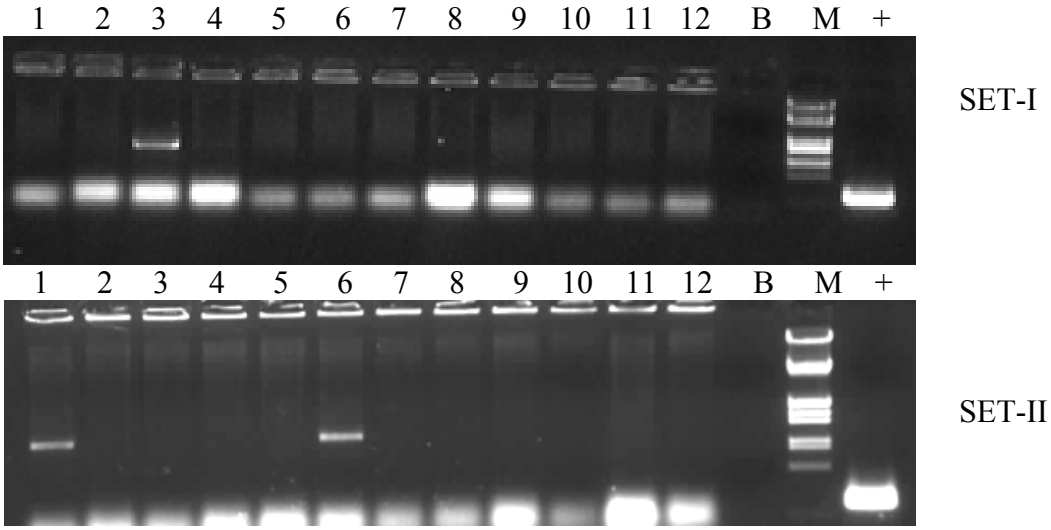


Fig.4.78.Colony PCR of EB double cassette vector clones Set-I and II using ACS5 FP/RP

Lane 1-12 Set-I and II: PCR of EB double cassette vector clones Set-I and II using ACS5 FP/RP
Lane B: PCR blank
Lane +: PCR positive
Lane M: Lambda *Hind*III/*EcoR*I double digest DNA ladder.

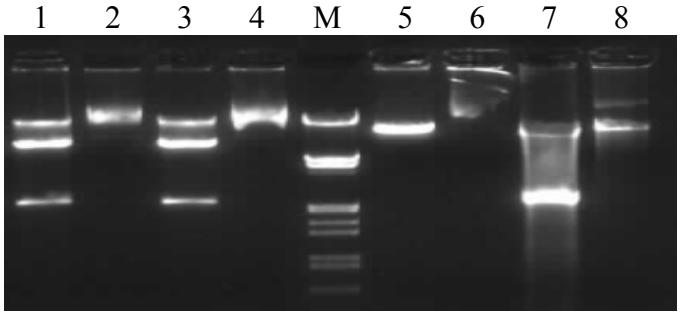


Fig.4.79. Restriction digestion check of EB double cassette vector # 1 and 6 with *Sac*I

Lane 1,3: EB double cassette vector # 1 and 6 digested with *Sac*I
Lane 2,4: uncut plasmid DNA of EB #1,6
Lane 5: pCAMBIA 1300-ACS4-*BIK1*-PolyA-P digested with *Sac*I
Lane 6: Uncut plasmid DNA of pCAMBIA 1300-ACS4-*BIK1*-PolyA-P
Lane 7: pCAMBIA 1300-ACS5-*ERF1*-PolyA-PS digested with *Sac*I
Lane 8: Uncut plasmid DNA pCAMBIA 1300-ACS5-*ERF1*-PolyA-PS
Lane M: Lambda *Hind*III/*Eco*RI double digest DNA ladder.

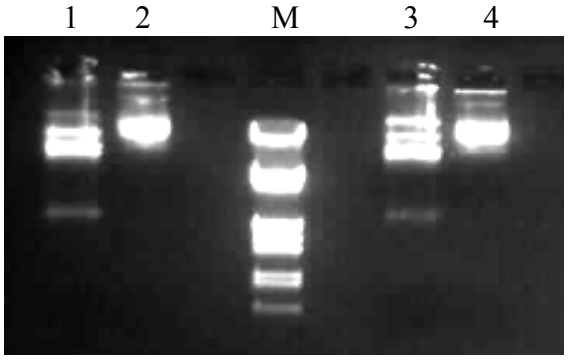


Fig.4.80. Restriction digestion check of EB double cassette vector # 1 and 6 with *Eco*RI

Lane 1,3: EB double cassette vector # 1 and 6 digested with ***Eco*RI**
Lane 2,4: uncut plasmid DNA of EB #1,6
Lane M: Lambda *Hind*III/*Eco*RI double digest DNA ladder.

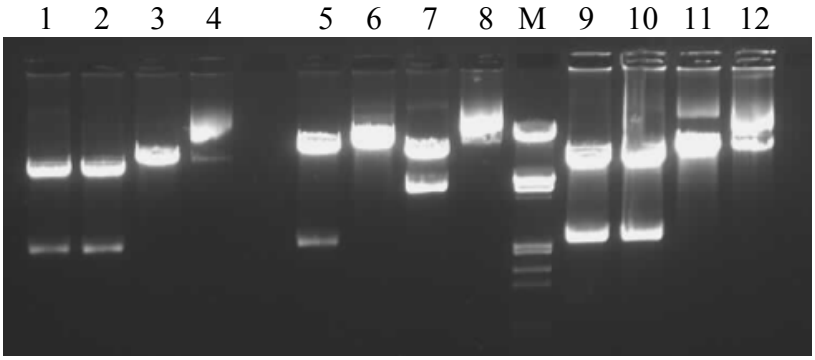


Fig.4.81. Orientation check through restriction digestion of EB#1,3 and 6 using *Sac*I, *Kpn*I and *Sal*I
Lane 1,5,9: EB#1,3,6 digested with *Sac*I
Lane 2,6,10: EB#1,3,6 digested with *Kpn*I
Lane 3,7,11: EB#1,3,6 digested with *Sal*I
Lane 4,8,12: Uncut plasmid DNA of EB#1,3,6
Lane M: Lambda *Hind*III/*EcoR*I double digest DNA ladder

4.4.2. Mobilization of the double gene cassette vector into *Agrobacterium tumefaciens* strain LBA4404

The confirmed double cassette vectors pCAMBIA 1300-EB#3 was mobilized into *Agrobacterium tumefaciens* strain LBA4404 through electroporation method of transformation (Glazebrooke *et al*., 2002). The *Agrobacterium* tranformants obtained on the selection medium were checked for the presence of both the gene cassettes *ACS4-BIK1*-polyA-P and *ACS5-ERF1*-polyA-PS within the T-DNA by colony PCR (Fig.4.82).

To confirm the individual gene cassettes by restriction digestion the plasmid DNA was isolated from PCR positive clones (Fig.4.83) of *Agrobacterium* and was retransferred into *E.coli*. The *E.coli* transformants obtained in the kanamycin selection plates were identified for the presence of the individual gene cassettes by colony PCR. The PCR positive *E.coli* clones were subjected to plasmid DNA isolation and further restriction digestions with different combinations of restriction enzymes to confirm the presence of two cassettes in the T-DNA.

The PCR identified positive *Agrobacterium* clones were tested for the purity by ketoglucosidase test. The colonies that showed the yellow colouration around itself in the medium were selected as pure culture of *Agrobacterium* to be used for the subsequent tobacco and castor transformations.

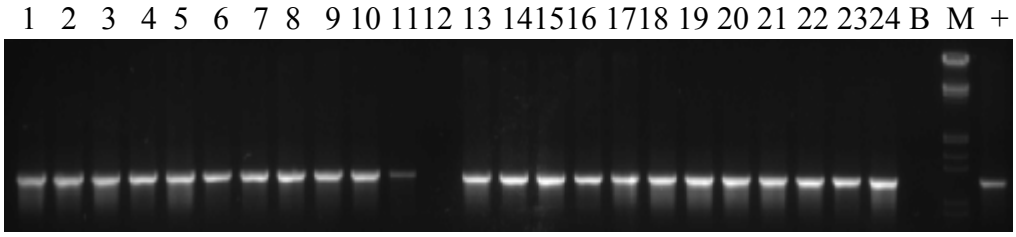


Fig.4.82. Colony PCR of LBA4404::EB#1-24 using ACS5 FP/RP
Lane 1-24: PCR of LBA4404::EB#1-24 using ACS5 FP/RP
Lane B: PCR Blank
Lane +: PCR Positive
Lane M: Lambda *Hind*III/*Eco*RI double digest DNA ladder

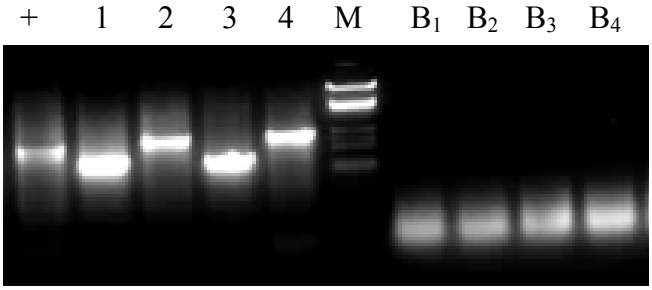
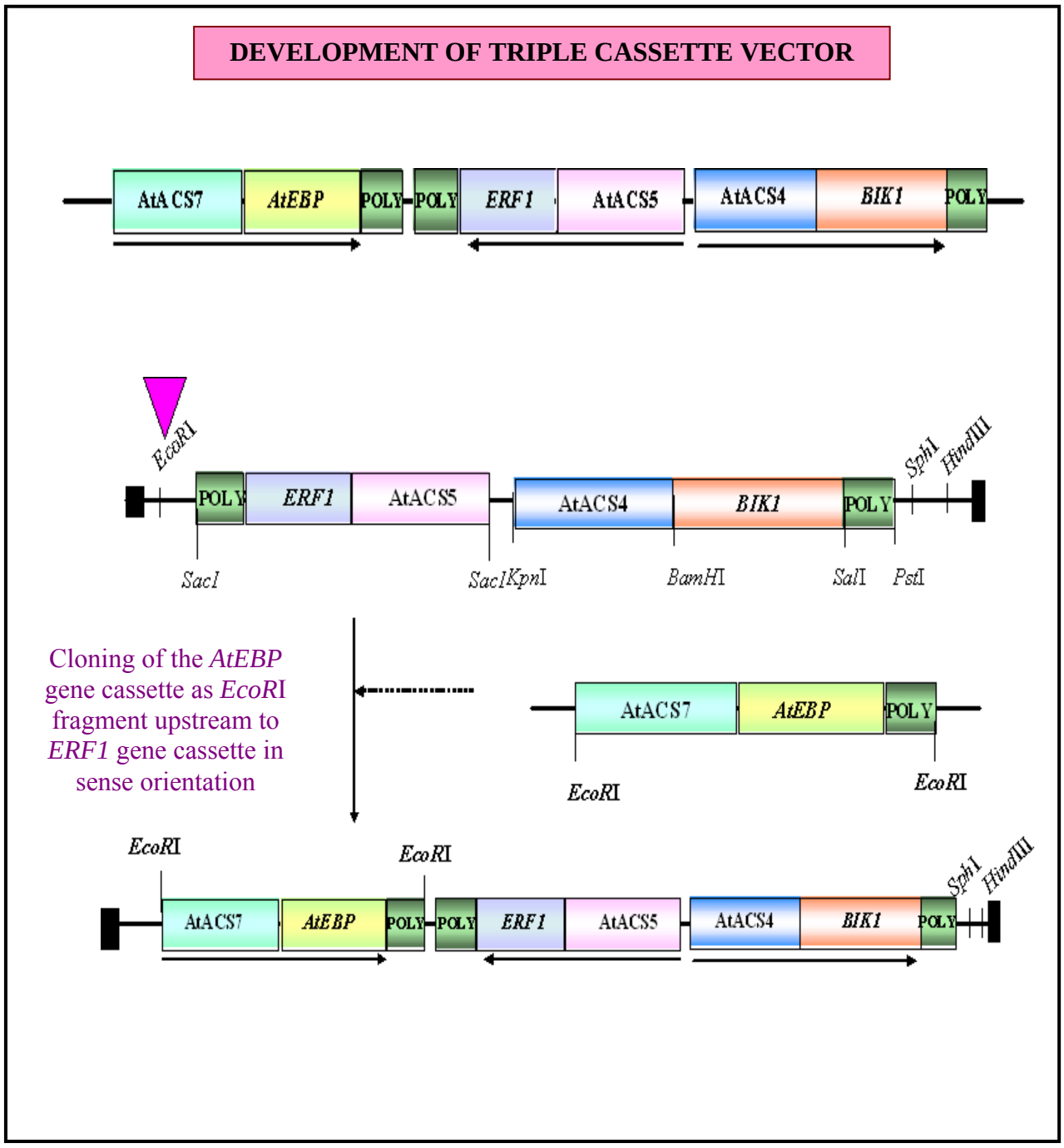


Fig.4.83.PCR confirmation of LBA4404::EB# 1 and 8 using promoter and gene specific primers
Lane 1: PCR of EB#1 using ACS5 FP/RP
Lane 2: PCR of EB#1 using ACS5 FP and *ERF1* RP
Lane 3: PCR of EB#8 using ACS5 FP/RP
Lane 4: PCR of EB#8 using ACS5 FP and *ERF1* RP
Lane +: PCR positive
Lane B_{1-4}: PCR Blank of primers used
Lane M: Lambda *Hind*III/*Eco*RI double digest DNA ladder

Fig .4.84. Schematic representation of development of triple gene cassette vector



4.4.3. Development of Triple Cassette vector *ACS7-AtEBP*-polyA + *ACS5-ERF1*-polyA + *ACS4-BIK1*-polyA (referred as AEB) in pCAMBIA 1300

The triple cassette vector *ACS7-AtEBP*-polyA + *ACS5-ERF1*-polyA + *ACS4-BIK1*-polyA (referred as AEB) was developed by cloning the *ACS7-AtEBP*-polyA gene cassette in a sense orientation upstream of the *ACS5-ERF1*-polyA gene cassette in the double cassette pCAMBIA 1300 binary vector. The schematic representation of developing the triple gene cassette vector is shown in Fig.4.84. The formation of the triple cassette vector was achieved by ligating the *EcoRI* fragment of the *ACS7-AtEBP*-polyA gene cassette in the *EcoRI* site present upstream to the *ACS5-ERF1*-polyA gene cassette in the same T-DNA of the double cassette vector developed earlier.

The ligated plasmid was transferred into *E.coli* and the positive colonies were identified by colony PCR using primers specific for *AtEBP*, *ERF1* and *BIK1* genes. The triple cassette binary plasmid DNA was isolated from the positive clones of *E.coli* and was confirmed for the presence and orientation of *ACS7-AtEBP*-polyA gene cassette with respect to other two cassettes already present in the double gene cassette by restriction digestion (Fig.4.85-87) and PCR (Fig.4.88).

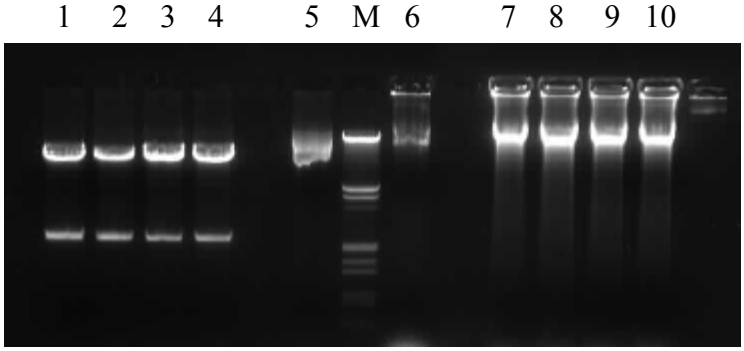


Fig.4.85. Restriction digestion of pCAMBIA 1300-ACS7-*AtEBP*-PolyA-PE#1 and double cassette vector EB#3 using *EcoR*I
Lane 1-4: pCAMBIA 1300-ACS7-*AtEBP*-PolyA-PE#1 digested with *EcoR*I
Lane 5: Uncut plasmid DNA pCAMBIA 1300-ACS7-*AtEBP*-PolyA-PE#1
Lane 6: Uncut plasmid DNA of double cassette vector EB#3
Lane 7-10: Double cassette vector EB#3 digested with *EcoR*I
Lane M: Lambda *Hind*III/*EcoR*I double digest DNA ladder

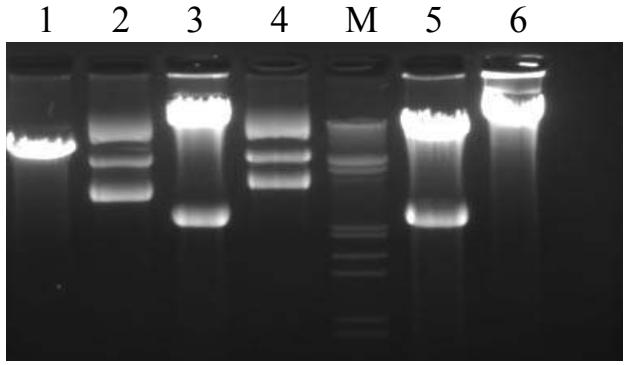


Fig 4.86. Restriction digestion of AEB binary vector with *EcoR*I
Lane 1,3,5: AEB binary plasmid #42,63 and 65 digested with *EcoRI*
Lane 2,4,6: AEB binary plasmid #42,63 and 65 uncut plasmid DNA
Lane M: Lambda *Hind*III/*EcoR*I double digest DNA ladder

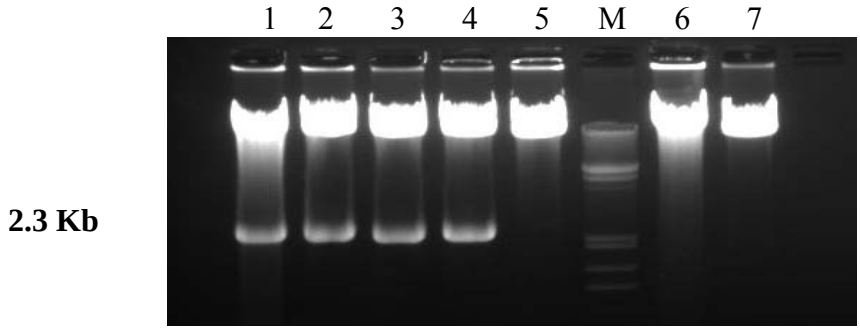


Fig 4.87. Restriction digestion of AEB binary vector with *Kpn*I
Lane 1-4: AEB binary plasmid #29,42,63,65 digested with *KpnI*
Lane 5: AEB binary plasmid uncut plasmid DNA
Lane 6: EB#3 binary plasmid digested with *KpnI*
Lane 7: EB#3 binary plasmid uncut plasmid DNA
Lane M: Lambda *Hind*III/*EcoR*I double digest DNA ladder

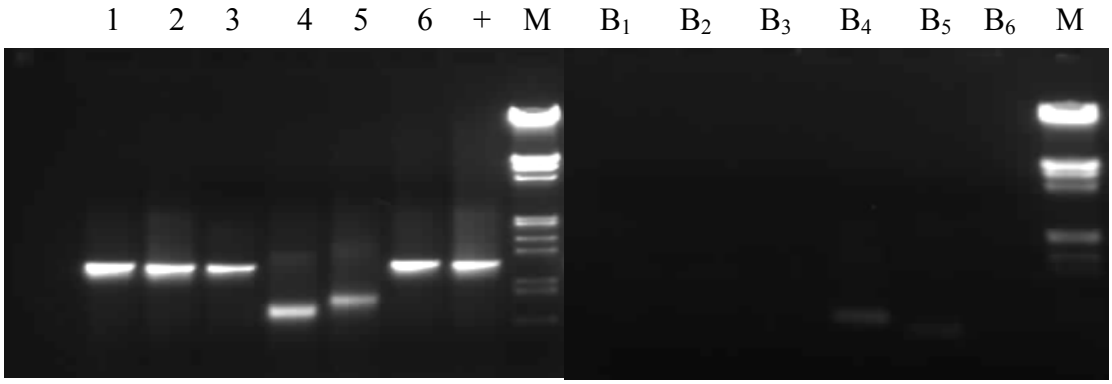


Fig 4.88. PCR confirmation of AEB #63 using promoter and gene specific primers
Lane 1: PCR amplification of AEB #63 using ACS4 FP/RP
Lane 2: PCR amplification of AEB #63 using ACS5 FP/RP
Lane 3: PCR amplification of AEB #63 using ACS7 FP/RP
Lane 4: PCR amplification of AEB #63 using *ERF1* FP/RP
Lane 5: PCR amplification of AEB #63 using *AtEBP* FP/RP
Lane 6: PCR amplification of AEB #63 using *BIK1* FP/RP
Lane B_1 to B_6: PCR Blank of each set of primer used
Lane +: PCR positive
Lane M: Lambda *Hind*III/*Eco*RI double digest DNA ladder

4.4.4. Mobilization of the triple gene cassette vector (AEB) vector into *Agrobacterium tumefaciens* LBA4404

The confirmed triple cassette vector was then mobilized into *Agrobacterium tumefaciens* strain LBA4404 by electroporation method of transformation carried out at 1700V for 5msec. The *Agrobacterium* transformants were selected on a YEP agar medium containing appropriate antibiotics. The *Agrobacterium* tranformants were checked for the presence of three cassettes viz. *ACS4-BIK1*-polyA, *ACS5-ERF1*-polyA and *ACS7-AtEBP*-polyA in the T-DNA of the binary vector by PCR (Fig.4.89). To confirm the triple cassette plasmid present in *Agrobacterium* transformants by restriction digestion, the plasmid DNA was isolated from the PCR positive clones of *Agrobacterium* and was retransferred into *E.coli*. The transformants obtained in the Kanamycin selection plates were identified for the presence of the triple cassette by colony PCR. LBA4404::AEB#10 was used for plasmid isolation and reverse transformation into *E.coli* competent cells. The PCR positive *E.coli* clones were subjected to plasmid DNA isolation and further restriction digestion with *EcoRI* to confirm the triple cassette (Fig.4.90).

The PCR identified positive *Agrobacterium* clones were tested for the purity by ketoglucosidase test. The colonies that showed the yellow coloration around the circumference in the medium were selected as pure culture of *Agrobacterium* to be used for the subsequent tobacco and castor transformations.

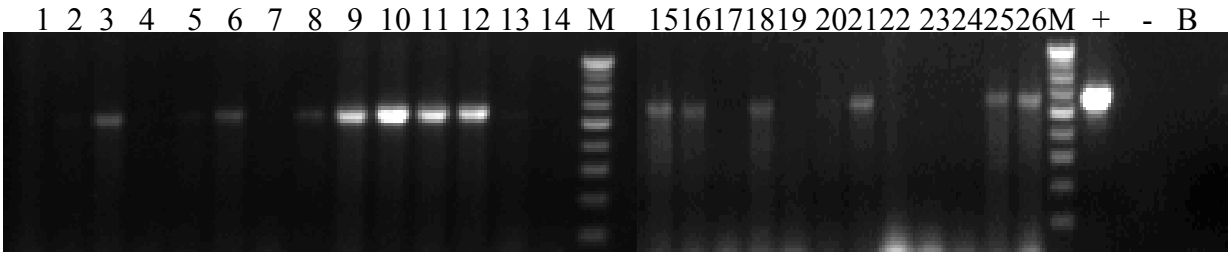


Fig 4.89. PCR check of LBA4404:: AEB clones using *ERF1* FP/RP
Lane 1-26: LBA 4404::AEB #1-26 PCR using *ERF1* FP/RP
Lane +: PCR positive
Lane -: PCR negative
Lane B: PCR Blank
Lane M: 100 bp DNA ladder

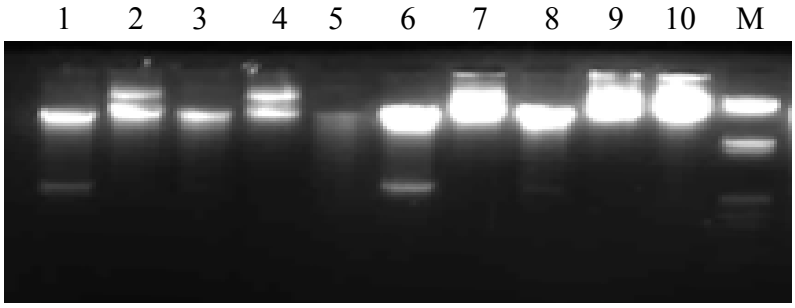


Fig4.90. Restriction digestion confirmation of reverse mated clones of AEB#1-4 using *EcoRI*
Lane 1,3,6,8: Restriction digestion of AEB#1-4 using *EcoRI*
Lane 2,4,5,7: Uncut plasmid DNA of AEB#1-4
Lane 9: Restriction digestion of EB#3 using *EcoRI*
Lane 10: Uncut plasmid DNA of EB#3
Lane M: Lambda *Hind*III/*Eco*RI double digest DNA ladder

To summarize, the first part of the research work intended to develop gene cassettes with the three chosen genes *biz.BIK1*, *ERF1* and *AtEBP*, selected promoters *AtACS4*, *AtACS5* and *AtACS7*. By adopting appropriate strategies, totally eight different vectors were developed. This included three promoter vectors (designated *AtACS4-GUS*-nos, *AtACS5-GUS*-nos and *AtACS7-GUS*-nos, three single gene cassettes *AtACS4-BIK1*-polyA (designated B), *AtACS5-ERF1*-polyA (designated E) and *AtACS7-AtEBP*-polyA (designated A), one double gene cassette carrying *AtACS4-BIK1*-polyA and *AtACS5-ERF1*-polyA is cloned in head to head fashion (designated EB) and one triple gene cassette *AtACS7-AtEBP*-polyA cassette upstream of 'B' cassette in EB vector in a tail to tail fashion (designated AEB). All these cassettes were confirmed for the presence and orientation of each component sequences in each of the cassettes, orientation of individual gene cassettes in the double and triple gene cassettes by adopting different strategies such as serial PCR, restriction digestion and full length cassettes PCR's. Each of the vectors developed were confirmed by sequencing the entire cassette. The confirmed vectors were mobilized into *Agrobacterium* strain and the presence of binary vectors in the resulted transformants was also confirmed for the intact vectors. Thus Agrobacterium with developed vectors was ready for developing transgenic plants.

4.5.0. Tobacco transformation using the single, double and triple gene constructs

The tobacco transgenics were produced using the developed individual, double and triple gene cassette vectors by leaf disc method of tobacco transformation (Plate.4.0). In this method the leaf explants from *Nicotiana tabacum* cv. *tabacum* were subjected to co-cultivation using the Agrobacterium strains LBA4404- harbouring promoter vectors, individual gene cassettes LBA4404-*ACS4-BIK1*-P#1, LBA4404-*ACS5-ERF1*-PS#1 and LBA4404-*ACS7-AtEBP*-PE#1, double gene cassette vector LBA4404-EB#8 and triple gene cassette vector LBA4404-AEB#27 were used.

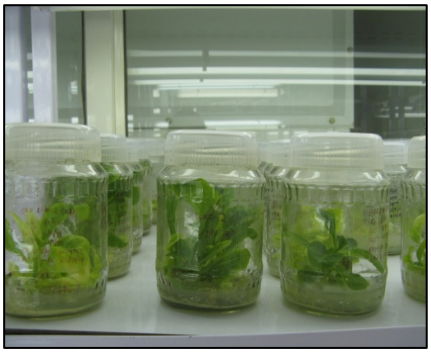
Plate 4.0. Scheme of Tobacco Transformation

Control seeds

Control tobacco plantlets

Co-cultivated leaf explants

Shoot regeneration and multiplication



Rooting

Hardening in vermiculite

Acclimatization in greenhouse

4.5.1. Morphological studies of the T_0 tobacco transgenic plants

The tobacco transgenics of the single gene cassette vectors pCAMBIA 1300-*ACS4*-*BIK1*-polyA, pCAMBIA 1300-*ACS5*-*ERF1*-polyA and pCAMBIA 1300-*ACS7*-*AtEBP*-polyA were scored based on their morphological characteristics. The *BIK1* transgenics showed wrinkled leaf appearance with tapered leaf tips, reduced leaf area and narrow when compared to the control with broad and flat leaves. The height of the transgenics was similar to that of the control plants. The *BIK1* transgenic plants showed branching at every node which was not seen in the control plants. The flowers showed reduced petal area and stunted anther filaments with reduced fertile pollen grains and petalloid sepal formation when compared to the controls. These characters did show some effect on the fertilization of the transgenic flowers forming capsules but the capsule size was much reduced when compared to the controls (Plate.4.1).

It was observed that the *ERF1* transgenics showed wrinkled leaf appearance with tapered leaf tips, reduced leaf area and narrow leaf blade when compared to the control plant that had broad and flat leaves. The height of the transgenics was also compromised with stunted growth and the plant showed branching at every node which was not seen in the control plants. The flowers showed reduced petal area and reduced anther filaments with reduced fertile pollen grains and petalloid sepal formation when compared to the controls. The capsules formed were with reduced size when compared to the controls (Plate.4.2).

The *AtEBP* tobacco transgenic plants were quite comparable to the control plants. The *AtEBP* plants showed very broad leaves, stunted growth and heavy branching at each node when compared to the controls. The flowers were similar to that of control plants except for the decolouration of the petals to white colour. The fertilization and seed set was equal to the control plants (Plate.4.3).

The seeds collected from all the three single gene cassette tobacco transgenic plants were studied for their germination on vermiculite and on selection medium with Hygromycin (30mg/L). The transgenic population that showed a germination rate of 3:1 when compared to the controls that showed nearly 100% germination and RT PCR positive transgenic plants were acclimatized in the green house for the T1 generation studies.

The double gene cassette vector EB#8 and the triple gene cassette vector AEB#27 showed delayed shoot regeneration after transformation with *Agrobacterium tumefacians*

LBA4404. The developed shoots could not elongate on elongation medium and no rooting was observed in the rooting medium even after prolonged incubation under normal tissue culture conditions (Plate.4.4 and 4.5).

Morphological Studies of *BIK1* Tobacco Transgenic Plants

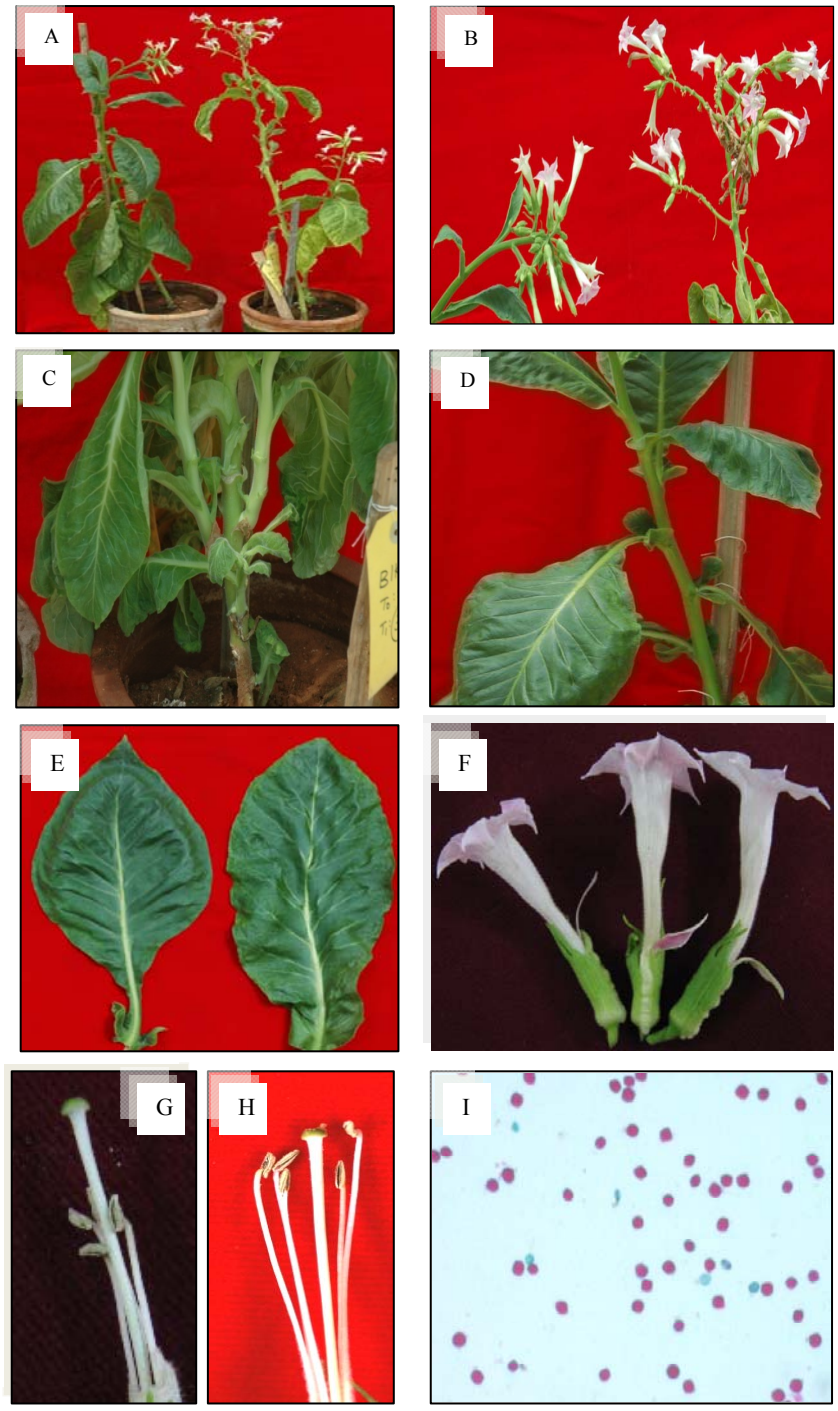


Plate 4.1. Morphological studies of *BIK1* transgenic vs. untransformed tobacco control plant

A. Control and *BIK1* plants. **B.** Inflorescence of control and *BIK1*. C. Branching in *BIK1*. D. Branching in control. E. Leaf morphology of control vs. *BIK1*. F. Petalloid sepals of *BIK1*. G and H. Anther filament morphology in *BIK1* and control respectively. I. Pollen viability study in the recessed anther of *BIK1* by Alexander Staining.

Morphological Studies of *ERF1* Tobacco Transgenic Plants

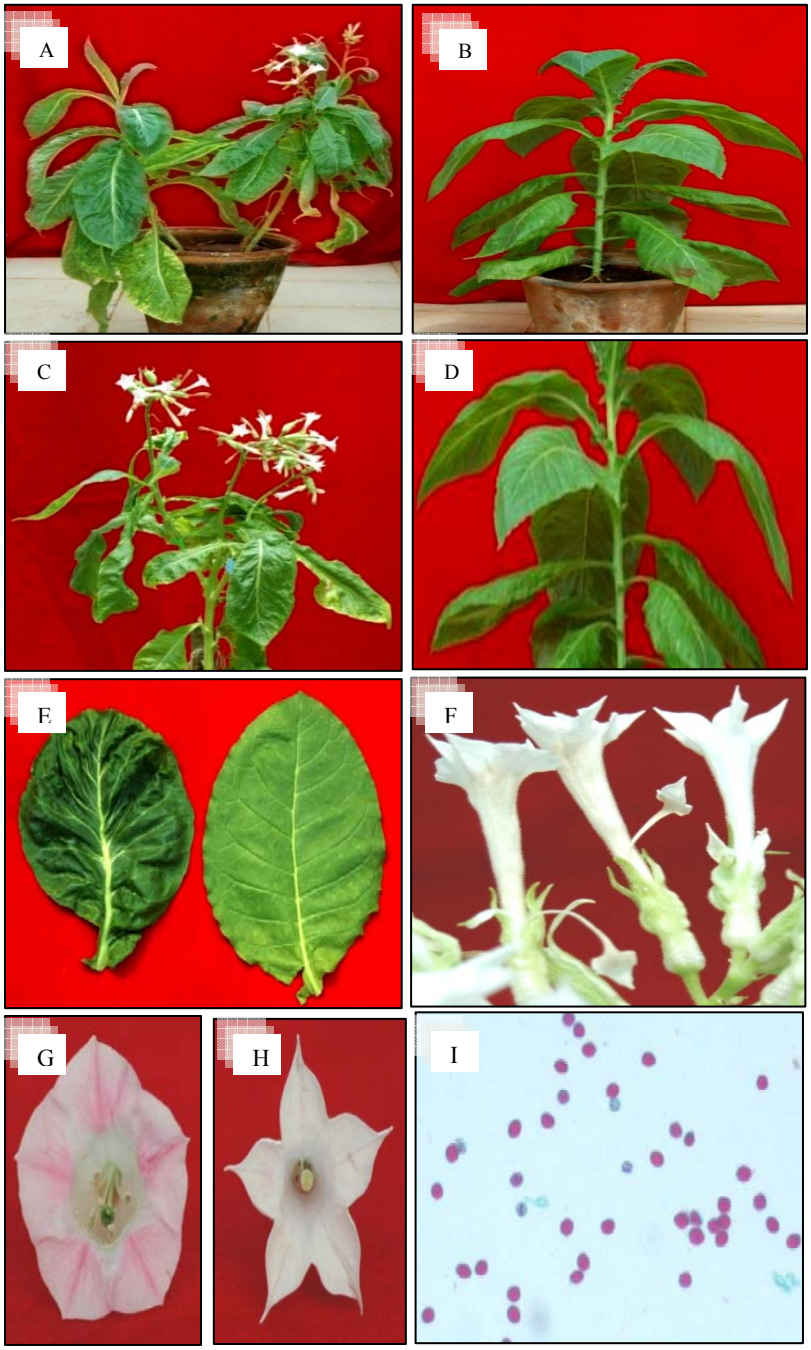


Plate.4.2. Morphological studies of *ERF1* transgenic vs. untransformed tobacco control plant

A. and **B.** *ERF1* and Control plants. **C.** Branching in *ERF1*. **D.** Branching in control. **E.** Leaf morphology of *ERF1* vs. control. **F.** Petalloid sepals of *ERF1*. **G and H.** Anther filament and floral morphology in control and *ERF1* respectively. **I.** Pollen viability study in the recessed anther of ERF*1* by Alexander Staining.

Morphological Studies of *AtEBP* Tobacco Transgenic Plants

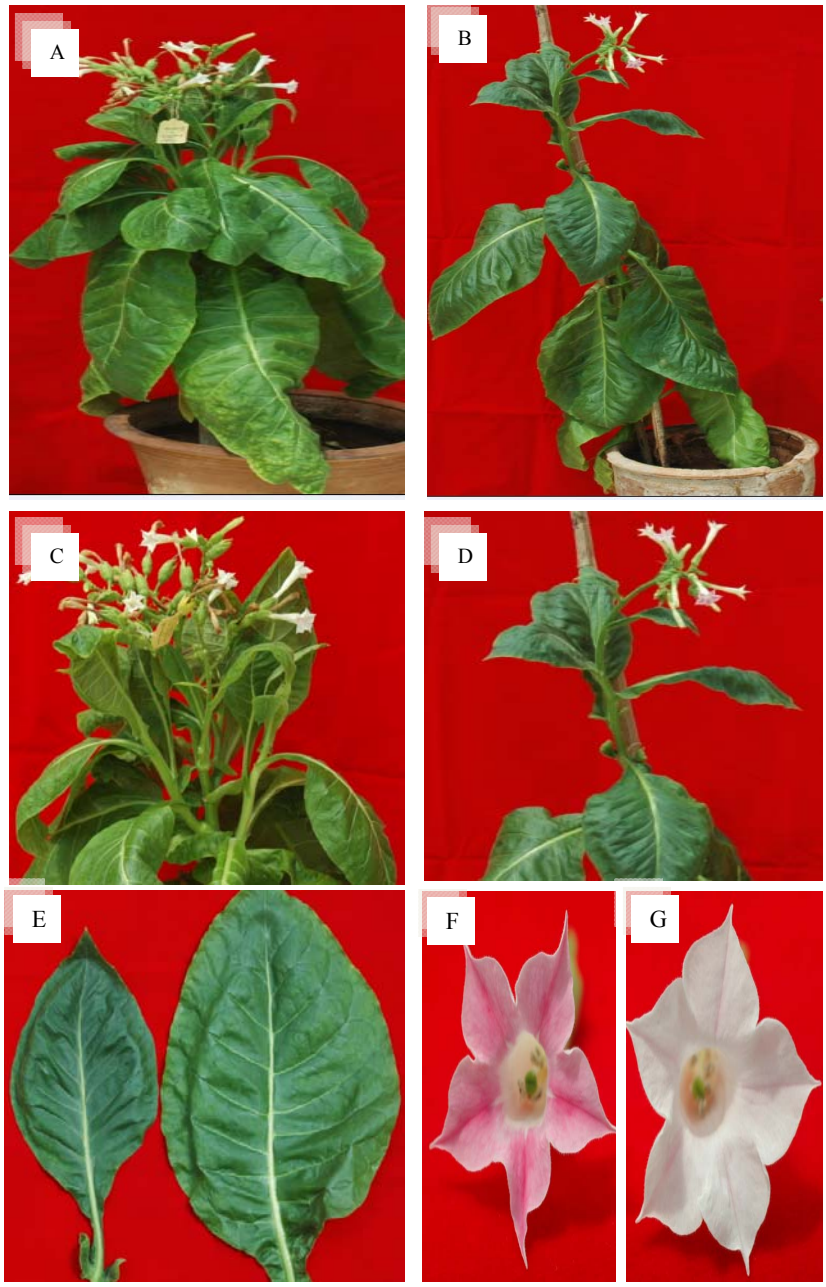


Plate.4.3. Morphological studies of *AtEBP* transgenic vs. untransformed tobacco control plant

A. *AtEBP* plant. **B.** Control plant. **C.** Branching in *AtEBP*. **D.** Branching in control. **E.** Leaf morphology of control vs. *AtEBP*. **F. and G.** Anther filament morphology in control and *AtEBP* respectively.

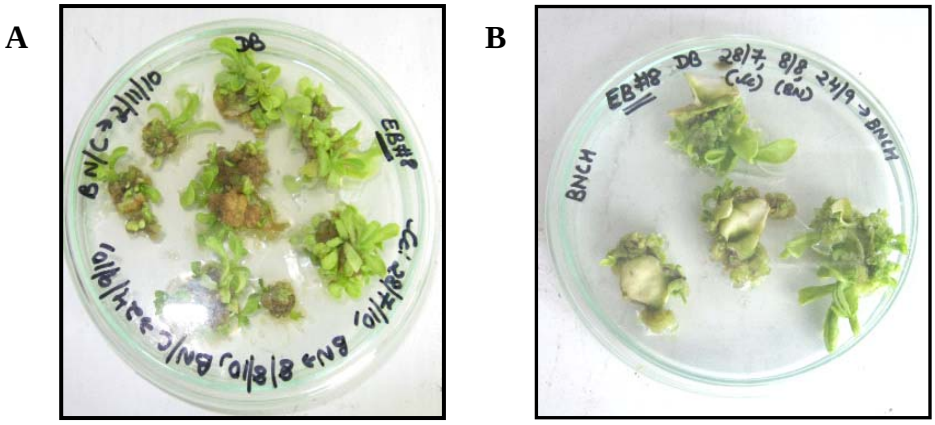


Plate.4.4. Transformation of tobacco using double gene cassette vector EB#8
A. and B. Shoot regeneration in double construct EB#8 tobacco explants

Plate.4.5. Transformation of tobacco using triple gene cassette vector AEB#27
A.B and C. Shoot regeneration in triple construct AEB#27 tobacco explants

4.5.2. Logistics of tobacco transformation

The tobacco transformations using the single gene cassette vectors **pCAMBIA1300-*ACS4*-*BIK1*-P, pCAMBIA1300-*ACS5*-*ERF1*-PS and pCAMBIA1300-*ACS7*-*AtEBP*-PE** resulted in many regenerated shoots which were elongated and rooted further before acclimatization in the greenhouse (Table 4.0- 4.2). The transformation experiments involving the double **pCAMBIA 1300-EB** and the triple gene construct **pCAMBIA 1300-AEB** resulted in a very low shoot regeneration and no further elongation of the shoots was observed (Table 4.3 and 4.4) indicating some deleterious effects of the combinations of genes on that could have interfered the development of the whole plants form the regenerated shoots.

Table.4.0. Transformation of the tobacco leaf explants using pCAMBIA1300-ACS4-*BIK1*-P

No. of co-cultivations	No. of explants	No. of shoots in 1st selection	No. of shoots in 2nd selection	No. of explants in elongation medium	No. of explants in rooting medium
2	60	50	43	36	32

Table.4.1. Transformation of the tobacco leaf explants using pCAMBIA1300-ACS5-*ERF1*-PS

No. of co-cultivations	No. of explants	No. of shoots in 1st selection	No. of shoots in 2nd selection	No. of explants in elongation medium	No. of explants in rooting medium
2	47	42	31	30	30

Table.4.2. Transformation of the tobacco leaf explants using pCAMBIA1300-ACS7-*AtEBP*-PE

No. of co-cultivations	No. of explants	No. of shoots in 1st selection	No. of shoots in 2nd selection	No. of explants in elongation medium	No. of explants in rooting medium
12	237	56	34	31	30

Table.4.3. Transformation using pCAMBIA 1300-EB double gene cassette vector

No. of co-cultivations	No. of explants	No. of shoots in 1st selection	No. of shoots in 2nd selection	No. of explants in elongation medium	No. of explants in rooting medium
25	875	430	173	-	-

Table .4.4. Transformation using pCAMBIA1300-AEB triple gene cassette vector

No. of co-cultivations	No. of explants	No. of shoots in 1st selection	No. of shoots in 2nd selection	No. of shoots in elongation medium	No. of shoots in rooting medium
19	570	432	143	-	-

4.6.0. PCR analysis of the developed (T_0) tobacco transgenics:

4.6.1. PCR analysis of *BIK1* (T_0) tobacco transgenics

Twenty three plants of *BIK1* (T_0) tobacco transgenic plants were acclimatized in the green house. These plants were checked for the presence of the ACS4-*BIK1*-polyA gene cassette in the gDNA through PCR. The plants were checked for the presence of all the components of the gene cassette by PCR using primers specific to the gene, promoter, terminator and for the complete gene cassette by serial PCR (Fig.4.91). Plants #1,2,3,4,7,8,9,10,11,13,15,16,17,18,20,21,22 and 23 showed positive with the different combinations of PCR reaction indicating the stable integration of the gene cassette.

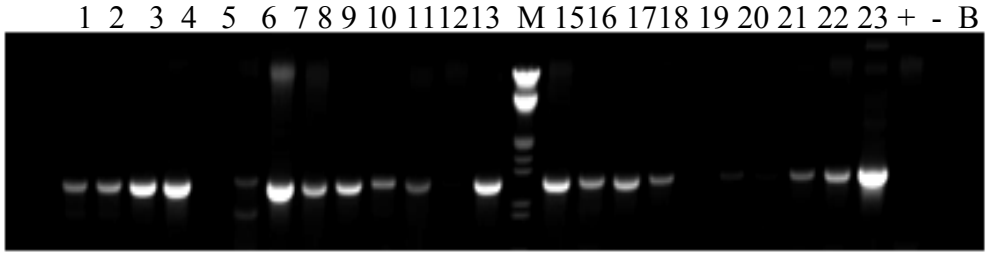


Fig 4.91.PCR amplification of *BIK* (T_0) tobacco plants
Lane 1-23 : PCR of *BIK1* (T0) #1-23 using ACS4 FP/ polyARP amplifying the *BIK1* gene cassette.
Lane M : Lambda DNA double digest
Lane + : PCR positive
Lane - : PCR negative
Lane B: PCR Blank

4.6.2. PCR analysis of *ERF1* (T_0) tobacco transgenics

Twenty plants of *ERF1* (T_0) tobacco transgenic plants were acclimatized in the green house. These plants were checked for the presence of the ACS5-*ERF1*-polyA gene cassette in the gDNA through PCR. The plants were checked for the presence of all the components of the gene cassette by PCR using primers specific to the gene, promoter, terminator and for the complete gene cassette by serial PCR (Fig.4.92). All plants (#1-20) showed positive with the different combinations of PCR reaction indicating the stable integration of the gene cassette.

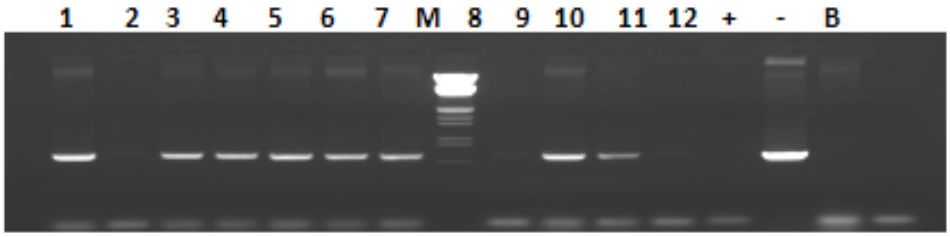


Fig 4.92.PCR amplification of *ERF1* (T_0) tobacco plants
Lane 1-12 : PCR of *ERF1* (T0) #1-20 using ACS5 FP/ polyARP amplifying the *ERF1* gene cassette.
Lane M : Lambda DNA double digest
Lane + : PCR positive
Lane - : PCR negative

4.6.3. PCR analysis of *AtEBP* (T_0) tobacco transgenics

Twenty plants of *AtEBP1* (T_0) tobacco transgenic plants were acclimatized in the green house. These plants were checked for the presence of the ACS7-*AtEBP1*-polyA gene cassette in the gDNA through PCR. The plants were checked for the presence of all the components of the gene cassette by PCR using primers specific to the gene, promoter, terminator and for the complete gene cassette by serial PCR (Fig 4.93). All plants (#1-20 except #10) showed positive with the different combinations of PCR reaction indicating the stable integration of the gene cassette.

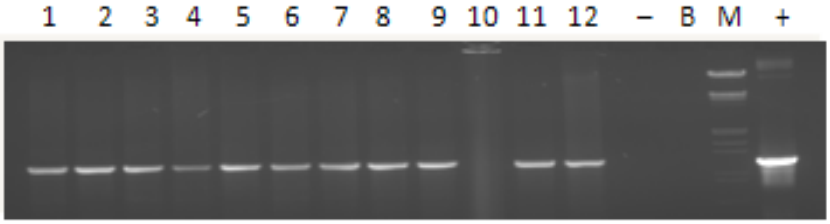


Fig4.93.PCR amplification of *AtEBP* (T_0) tobacco plants
Lane 1-12 : PCR of *AtEBP* (T0) #1-20 using ACS7 FP/ polyARP amplifying the *AtEBP* gene cassette.
Lane M : Lambda DNA double digest
Lane + : PCR positive
Lane - : PCR negative

4.7.0. PCR analysis of the developed (T_1) tobacco transgenics:

The T_0 plants showing positive with PCR, Dot Blot and RT-PCR with respect to the exrpession of the transgene were used for raising the T_1 generation.

4.7.1. PCR analysis of *BIK1* (T_1) tobacco transgenics

Three populations of T_1 plants of (T_0 # 4, 5 and 12) were raised containing thirty six plants in each population. The plants were checked for the presence of all the components of the gene cassette by PCR using primers specific to the gene, promoter, terminator and for the complete gene cassette by serial PCR (Fig4.94). Plants (# 1,2,3,4,5,13,14,15,17,18,19,20,24,25,26,27,28,29,33,35 and 36) in population of (T_0 # 4), plants (# 2,3,4,5,6, ,14,15,17,18,19,20,24,25,26,34 and 35) in population of (T_0 # 5) and plants (#4,5,13,14,15,17,18,19,20,24,25,27,31,32,33,35 and 36) in population of (T_0#12) showed positive with the different combinations of PCR reaction indicating the stable integration of the gene cassette.

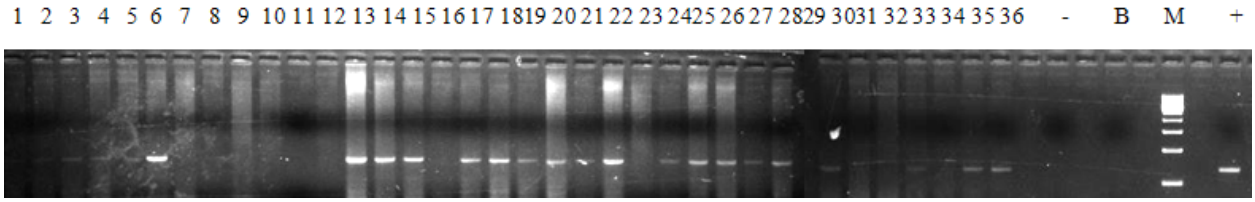


Fig 4.94.PCR of *BIK1* (T_1) tobacco transgenic plants
Lane 1-36 : *BIK1*(T_1) PCR using *ACS4* FP/polyA RP
Lane -: Untransformed control
Lane B: PCR Blank
Lane M: DNA ladder
Lane +: PCR positive

4.7.2. PCR analysis of *ERF1* (T_1) tobacco transgenics

Three populations of T_1 plants of (T_0 # 13, 16 and 29) were raised containing thirty plants in each population. The plants were checked for the presence of all the components of the gene cassette by PCR using primers specific to the gene, promoter, terminator and for the complete gene cassette by serial PCR (Fig 4.95). Plants (# 1,2,3,4,7,9,13,14,15,16,18,20,21,22,23,24,25,26,27,29,32 and 34) in population of (T_0#4), plants (#2,4,5,6,7,9,14,16,17,18,22,25,26,29 and30) in population of (T_0#5) and plants (#,7,9,13,14,15,16,18, 22,23,24 and 28) in population of (T_0 # 12) showed positive with the different combinations of PCR reaction indicating the stable integration of the gene cassette.

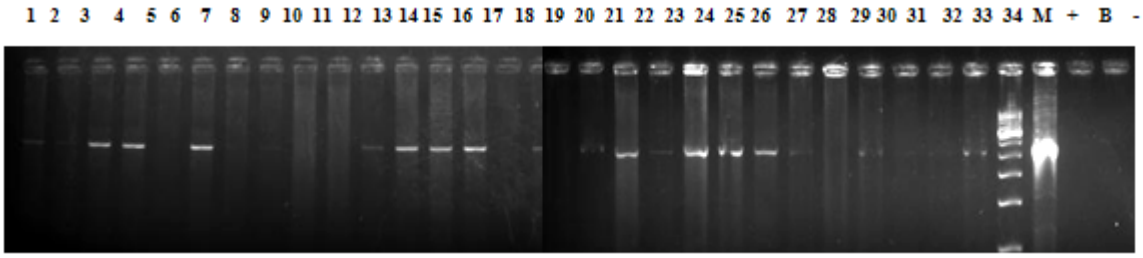


Fig 4.95. PCR of *ERF1* (T_1) tobacco transgenic plants
Lane 1-34 : *ERF1* (T1) Tobacco transgenic plants PCR with *ACS5* FP and polyA RP complete cassette specific primers
Lane M : 500 bp Molecular size marker
Lane +ve : PCR positive
Lane B : PCR Blank
Lane –ve : Untransformed Tobacco plant PCR

4.7.3. PCR analysis of *AtEBP* (T_1) tobacco transgenics

One population of T_1 plants of (T_0 # 1) were raised containing thirty plants in each population. The plants were checked for the presence of all the components of the gene cassette by PCR using primers specific to the gene, promoter, terminator and for the complete gene cassette by serial PCR (Fig 4.96). Plants (#1,3,5,7,8,9,11,12,15,19,21 and 22) in the population of (T_0#1) showed positive with the different combinations of PCR reaction indicating the stable integration of the gene cassette.

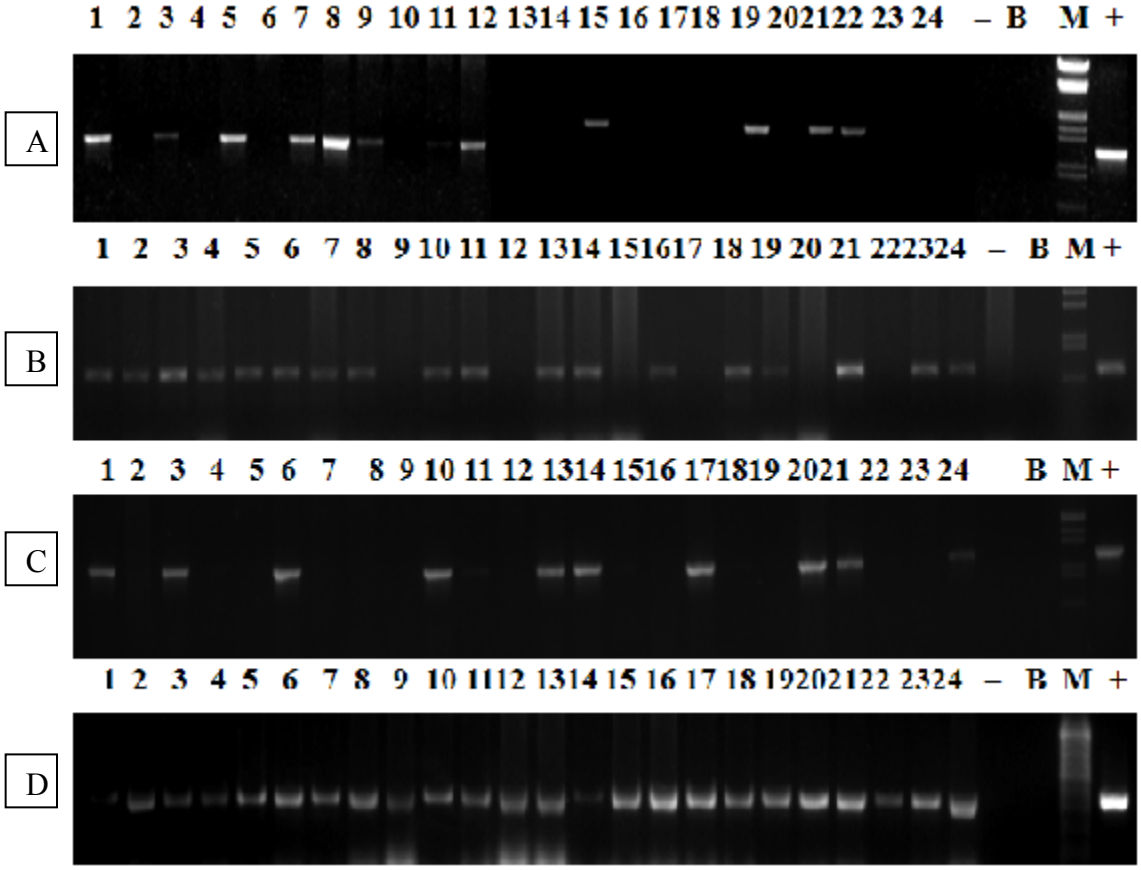


Fig 4.96.PCR amplification check of *AtEBP* (T1) # 1-24
Panel A: PCR of *AtEBP* (T1) # 1-24 using ACS7 FP/RP
Panel B: PCR of *AtEBP* (T1) # 1-24 using *AtEBP* FP/RP
Panel C: PCR of *AtEBP* (T1) # 1-24 using ACS7 FP /*AtEBP* RP
Panel D: PCR of *AtEBP* (T1) # 1-24 using *Actin* FP/RP
Lane + : PCR of *AtEBP* binary lasmid DNA using respective primers
Lane -: PCR of untransformed tobacco gDNA using respective primers
Lane M: DNA ladder Lane B : PCR Blank

4.8. PCR analysis of crossed progeny of single gene cassette plants

As the triple gene cassette transformation in tobacco was not successful in producing complete plants for analyzing the pyramided effect of three genes, crossing of three single gene cassette plants was carried out. The plants selected for crossing were confirmed to carry and express the transgenes as revealed by positive PCR and RT-PCR results. The seeds collected from the crossed plants were raised and forty plants of each cross were acclimatized and analyzed for the presence of the single gene cassettes using PCR.

4.8.1. PCR analysis of *AtEBP* x *BIK1* single gene cassette tobacco plants

The *AtEBP* single gene tobacco transgenic plant #1 was crossed with *BIK1* #3 and the resultant progeny was designated as AB. The seeds were germinated and forty plants were grown in the green house and checked for the presence of both the single gene cassettes by PCR using primers specific to each gene cassette (Fig4.97). The plant # 2,3,4,5,8,9,11,13,14,21 and 23 showed positive for both the gene cassettes.

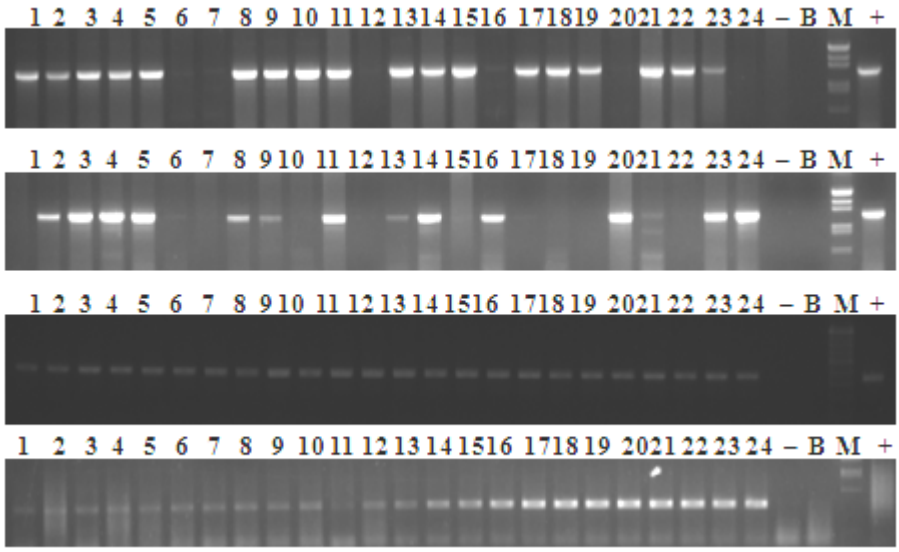


Fig 4. 97.PCR analysis of AB tobacco plants

Panel A: PCR of AB gDNA using ACS4 FP/poly RP amplifying the *BIK1* gene cassette
Panel B: PCR of AB gDNA using ACS7 FP/poly RP amplifying the *AtEBP* gene cassette
Panel C: PCR of AB gDNA using *Actin* FP/ RP
Panel D: PCR of AB gDNA using *Hygromycin* FP/ RP
Lane + : PCR positive, Lane -: PCR of untransformed tobacco gDNA using respective primers
Lane M: DNA ladder and Lane B : PCR Blank

4.8.2. PCR analysis of *AtEBP* x *ERF1*single gene cassette tobacco plants:

The *AtEBP* single gene tobacco transgenic #1 was crossed with *ERF* #11. The seeds were germinated and forty plants were grown until flowering and checked for the presence of both the single gene cassettes by PCR using primers specific to each gene cassette (Fig 4.98). The plant # 1,3,6,7,8,10,13,14,17,20,21 and 24 showed positive for both the gene cassettes.

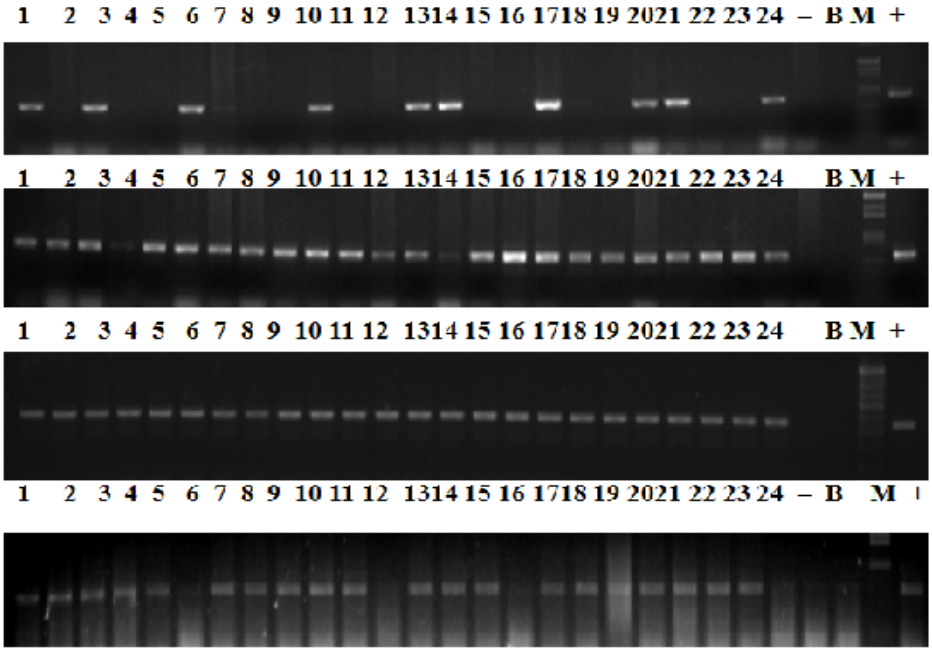


Fig 4.98. PCR analysis of AE tobacco plants
Panel A: PCR of AE gDNA using ACS7 FP/poly RP amplifying the *AtEBP* gene cassette
Panel B: PCR of AE gDNA using ACS5 FP/poly RP amplifying the *ERF1* gene cassette
Panel C: PCR of AE gDNA using Actin FP/ RP
Panel D: PCR of AE gDNA using Hygromycin FP/ RP
Lane + : PCR positive, Lane -: PCR of untransformed tobacco gDNA using respective primers ,Lane M: DNA ladder and Lane B : PCR Blank

4.8.3. PCR analysis of *BIK1* x *ERF1*single gene cassette tobacco plants:

The *BIK1* single gene tobacco transgenic #3 was crossed with *ERF* #9. The seeds were germinated and fourty plants were grown in the green house and checked for the presence of both the single gene cassettes by PCR using primers specific to each gene cassette (Fig 4.99). The plant # 1,2,3,4,5,6,7,8,9,11,12,14,16,21,23 and 24 showed positive for both the gene cassettes.

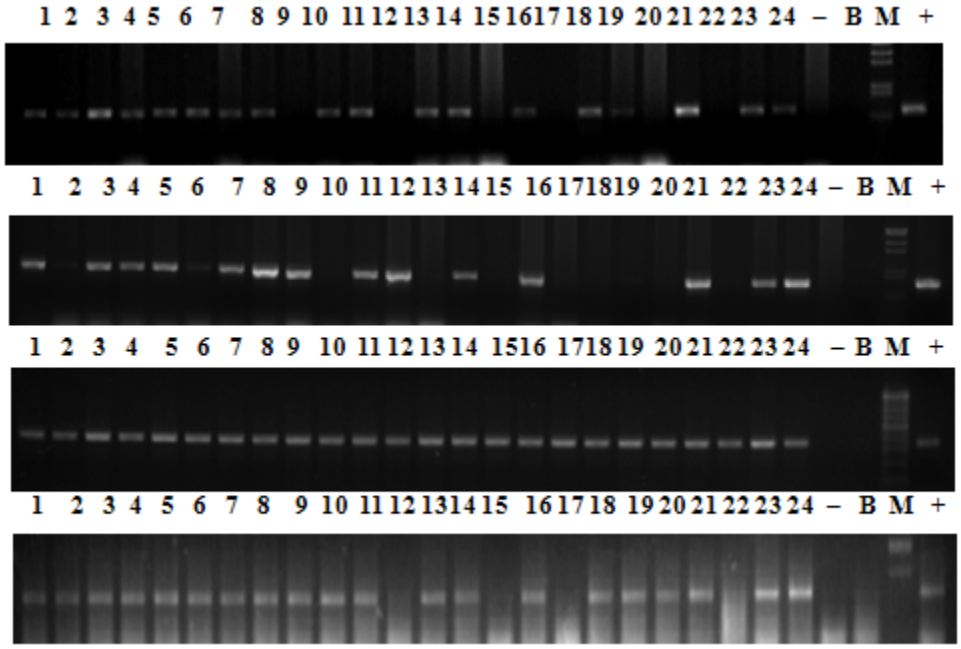


Fig 4.99. PCR analysis of BE tobacco plants
Panel A: PCR of BE gDNA using ACS4 FP/poly RP amplifying the *BIK1* gene cassette
Panel B: PCR of BE gDNA using ACS5 FP/poly RP amplifying the *ERF1* gene cassette
Panel C: PCR of BE gDNA using Actin FP/ RP
Panel D: PCR of BE gDNA using Hygromycin FP/ RP
Lane + : PCR positive, Lane -: PCR of untransformed tobacco gDNA using respective primers
Lane M: DNA ladder and Lane B : PCR Blank

To summarize the second part of the results the crossing of the single gene tobacco transgenics was carried out to stack the expression of the individual genes in a single plant. This exercise was the best way to achieve the aim of pyramiding the genes as the regeneration of the transformed tobacco explants was not successful in case of double and triple gene cassette vectors. This could be attributed to the specific interaction among the gene cassettes present in

them because individually the single gene cassettes did not have deleterious effects on regeneration and also when the gene cassettes were brought together in the same plant by crossing the appropriate transgenic plants. Hence, once the plants carrying two gene cassettes start flowering the expression of both the genes will be analyzed in these plants. The plants showing stable expression of either of the two transgenes would be further crossed with the plant carrying the third gene cassette to complete the pyramiding of all three genes in one single plant. All the double and triple stacked tobacco plants obtained would be assessed for their tolerance against *Botrytis* spp.

4.9. RT PCR analysis of the (T_0) transgenic plants

4.9.1. RT PCR of T_0 transgenic tobacco plants carrying *ACS4-BIK1*-polyA cassette

The PCR positive T_0 transgenic plants (# 4,5,12,16,28) carrying *ACS4-BIK1*-polyA-P cassette were studied for their morphological characters (Plate.4.1) and were confirmed for the expression of the inserted gene through RT PCR. The RNA samples (Fig.4.100) were first checked for any DNA contamination by using the RNA samples directly (minus RT) in PCR reaction using *BIK1* specific primers and *Actin* primers. The cDNA obtained was subjected to PCR analysis using *BIK1* forward and reverse primers (Fig 4.101). Expression of the transgene was observed only in flowers and not in the leaves of plant# 4, 5 and 12 and the seeds of these three plants were selected for the T_1 generation studies. The results clearly indicated that *BIK1* expression was more in flowers compared to leaves.

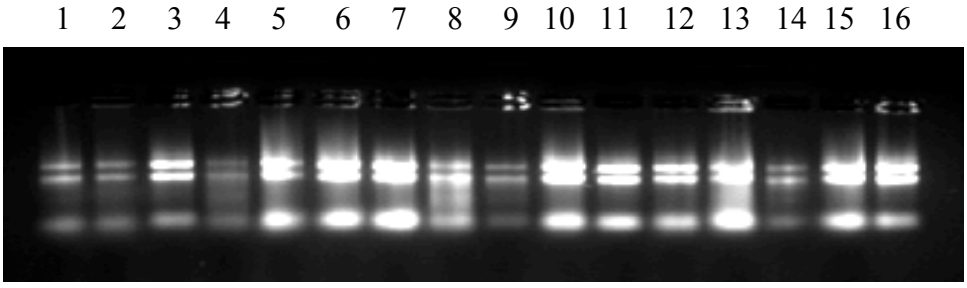


Fig 4.100.Quantification of RNA from the *BIK1* leaf and flower tissues
Lane 1,3,5,7,9,11,13: RNA isolated from leaf tissue of *BIK1* tobacco transgenics
Lane 2,4,6,8,10,12,14: RNA isolated from flower tissue of *BIK1* tobacco transgenics
Lane 15: RNA isolated from leaf tissue of untransformed tobacco
Lane 16: RNA isolated from flower tissue of untransformed tobacco

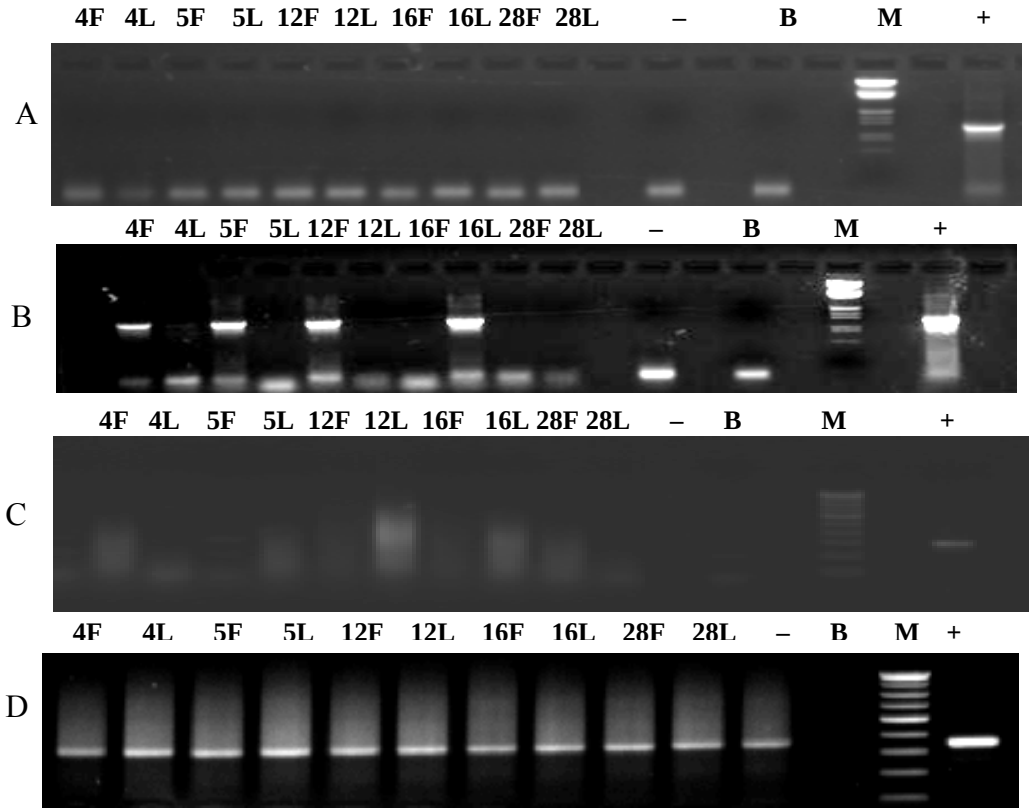


Fig 4.101. RT PCR of *BIK1*(T0) tobacco transgenic plants leaf and flower
Pane A: Lane 1-10: Minus-RT PCR of *BIK1*(T0) using *BIK1* FP/RP
Pane B: Lane 1-10: RT PCR of *BIK1*(T0) tobacco transgenic leaf and flowers using *BIK1* FP/RP
Pane C: Lane 1-10: Minus-RT PCR of *BIK1*(T0) using *Actin* FP/RP
Pane D: Lane 1-10: RT PCR of *BIK1*(T0) tobacco transgenic leaf and flower using *Actin* FP/RP
Lane - : PCR negative
Lane +: PCR positive
Lane B: PCR Blank
Lane M: Lambda *Hind*III/*Eco*RI double digest DNA ladder

4.9.2. RT PCR of T_0 transgenic tobacco plants carrying *ACS5-ERF1*-polyA cassette

The PCR positive T_0 transgenic plants (# 1,2,4,6,8,13,15,16,29,30) carrying *ACS5-ERF1*-polyA-PS cassette and untransformed tobacco plants were studied for their morphological characters (Plate.4.2). PCR positive transgenic plants were analyzed for the expression of the inserted gene through RT-PCR with the RNA isolated from flowers and leaves. The qualitative and quantitative analysis of the isolated RNA was done using agarose gel electrophoresis. The RNA samples were first checked for any DNA contamination through direct PCR analysis using *ERF1* specific primers and *Actin* primers. The cDNA obtained was further subjected to PCR analysis using *ERF1* forward and reverse primers. Expression pattern of *Actin* was used as internal control (Fig 4.102). The expression of the transgene *ERF1* was observed only in the flowers and not in the leaves of plant# 13, 16 and 29 (Fig 4.103). To check the tissue specific expression of the *ERF1* gene in the flowers of plant# 13, 16 and 29 the RT PCR was carried out using the RNA isolated from different parts of the flower sepals, petals, stamens and stigma. The expression of *ERF1* was observed in the floral parts and not in the leaves. Seeds of these three plants were selected for the T_1 generation studies.

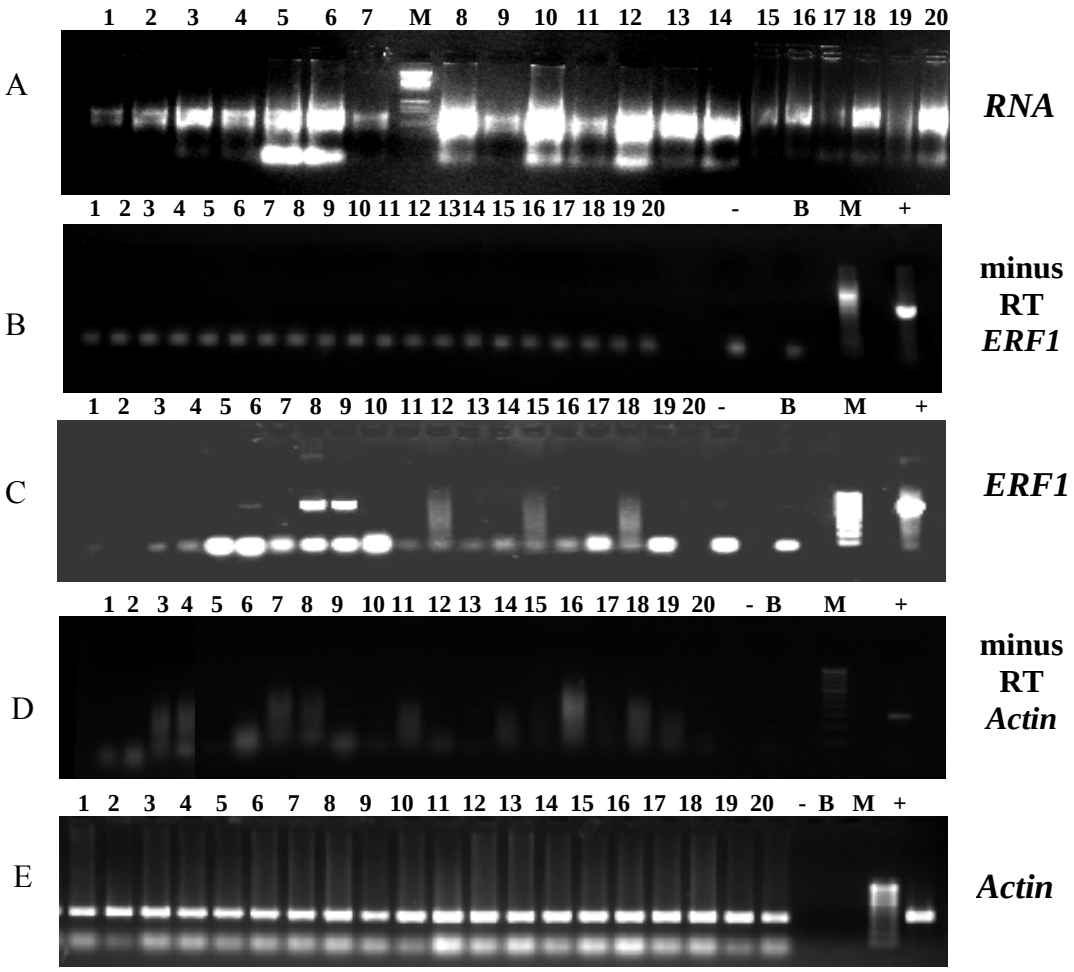


Fig 4.102. RT PCr of *ERF1*(T_0) tobacco transgenic plants
1,2,4,6,8,13,15,16,29,30
Pane A: Lane 1-20: RNA isolated from *ERF1* tobacco transgenics leaf and flower of 1,2,4,6,8,13,15,16,29,30
Pane B: Lane 1-10: minus RT PCR of the RNA from flowers of 1,2,4,6,8,13,15,16,29,30 using *ERF1* FP/RP
Pane B: Lane 11-20: minus RT PCR of the RNA from leaves of 1,2,4,6,8,13,15,16,29,30 using *ERF1* FP/RP
Pane C: Lane 1-10: RT PCR of the RNA from flowers of 1,2,4,6,8,13,15,16,29,30 using *ERF1* FP/RP
Pane C: Lane 11-20: RT PCR of the RNA from leaves of 1,2,4,6,8,13,15,16,29,30 using *ERF1* FP/RP
Pane D: Lane 1-20: minus RT PCR of the RNA from leaf and flower of 1,2,4,6,8,13,15,16,29,30 using *Actin* FP/RP
Pane E: Lane 1-20: RT PCR of cDNA of leaf and flower of 1,2,4,6,8,13,15,16,29,30 using *Actin* FP/RP
Lane - : PCR negative
Lane B: PCR Blank
Lane M: 100 bp DNA ladder
Lane +: PCR positive

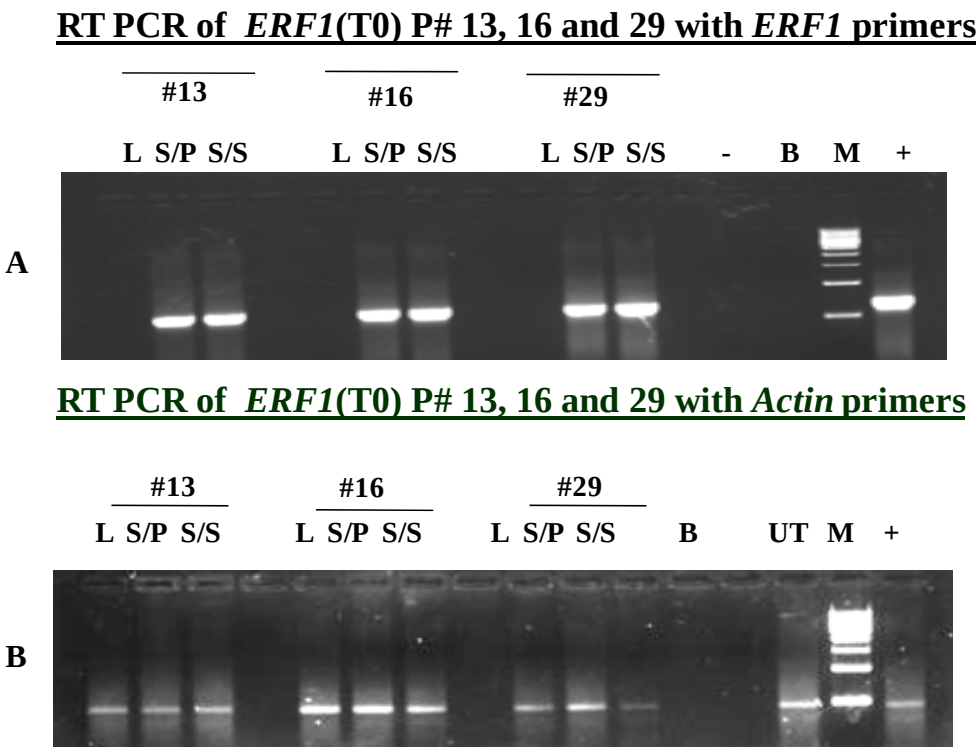


Fig 4.103. RT PCR of *ERF1* tobacco plant # 13,16 and 29 using RNA of Leaf (L), Sepals+Petals (S/P) and Stamens+Stigma (S/S)
Pane A: RT PCR of *ERF1* tobacco plant # 13,16 and 29 using RNA of Leaf (L), Sepals+Petals (S/P) and Stamens+Stigma (S/S) using *ERF1* FP/RP
Pane B: RT PCR of *ERF1* tobacco plant # 13,16 and 29 using RNA of Leaf (L), Sepals+Petals (S/P) and Stamens+Stigma (S/S) using *Actin* primers
Lane -/UT: PCR negative/ Untransformed tobacco RT PCR
Lane B: PCR Blank
Lane +: PCR positive
Lane M: 500bp DNA ladder

4.9.3. RT PCR of T_0 transgenic plants carrying *ACS7-AtEBP*-polyA

The PCR positive T_0 transgenic plants carrying ***ACS7-AtEBP*-polyA-PE** gene cassette were studied for their morphological characters (Plate.4.3) and were confirmed for the expression of the inserted gene through RT PCR. To confirm the expression of the transgene, RNA was isolated from the PCR positive T_0 plants, and checked for any DNA contamination by direct PCR (minus RT reaction) using *AtEBP* and *Actin* specific primers. The cDNA thus obtained was further subjected to PCR analysis using *AtEBP* forward and reverse primers to check the expression of the transgene and *Actin* was used as an endogenous control (Fig.4.104). *AtEBP* gene expression was observed both in the flower and the leaves of ten plants chosen for the RT-

PCR analysis. The samples were also positive with the endogenous control *Actin*. Three RT-PCR positive plant# 1,2 and 3 were chosen for the T_1 generation studies in the next generation.

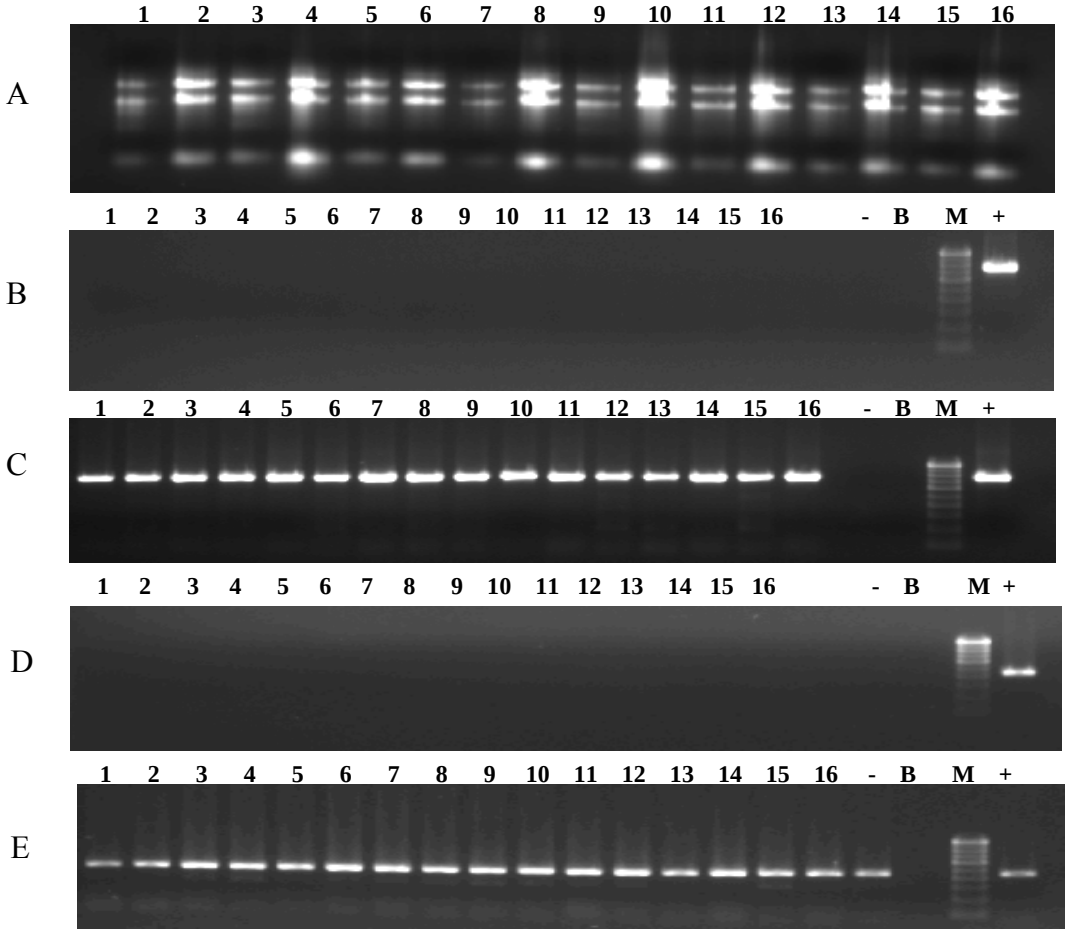


Fig4.104. RT PCR of *AtEBP*(T_0) tobacco transgenic plants
Pane A: Lane 1-16: RNA isolated from *AtEBP*1 tobacco transgenics (P#1-8) leaf and flower
Pane B: Lane 1-16: minus RT PCR of the RNA of (P#1-8) leaf and flower using *AtEBP* FP/RP
Pane C: Lane 1-16: RT PCR of cDNA of (P#1-8) leaf and flower using *AtEBP* FP/RP
Pane D: Lane 1-16: minus RT PCR of the RNA of (P#1-8) leaf and flower using *Actin* FP/RP
Pane E: Lane 1-16: RT PCR of cDNA of (P#1-8) leaf and flower using *Actin* FP/RP
Lane - : PCR negative
Lane B: PCR Blank
Lane +: PCR positive
Lane M: 100 bp DNA ladder

4.10. RT PCR analysis of the T_1 transgenic plants carrying BIK1 or ERF1 constructs

4.10.1. RT PCR analysis of *BIK1* (T_1) transgenic plants:

Thirty T_1 generation plants of T_0 plants #4,5 and 12 carrying *ACS4-BIK1*-P cassette were raised in the transgenic green house. The plants were confirmed for the presence of the gene cassette by PCR using gene, promoter and terminator specific primers. This analysis confirmed single gene insertions in the respective T_0 plants as they segregated in Mendelian monogenic ration (3:1). A few plants that were positive for PCR were selected for RT PCR analysis. The RNA was isolated from the leaves and flowers of randomly selected PCR positive *BIK1* plants from the population and quantified using agarose gel electrophoresis. The cDNA was checked for the transgene expression using *BIK1* and *Actin* FP and RP. The RT PCR positive plants of T_0 # 4 plant included #1-5, of T_0# 5 plant included #1,2,3,4,5,6,7,9,12 and of T_0 #12 included # 1,3,13,14,18,19,20,21 and 25 . These plants showed morphological variations corresponding to the expression of the transgene as observed in the T_0 plants. All the T_1 plants showed the expression of *BIK1* gene both in the leaves and in the flowers in all the three selected populations (Fig 4.105-107) indicating the promoter expression in both the leaf and in the flowers. The plants that showed RT PCR positive were subjected to *Botrytis* infection study.

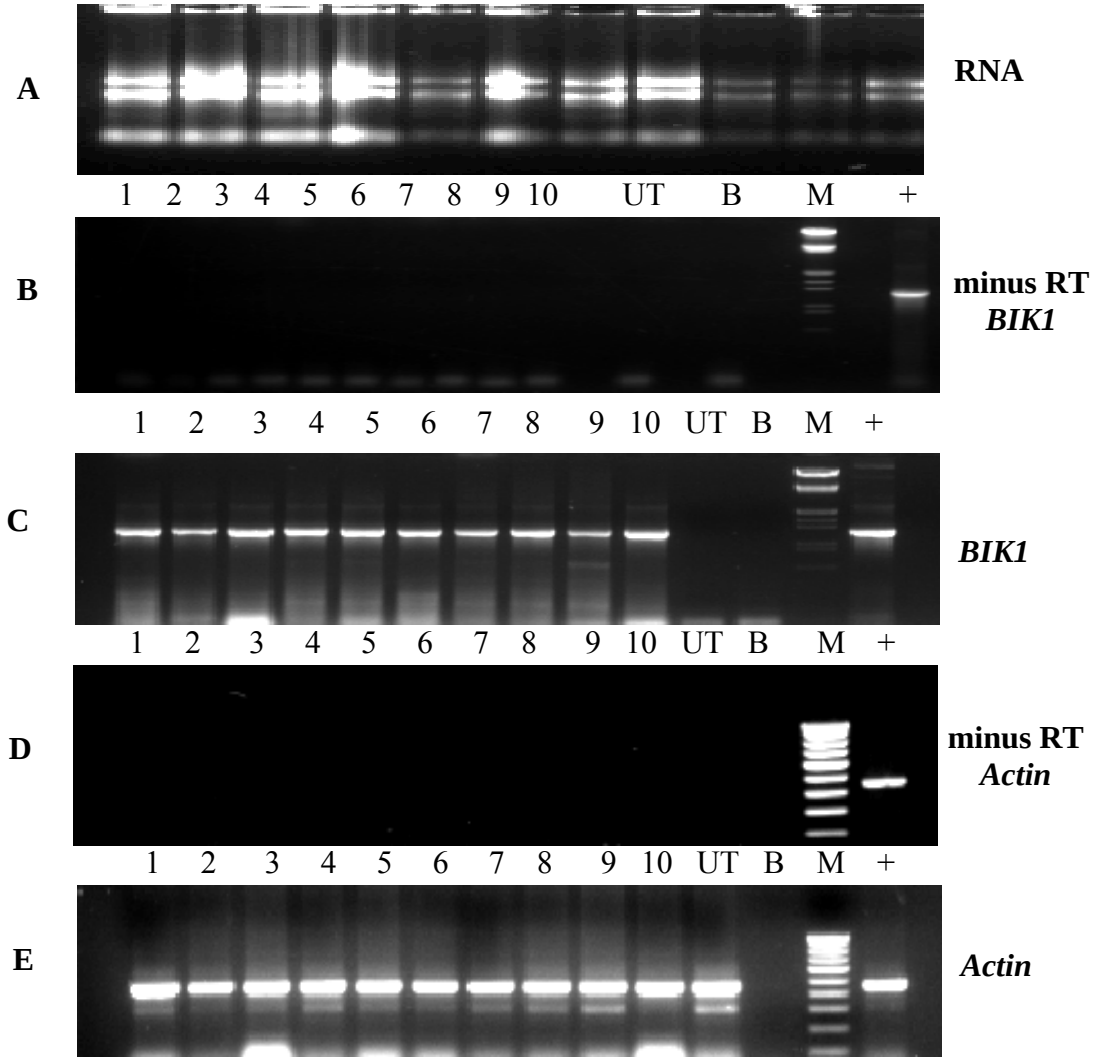


Fig 4.105. RT PCR of *BIK1*(T1) of T0 plant#4 tobacco transgenics of leaf and flower
Pane A. Lane 1-11: *BIK1*(T1) tobacco leaf and flower (P#1-5) and untransformed RNA
Pane B. Lane 1-10: minus RT PCR of *BIK1*(T1) tobacco leaf and flower of (P#1-5) with *BIK1* FP/RP RT
Pane C. Lane 1-10: RT PCR of *BIK1*(T1) tobacco leaf and flower of (P#1-5) with *BIK1* FP/RP
Pane D. Lane 1-10: minus RT PCR of *BIK1*(T1) tobacco leaf and flower of (P#1-5) with *Actin* FP/RP
Pane E. Lane 1-10: PCR of *BIK1*(T1) tobacco leaf and flower of (P#1-5) with *Actin* FP/RP
Lane M: Lambda *Hind* III/*Eco*RI double digest
Lane +: PCR positive
Lane B: PCR blank
UT: Untransformed tobacco control cDNA

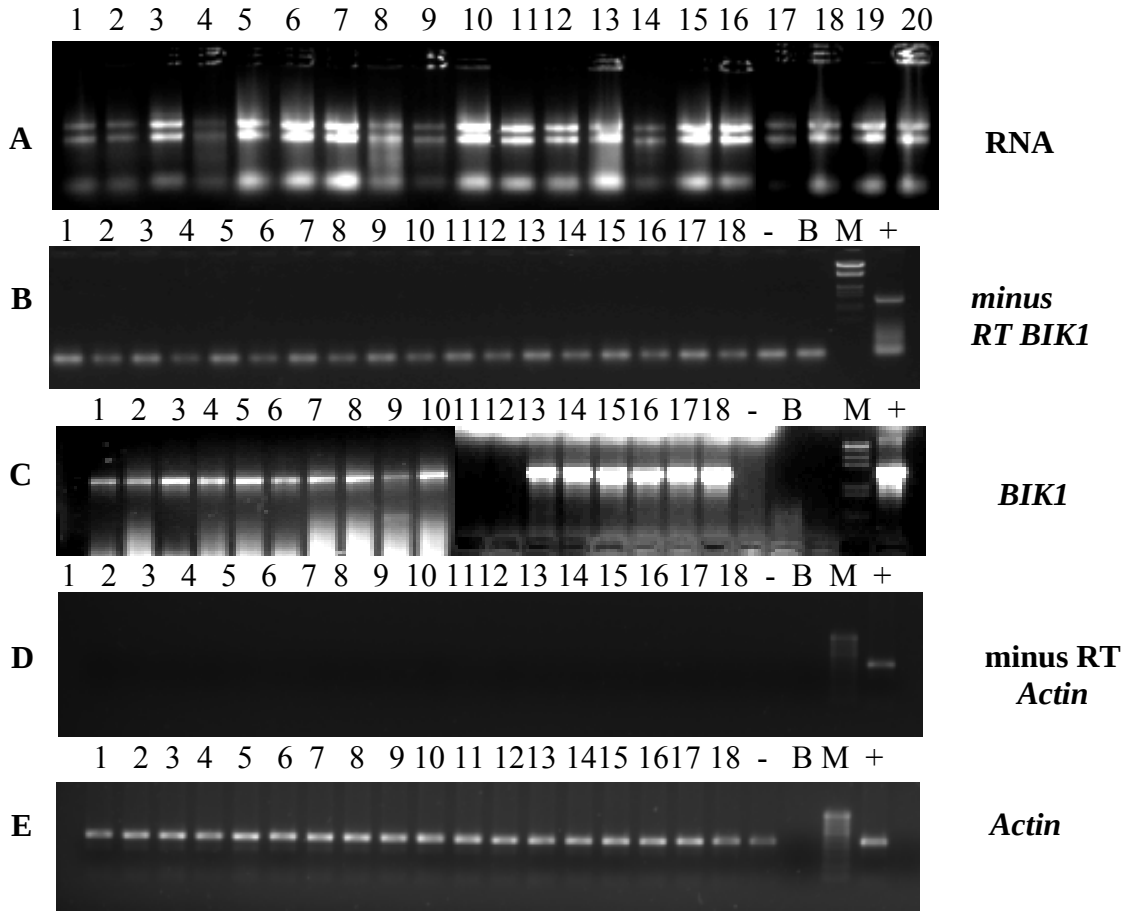


Fig4.106.RT PCR of *BIK1*(T1) of T0 plant # 5 tobacco transgenics of leaf and flower
Pane A. Lane 1-20: *BIK1*(T1) tobacco leaf and flower (p# 1,2,3,4,5,6,7,9,12) and untransformed RNA
Pane B. Lane 1-18: minus RT PCR of *BIK1*(T1) tobacco leaf and flower of (p# 1,2,3,4,5,6,7,9,12) with *Actin* FP/RP
Pane C. Lane 1-18: RT PCR of *BIK1*(T1) tobacco leaf and flower of (p# 1,2,3,4,5,6,7,9,12) with *Actin* FP/RP
Pane D. Lane 1-18: minus RT PCR of *BIK1*(T1) tobacco leaf and flower of (p# 1,2,3,4,5,6,7,9,12) with *BIK1* FP/RP
Pane E. Lane 1-18: RT PCR of *BIK1*(T1) tobacco leaf and flower of (p# 1,2,3,4,5,6,7,9,12) with *BIK1* FP/RP
Lane M: Lambda *Hind* III/*Eco*RI double digest
Lane +: PCR positive
Lane B: PCR blank
UT: Untransformed tobacco control cDNA

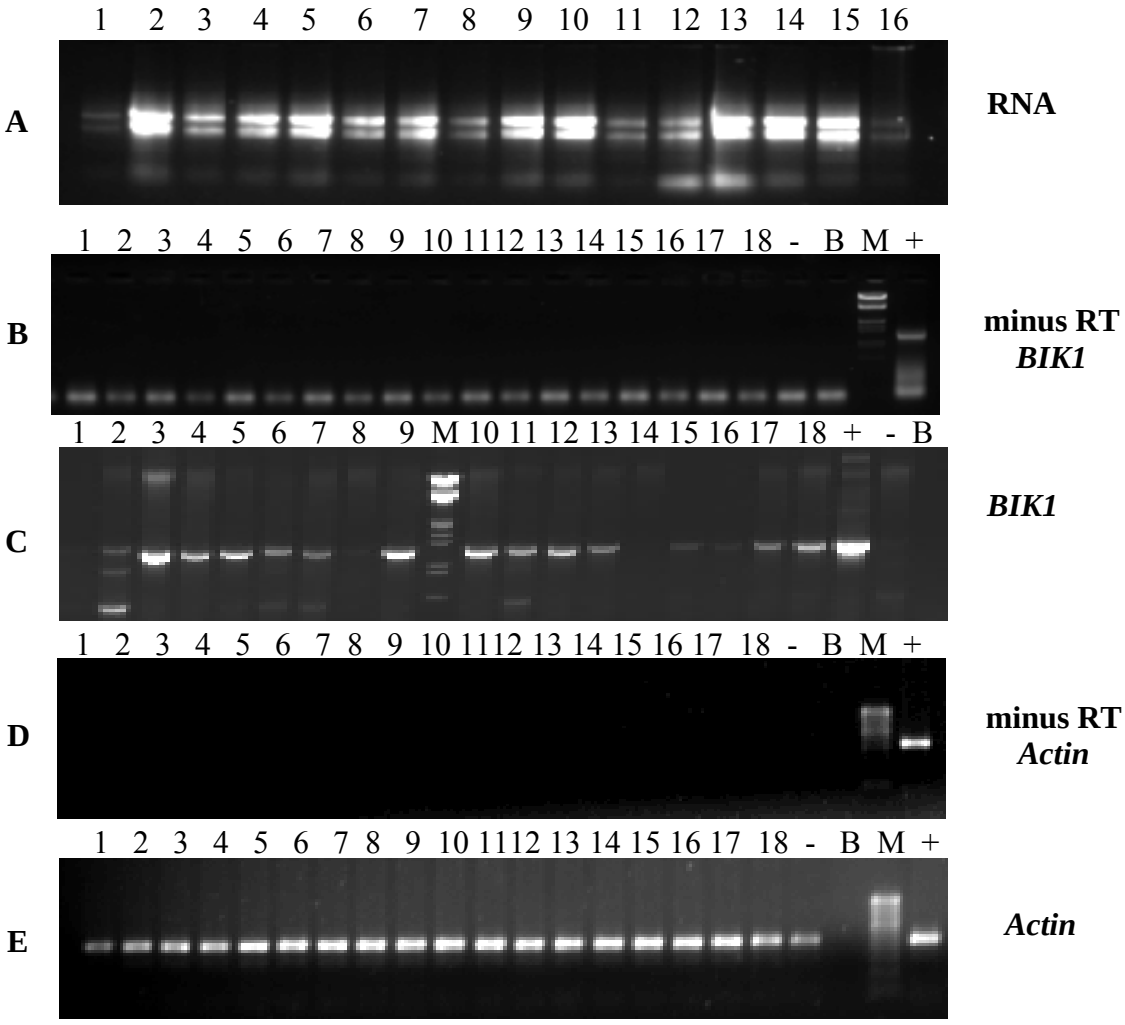


Fig 4.107. RT PCR of *BIK1*(T1) of T0 plant # 12 tobacco transgenics of leaf and flower

Pane A. Lane 1-20: *BIK1*(T1) tobacco leaf and flower of (# 1,3,13,14,18,19,20,21, 25 and untransformed RNA

Pane B. Lane 1-18: minus RT PCR of *BIK1*(T1) tobacco leaf and flower with *BIK1* FP/RP

Pane C. Lane 1-18: RT PCR of *BIK1*(T1) tobacco leaf and flower of (# 1,3,13,14,18,19,20,21, 25) with *BIK1* FP/RP

Pane D. Lane 1-18: minus RT PCR of *BIK1*(T1) tobacco leaf and flower of (# 1,3,13,14,18,19,20,21, 25) with *Actin* FP/RP

Pane E. Lane 1-18: RT PCR of *BIK1*(T1) tobacco leaf and flower of (# 1,3,13,14,18,19,20,21, 25) with *Actin* FP/RP

Lane M: Lambda *Hind* III/*Eco*RI double digest

Lane +: PCR positive

Lane B: PCR blank

UT: Untransformed tobacco control cDNA

4.10.2. RT PCR analysis of *ERF1* (T_1) transgenic plants:

Thirty T_1 generation plants of T_0 plants # 13, 16 and 29 carrying *ACS5*-*ERF1*-PS cassette were raised in the transgenic green house. The T_0 plant#13 produced were very few in number and only 1 plant out of 8 seeds could be recovered. The plants were confirmed for the presence of the gene cassette by PCR using gene, promoter and terminator specific primers. This analysis confirmed single gene insertions in the respective T_0 plants as they segregated in Mendelian monogenic ration (3:1). From each of the three populations, a few plants that were positive for PCR were selected for RT PCR analysis. The RNA was isolated from the leaves and flowers of the selected PCR positive *ERF1* plants from the population and quantified using agarose gel electrophoresis. The cDNA was checked for the transgene expression using *ERF1* and *Actin* specific primers. The T_1 plants showed morphological variations (Plate 4.2) corresponding to the expression of the transgene. The plant showed stunted growth, flower drop and very less frequency of fertilization due to malformed anthers and short anther filaments. Hence the T_1 of plant#13 did not give rise to any seed formation. All the PCR positive T_1 plants of T_0 plants# 13 (#1), 16 (#1,2,3,5,6,7,9,11,12,13) and 29 (#4,6,8,11,12,13,15,18,19,20) showed the expression of *ERF1* gene both in the leaves and in the flowers indicating the promoter expression in both the leaf and in the flowers (Fig 4.108-110). The plants that showed RT PCR positive were subjected to *Botrytis* infection study.

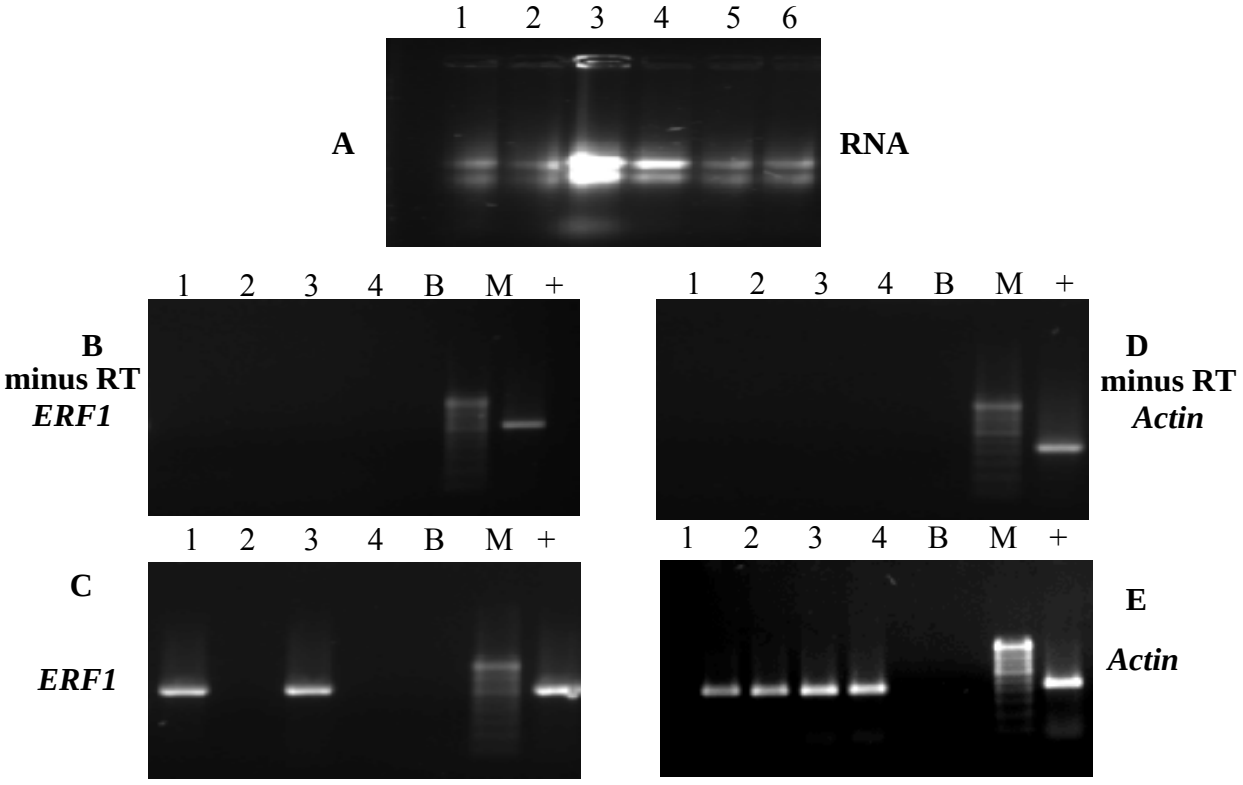


Fig 4.108.RT PCR of *ERF1*(T1) of plant # 13 (T0) using *ERF1* and *Actin* primers

Pane A: *ERF1*(T1) tobacco leaf and flower of P# 1and untransformed RNAof
Pane B and C:
Lane 1: PCR of *ERF1* (T1) leaf cDNA of P# 1 using *ERF1* FP/RP
Lane 2: PCR of untransformed leaf cDNA using *ERF1* FP/RP
Lane 3: PCR of *ERF1* (T1) flower cDNA of P# 1 using *ERF1* FP/RP
Lane 4: PCR of untransformed flower cDNA using *ERF1* FP/RP
Pane D and E:
Lane 1: PCR of *ERF1* (T1) leaf cDNA of P# 1 using *Actin* FP/RP
Lane 2: PCR of untransformed leaf cDNA using *Actin* FP/RP
Lane 3: PCR of *ERF1* (T1) flower cDNA of P# 1 using *Actin* FP/RP
Lane 4: PCR of untransformed flower cDNA using *Actin* FP/RP

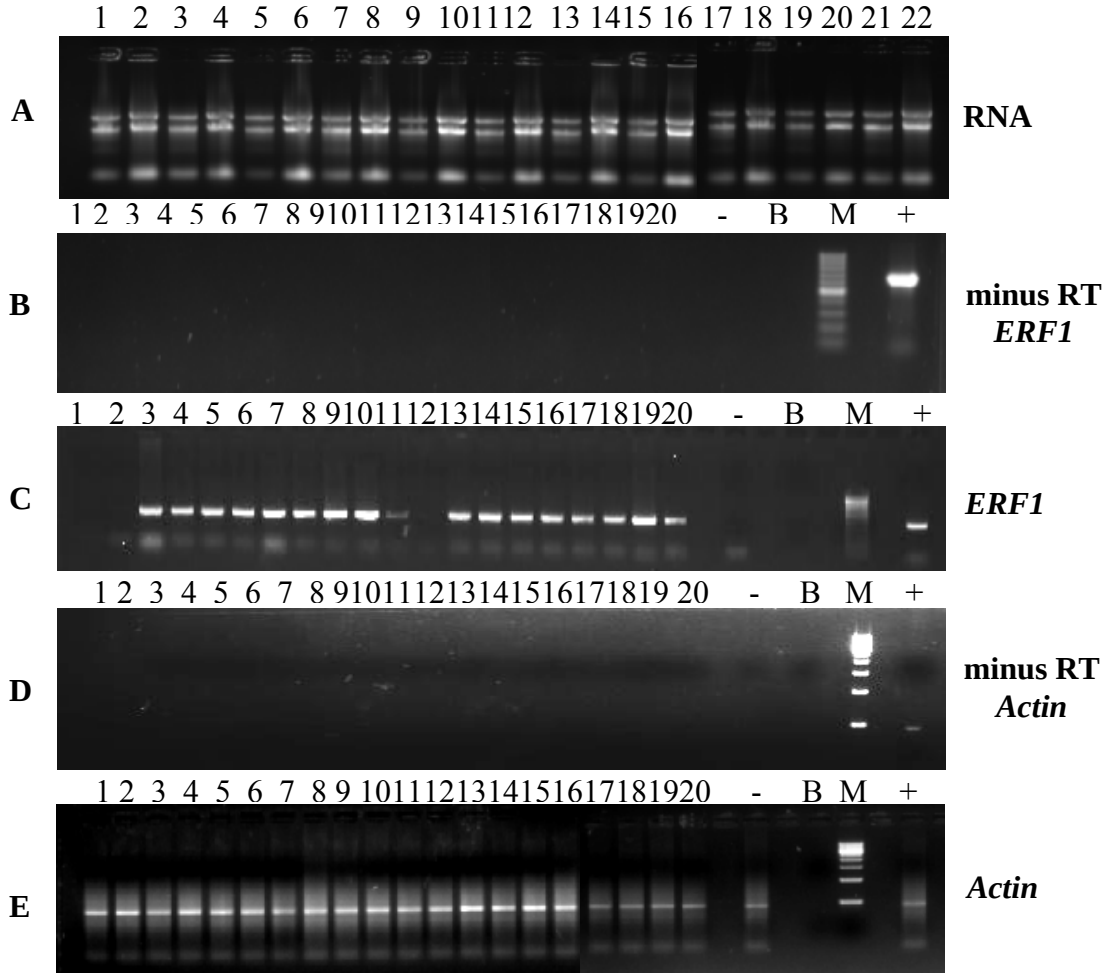


Fig 4.109.RT PCR of *ERF1*(T1) of T0 plant # 16 tobacco transgenics of leaf and flower
Pane A. Lane 1-22: *ERF1*(T1) tobacco leaf and flower of (# 1,2,3,5,6,7,9,11,12,13) and untransformed RNA
Pane B.Lane 1-20: minus RT PCR of *ERF1* (T1) tobacco leaf and flower of (# 1,2,3,5,6,7,9,11,12,13) with *ERF1* FP/RP
Pane C. Lane 1-20: RT PCR of *ERF1* (T1) tobacco leaf and flower of (# 1,2,3,5,6,7,9,11,12,13) with *ERF1* FP/RP
Pane D. Lane 1-20: minus RT PCR of *ERF1* (T1) tobacco leaf and flower of (# 1,2,3,5,6,7,9,11,12,13) with *Actin* FP/RP
Pane E. Lane 1-20: RT PCR of *ERF1* (T1) tobacco leaf and flower of (# 1,2,3,5,6,7,9,11,12,13) with *Actin* FP/RP
Lane M: 100bp and 500bp DNA ladder
Lane +: PCR positive
Lane B: PCR blank
Lane - : Untransformed tobacco control cDNA

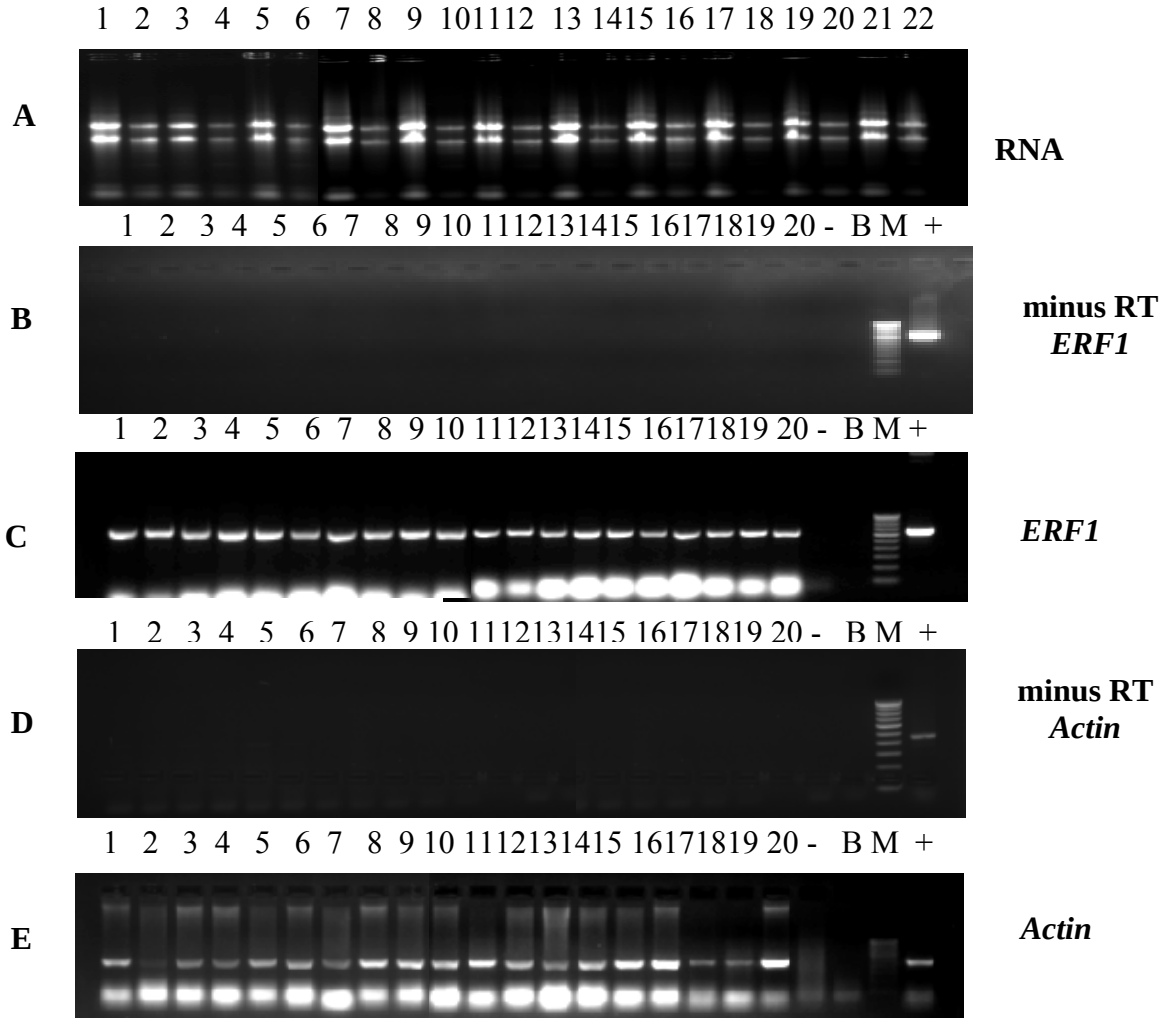


Fig 4.110.RT PCR of *ERF1*(T1) of T0 plant # 29 tobacco transgenics of leaf and flower
Pane A. Lane 1-22: *ERF1*(T1) tobacco leaf and flower of (P#4,6,8,11,12,13,15,18,19,20) and untransformed RNA
Pane B.Lane 1-20: minus RT PCR of *ERF1* (T1) tobacco leaf and flower of (P#4,6,8,11,12,13,15,18,19,20) with *ERF1* FP/RP
Pane C. Lane 1-20: RT PCR of *ERF1* (T1) tobacco leaf and flower of (P#4,6,8,11,12,13,15,18,19,20) with *ERF1* FP/RP
Pane D. Lane 1-20: minus RT PCR of *ERF1* (T1) tobacco leaf and flower of (P#4,6,8,11,12,13,15,18,19,20) with *Actin* FP/RP
Pane E. Lane 1-20: RT PCR of *ERF1* (T1) tobacco leaf and flower of (P#4,6,8,11,12,13,15,18,19,20) with *Actin* FP/RP
Lane M: 100bp DNA ladder
Lane +: PCR positive
Lane B: PCR blank
Lane - : Untransformed tobacco control cDNA

4.10.3. RT PCR analysis of *AtEBP* (T_1) transgenic plants:

Thirty T_1 generation plants of T_0 plants # 1 carrying *ACS7-AtEBP*-PE cassette were raised in the transgenic green house. The plants were confirmed for the presence of the gene cassette by PCR using gene, promoter and terminator specific primers. This analysis confirmed single gene insertions in the respective T_0 plants as they segregated in Mendelian monogenic ration (3:1). Plants that were positive for PCR were selected for RT PCR analysis. The RNA was isolated from the leaves and flowers of the selected PCR positive *AtEBP* plants from the population and quantified using agarose gel electrophoresis. The cDNA was checked for the transgene expression using *AtEBP* and *Actin* specific primers. The T_1 plants showed morphological variations (Plate 4.3) corresponding to the expression of the transgene. The plant showed stunted growth, broad and thick leaves, normal fertilization and seed set. All the PCR positive T_1 plants of T_0 plants#1(#1,3,5,7,8,9,11,12,15 and 19) showed the expression of *AtEBP* gene both in the leaves and in the flowers indicating the promoter expression in both the leaf and in the flowers (Fig4.111). The plants that showed RT PCR positive were subjected to *Botrytis* infection study.

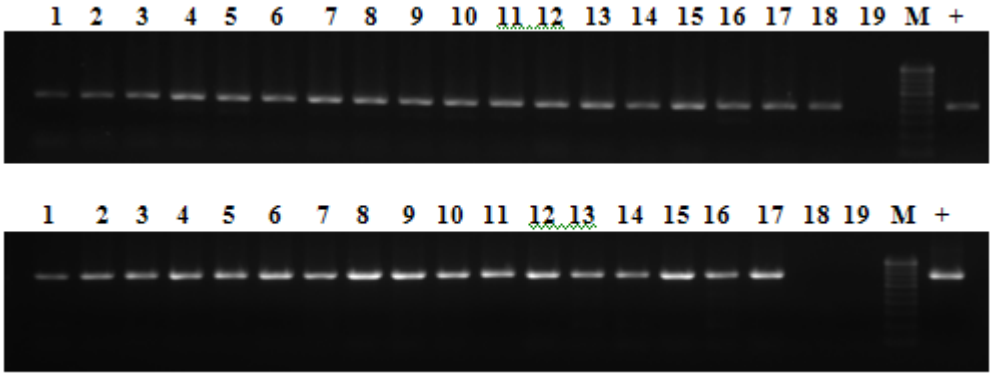


Fig 4.111. RT-PCR of the *AtEBP* (T_1) tobacco leaf and flowers
Lane# 1,3,5,7,9,11,13,15and 17 : RT PCR of *AtEBP* (T_1) using *Actin* FP/RP
Lane# 2,4,6,8,9,10,12,14and 16 : RT PCR of *AtEBP* (T_1) using *AtEBP* FP/RP
Lane# 18 : Untransformed control
Lane# 19 : PCR Blank
Lane# M : Marker
Lane# + : Positive control –PCR of *AtEBP* gDNA

4.11.0. Dot Blot analysis of the single gene transgenics

The single gene transgenics of pCAMBIA 1300- *ACS4-BIK1*-polyA-P (P#1, 2, 3,4,5,10,12), pCAMBIA 1300-*ACS5-ERF1*-PS (#2,4,5,11.13.16.29) and pCAMBIA 1300-*ACS7-AtEBP*-PE (#1,2,13,16,17,21,26) were analyzed for the stable integration of the transgene cassette into the plant genome by Dot Blot analysis. In this method the genomic DNA (1ug) of the PCR and RT PCR positive tobacco transgenics were considered. The gDNA in each case was denatured and spotted on to N+ Hybond membrane and was hybridized with alkaline phosphatase probes specific to individual genes in the gene cassette. The blot was then subjected to a detection of the hybridized probes using *CDP star* detection system and further developing the blot on an X-Ray film.

In each case the seven PCR and RT PCR positive plants were taken for the analysis and the untransformed tobacco plant genomic DNA acted as the negative control and the plasmid DNA harbouwas used as the positive control for the gene specific blot. The gDNA spots hybridized using *Actin* probes was also performed to ensure the authenticity of the gDNA samples taken for the analysis and PCR product of 200 picograms of *Actin* was taken as the positive control. The dot blots of the gDNA samples showed a positive hybridization both with *Actin* and gene specific probes indicating the stable integration of the transgene cassette into the tobacco transgenic plants. The gene specific probes did not show any hybridization with the untransformed control gDNA (Fig 4.112). The positive plants were further studied for their segregation in the subsequent generation.

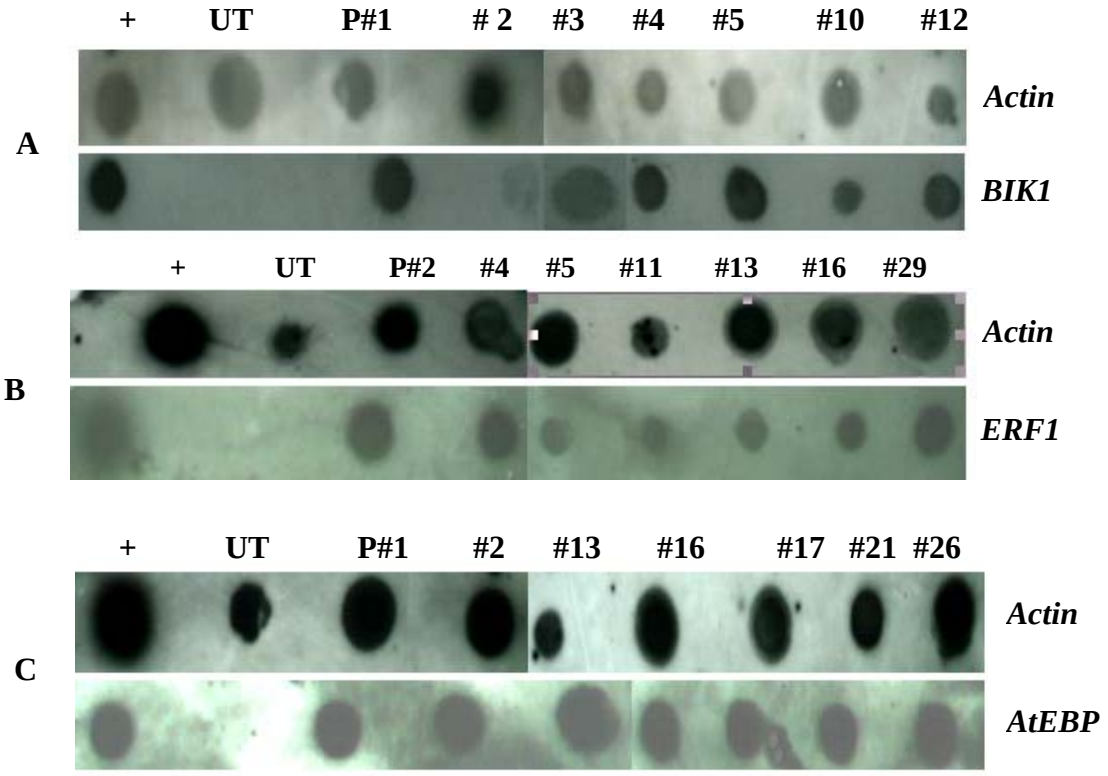


Fig4.112. Dot Blot analysis of the single gene tobacco transgenics
Panel A: Dot blot of *BIK1* tobacco transgenics using *Actin* and *BIK1* specific probes
Panel B: Dot blot of *ERF1* tobacco transgenics using *Actin* and *ERF1* specific probes
Panel C: Dot blot of *AtEBP* tobacco transgenics using *Actin* and *AtEBP* specific probes
Lane UT: Untransformed tobacco gDNA
Lane +: Positive

4.12.0. Study of genes upregulated in *BIK1, ERF1* and *AtEBP* tobacco transgenics

As it is well known that the expression of the defense regulatory genes in plants would subsequently switch on/off many downstream genes involved in defense process, the regulation of such genes in the transgenic plants developed under the present investigation were investigated. Downstream defense regulatory or the pathogenesis related (PR) gene expression pattern was studied by RT PCR using the cDNA synthesized from the RNA of flowers in each case. The PR genes like *prb-1b* (PR1), *GLA* (PR2), *CHN50* (PR3) and *Osmotin* (PR5) and the defense regulatory gene (Nt_*ERF1*) and the internal control *Actin* were studied for their levels of gene expression in the single gene transgenics.

It was observed that in *BIK1* transgenics (Fig4.113) the expression of PR2 and 3 was up regulated and increased by 2 folds, in *ERF1* transgenics (Fig4.114) the expression of PR1 and 3 increased by 4 folds and PR5 was increased by 2 folds and the *AtEBP* transgenics (Fig4.115) showed a 2 fold increase in the expression of PR 2, 3 and 5 genes. The innate tobacco Nt_*ERF1* gene showed an increased expression in all the three single gene tobacco transgenics indicating the crosstalk between these pathogen induced defense regulatory genes in the developed tobacco transgenics. Hence, this data supported the hypothesis of using defense regulatory genes to up-regulate the expression of many defense related genes simultaneously and thus increase the probability of getting better disease tolerance in such plants.

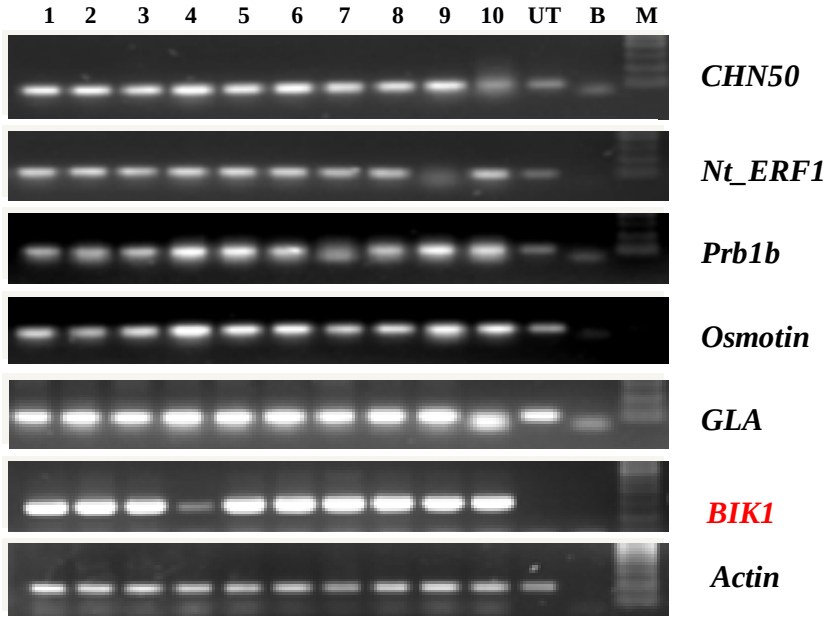


Fig 4.113. Expression study of different upregulated defense regulatory genes in *BIK1* single gene tobacco transgenics

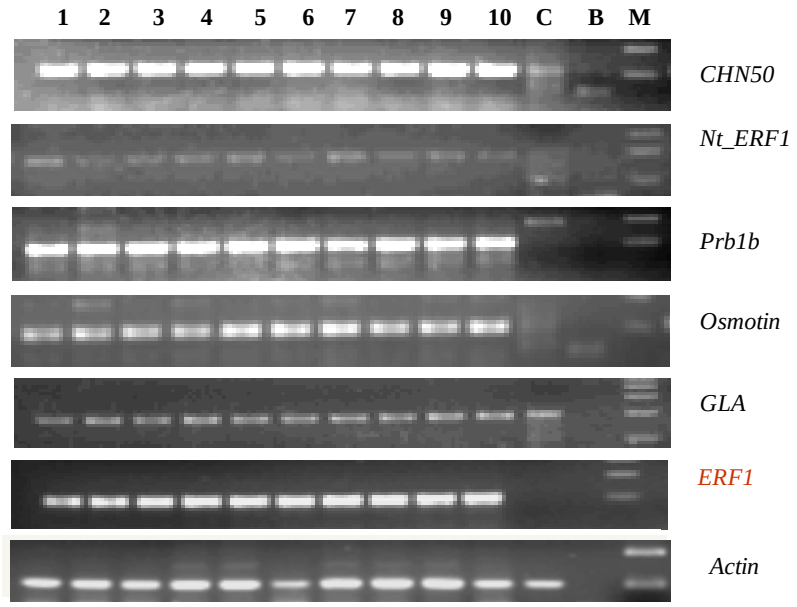


Fig 4.114. Expression study of different upregulated defense regulatory genes in *ERF1* single gene tobacco transgenics

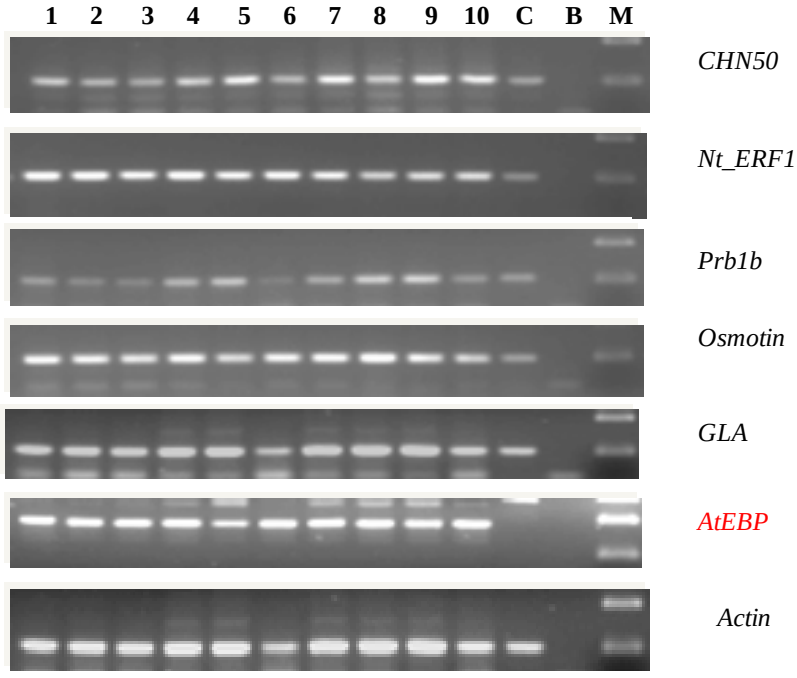


Fig 4.115. Expression study of different upregulated defense regulatory genes in *AtEBP* single gene tobacco transgenics

4.13.0. Infection assays of the developed tobacco transgenics

The T_1 generation single gene transgenic plants (*BIK1*#3, *ERF1*#4 and *AtEBP*#2) of all three gene cassettes showing positive reaction with PCR and RT-PCR were selected for the infection assays using two necrotrophic fungi *Botrytis cinerea* and *Alternaria alternata*. As the expression of the gene cassettes was observed both in the leaves and flower through RT-PCR analysis, both leaves and flower were studied for tolerance against both the necrotrophs.

4.13.1. Excised leaf infection assays

The excised leaves on inoculation with the *Botrytis cinerea* (Fig 4.116) and *Alternaria alternata* (Fig 4.117) separately, with a concentration of 1x 10^6 of spore suspension and incubation at 22^0C, 90% humidity and with a photoperiod of 18hrs dark and 6 hrs of light was observed for 7days post infection. The necrotic lesions were observed in the control leaves and flowers from day 4 onwards and the necrosis increased until day7. The necrosis was more prominent in the control tissues and was reduced in *BIK1* and *AtEBP* and highly reduced in *ERF1* tobacco. These results indicated *ERF1* transgenic plants to be more tolerant than *BIK1* and then followed by *AtEBP* when compared to control plants. This also indicated the varying degree of tolerance exhibited by three defense regulatory genes against *B. cinerea* and *Alternaria alternata.*

4.13.2. Excised inflorescence infection assays

As the expression of all the three defense regulator genes *BIK1, ERF1* and *AtEBP* was driven by promoters with elevated expression in the inflorescence which was proven by RT-PCR analysis, the excised inflorescence infection assays were carried out to study the tolerance exhibited by the transgenics. The excised inflorescences from the same plants as used for leaf assay (*BIK1*#3, *ERF1*#4 and *AtEBP*#2) were subjected to infection assays using necrotrophic fungi *Botrytis cinerea.* The excised inflorescence of both the transgenic and control plants was sprayed using 1x10^6 sore suspension of *B. cinerea* and incubated at 22^0C, 90% humidity and with a photoperiod of 18hrs dark and 6 hours of light for 7 days. The browning of the floral tissues was observed from 2 days post infection until 7 days. The flowers of control plants showed complete browning on day 4 when compared to the water inoculated control flowers. The *BIK1*, *ERF1* and *AtEBP* flowers showed considerably reduced browning than the untransformed control indicating the tolerance imparted by the transgenes against the necrotrophic fungi.

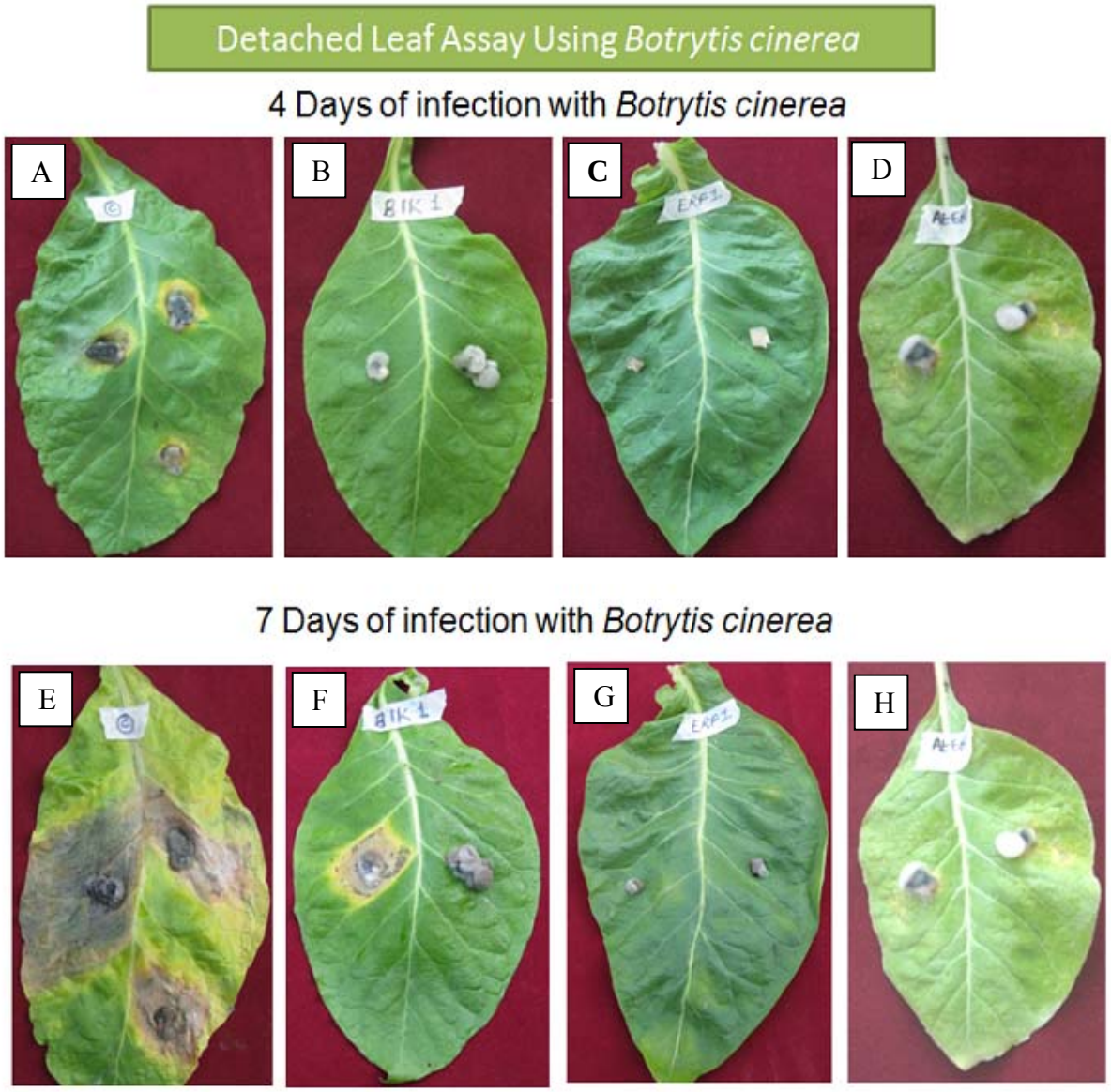


Fig 4.116. Excised leaf assay of tobacco control and transgenic plants using *Botrytis cinerea* 4days and 7days post infection studies
Pane A and E: Tobacco control leaf 4 and 7 days post infection
Pane B and F: *BIK1* tobacco leaf 4 and 7 days post infection
Pane C and G: *ERF1* tobacco leaf 4 and 7 days post infection
Pane D and H: *AtEBP1* tobacco leaf 4 and 7 days post infection

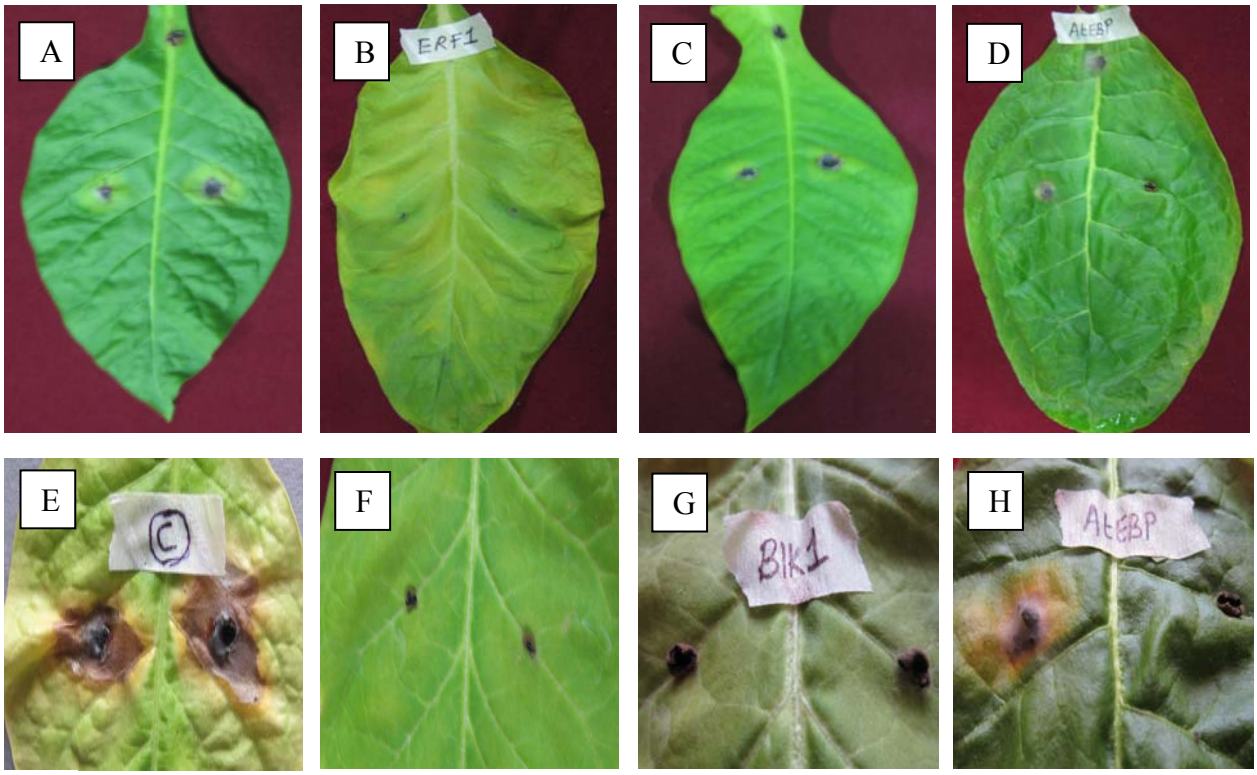


Fig 4.117. Excised leaf assay of tobacco control and transgenic plants using *Alternaria alternata* 4days and 7days post infection studies
Pane A and E: Tobacco control leaf 4 and 7 days post infection
Pane B and F: *BIK1* tobacco leaf 4 and 7 days post infection
Pane C and G: *ERF1* tobacco leaf 4 and 7 days post infection
Pane D and H: *AtEBP1* tobacco leaf 4 and 7 days post infection

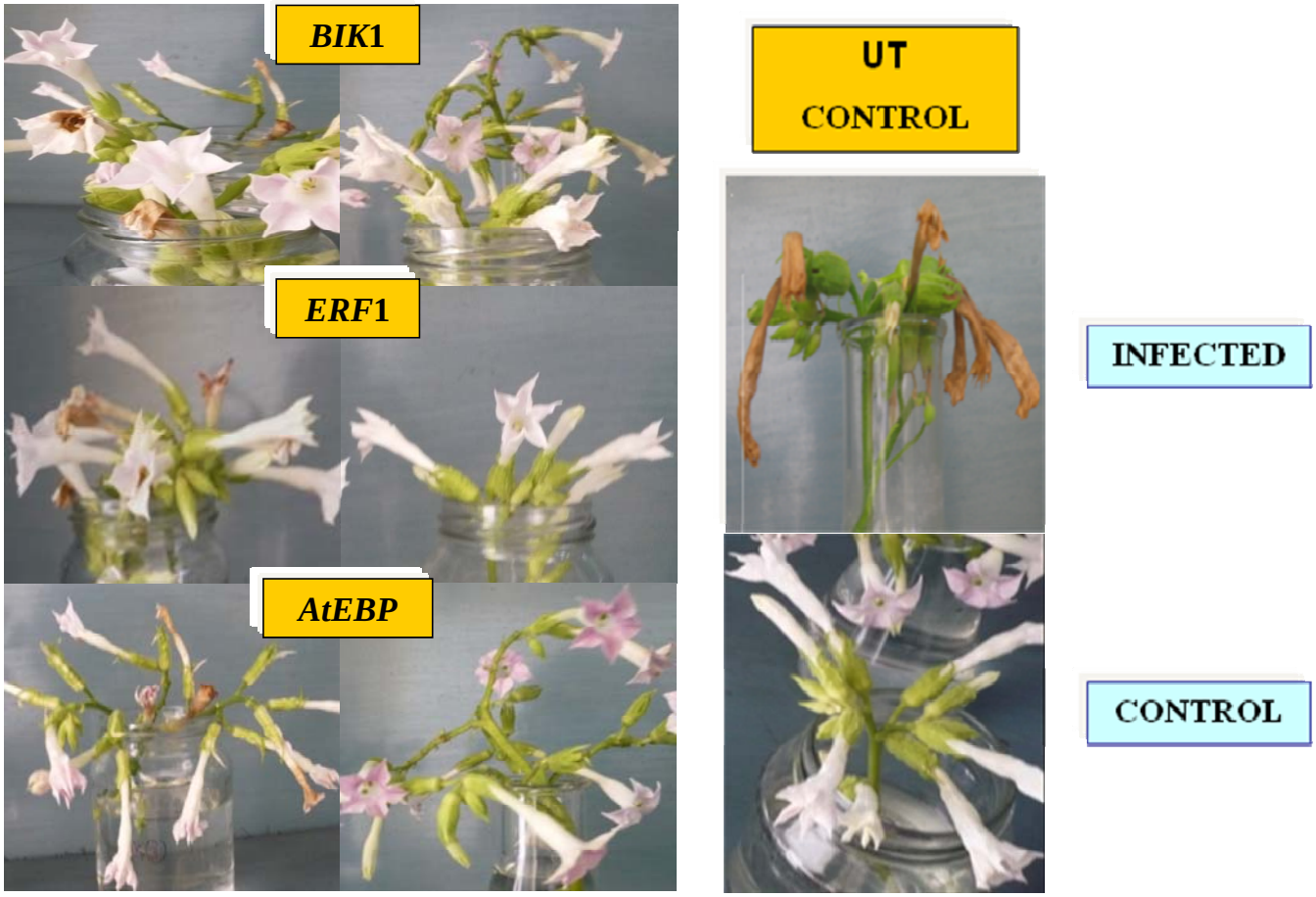


Fig 4.118. Excised inflorescence assay of tobacco control and transgenic flowers using *Botrytis cinerea* 4 days post infection studies
Pane A: Tobacco control flower 4 days post infection
Pane B: *BIK1* tobacco flower 4 days post infection
Pane C: *ERF1* tobacco flower 4 days post infection
Pane D: *AtEBP1* tobacco flower 4 days post infection

4.14 Castor transformation

4.14.1 Meristem based transformation

The meristem based castor transformation method developed at DOR (Sujatha *et al*., 2004 and Athma *et al*., 1989) was tried in the present investigation. The embryo axes of castor DCS-9 was subjected to transformation using *Agrobacterium* LBA4404 harbouring double and triple gene cassette vectors. The shoot regeneration was observed until third round of selection. But, the shoots could not elongate once out of selection pressure and thus could not be taken further for rooting and acclimatization. The details of tissue culture based shoot regeneration is shown in Fig 4.119 and 4.120. The logistics of meristem based transformation is shown in Table 4.5.

4.14.2 *In planta* transformation

Owing to the problems of tissue culture mediated transformation of castor, alternate non-tissue culture based methods have been developed and used in castor. In the present investigation, as transgenic plants could not be realized through tissue culture based methods, *in planta* transformation protocol as reported by (Kumar *et al*., 2011) was used for realizing transformants in castor. Germinated seedlings of castor on infection with the triple gene cassette vector yielded hundered seeds on acclimatization. But, none of them showed positive reaction in the transgene specific PCR. The logistics of *in planta* based transformation is shown in Table 4.6. The trials for obtaining transgenics using *in planta* are in progress using the triple gene and the concoction of three single gene constructs.

Fig 4.119. Castor Meristum based transformation
Pane A: Castor seeds DCS-9
Pane B: Decoated Castor seeds DCS-9
Pane C: Excised embryos of Castor
Pane D: Cocultivated embryoids
Pane E: Shoot Regeneration from Cocultivated embryos

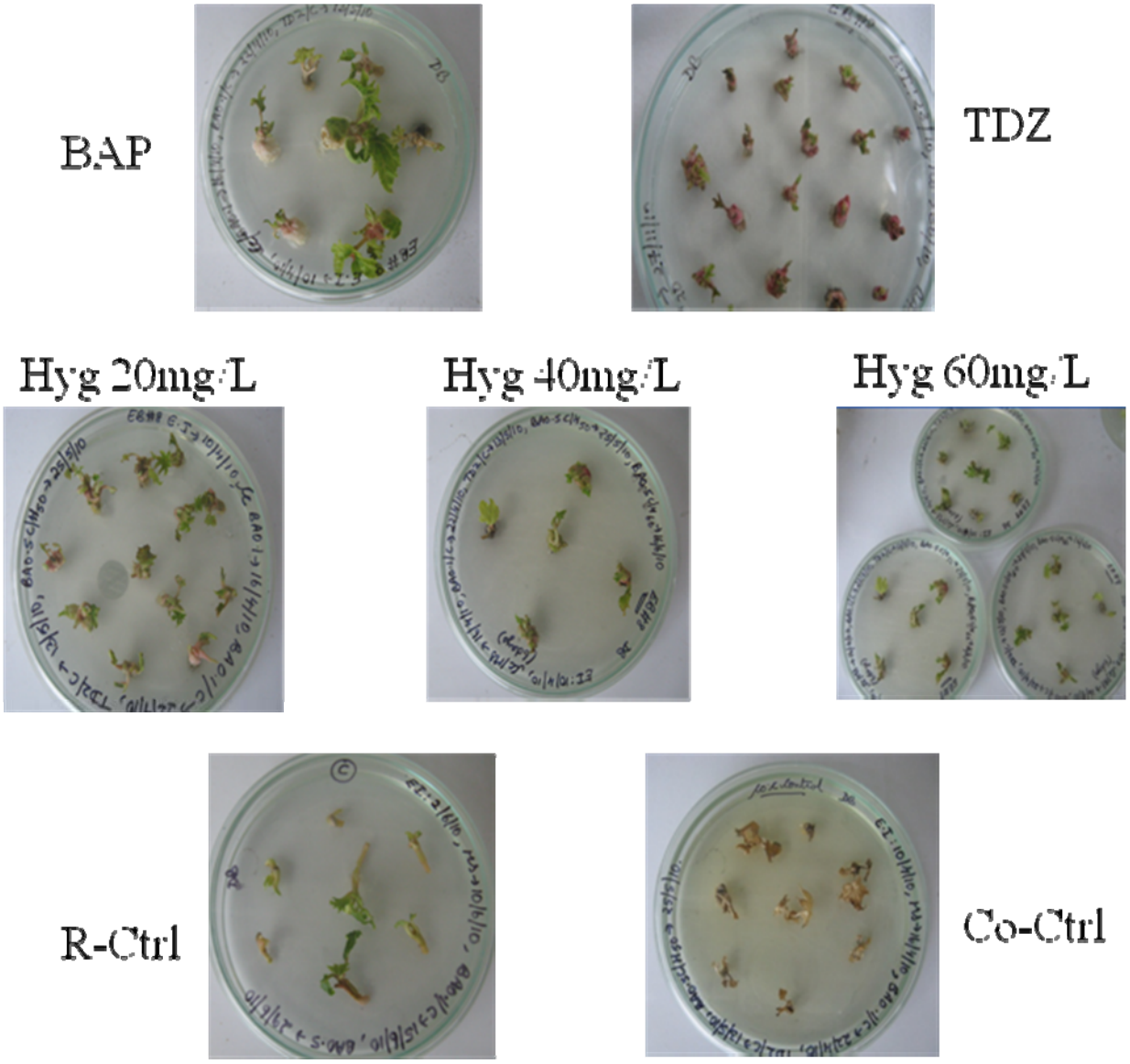


Fig4.120. Castor Meristum based transformation
Pane A: Castor seeds DCS-9
Pane B: Decoated Castor seeds DCS-9
Pane C: Excised embryos of Castor
Pane D: Cocultivated embryoids
Pane E: Shoot Regeneration from Cocultivated embryos

In summary, of the research work, to validate the developed vectors, tobacco was transformed and the transgenic plants were analyzed for the presence and expression of the transferred gene cassettes. Though transgenic tobacco plants were realized with the single gene cassettes, no plants could be obtained with the double and triple gene cassettes. But this issue was not investigated further. However, to study the cumulative effects of the two gene cassettes in conferring resistance, the transgenic plants carrying single genes were crossed and the progeny plants were obtained. Analysis of these plants could form future line of work. Confirmed transgenic tobacco plants carrying single gene cassettes were subjected to disease tolerance test by detached leaf and inflorescence assays. These results clearly indicated that expression of regulatory genes increased the disease tolerance substantially. RT-PCR analysis with the transgenic plants indicated that pathogenesis related genes were up-regulated due to the expression of introduced transgenes. Though several attempts were made to realize transgenic castor plants with the developed constructs, there was no success. This shall also become the future line of work.

Table 4.1: Details of constructs/clones developed during the present investigation

Insert	Source for insert isolation	Size of Insert (bp)	Enzymes used for cloning	Vector into which insert was cloned	Constructs / Clones generated
PolyA	T/A PolyA	250bp	*Sal*I/*Pst*I	pCAMBIA 1300	p1300-polyA-P
PolyA	T/A PolyA	250bp	*Sal*I/*Pst*I	pCAMBIA 1300	p1300-polyA-PS
PolyA	T/A PolyA	250bp	*Sal*I/*Pst*I	pCAMBIA 1300	p1300-polyA-PE
AtACS4 promoter	*A.thaliana* genomic DNA	1.2 kb	*Kpn*I/*BamH*I	pCAMBIA 1300	p1300-ACS4-P
AtACS5 promoter	*A.thaliana* genomic DNA	1.24 kb	*Kpn*I/*BamH*I	pCAMBIA 1300	p1300-ACS5-PS
AtACS7 promoter	*A.thaliana* genomic DNA	1.22 kb	*Kpn*I/*BamH*I	pCAMBIA 1300	p1300-ACS7-PE
BIK1	*A.thaliana* genomic DNA	1188bp	*BamH*I/ *Sal*I	pCAMBIA 1300	p1300-ACS4-*BIK1*-P
ERF1	*A.thaliana* genomic DNA	650bp	*BamH*I/ *Sal*I	pCAMBIA 1300	p1300-ACS5-*ERF1*-PS
AtEBP	*A.thaliana* genomic DNA	747bp	*BamH*I/ *Sal*I	pCAMBIA 1300	p1300-ACS7-*AtEBP*-PE
AtACS4 promoter	*A.thaliana* genomic DNA	1.2 kb	*Hind*III/*Eco*RI	pCAMBIA 1381z	p1381z-ACS4
AtACS5 promoter	*A.thaliana* genomic DNA	1.24 kb	*Hind*III/*Eco*RI	pCAMBIA 1381z	p1381z-ACS5
AtACS7 promoter	*A.thaliana* genomic DNA	1.22 kb	*Hind*III/*Eco*RI	pCAMBIA 1381z	p1381z-ACS7

Table 4.6. Logistics of the castor transformation work carried out during the investigation

No. of embryos	No. of explants co-cultivated	No. of explants on recovery medium	No.of explants on Proliferation medium	No.of explants on sel I	No.of explants on sel II	No.of explants on sel III	No.of explants on 1st cycle of proliferation	No.of explants on2nd cycle of proliferation
20,400 (AEB)	16,320	15,360	7,400	3,452	1,750	456	600	-
(EB)	2,000	1,136	546	327	116	71	-	-
AtEBP	823	773	331	178	56	40	-	-

Table.4.7. Logistics of the castor *in planta* transformation carried out during the investigation

Construct used inplanta experiments	Genotype	No.of inplanta experiments	No.of Seeds used for germination	No.of seeds germinated and subjected to Agro-infection	No. of plants transferred to vermiculite	No. of plants transferred to Soil	No.of seeds harvested	No.of seeds transformed
pCAMBIA 1300-AEB#27	**DCS9 (Jyoti)**	**2**	**120**	**102**	**64**	28	100	**None (Trials in progress)**

CHAPTER V

DISCUSSION

Recent progress in the genetic dissection of plant disease resistance signaling pathways has opened a number of new avenues towards engineering pathogen resistance in crops. Genes regulating race-specific and broad-spectrum resistance responses have been cloned, and novel induced resistance pathways have been identified in model and crop systems (**Glazebrook.** (2005)). Advances continue to be made in identification of host-expressed antifungal proteins with effects inhibitory to either pathogen development or to the accumulation of pathogen associated mycotoxins. As enumerated in the review of literature chapter, transgenic plants have been produced using different types of genes such as antifungal, regulatory transcription factor encoding genes, etc., to alleviate the deleterious effects of necrotrophic fungi. However, expression of such genes singly has led only to partial resistance in the transgenic plants. The present investigation was carried out to obtain leads and identify appropriate strategies to obtain transgenic castor plants with improved resistance against Botrytis grey mold disease and it included the main objectives of developing constructs with logically chosen genes and promoters, validate the developed constructs in a model plant system such as tobacco and attempt transformation of castor with the gene construct(s). The hypothesis, the rationale and logic adopted in designing the gene constructs, the strategies used for developing the constructs, results obtained from validation experiments of the developed vectors as well as the results of the attempts to transform castor are discussed here in light of the available literature.

5.0. Rationale in designing the gene constructs

The necrotrophic fungal species, *Botrytis,* uses multiple strategies to subdue its host plants. The ultimate goal of a necrotrophic plant pathogen is not to kill its host plant *per se* but to decompose plant biomass and convert it into fungal mass. Infection of the host plant is a highly

regulated process in which the pathogen must decide whether or not to germinate, when and where to develop an infection structure (appresorium) or produce enzymes and metabolites for its further growth on the host tissues. The infection process is based on the environmental conditions and the pathogen's adaptability to various host systems. Increasing our knowledge on the infection strategies of necrotrophic pathogens in general, and more specifically on *Botrytis* should be helpful in designing novel rational disease control strategies.

Botrytis ricini is a necrotrophic fungus causing grey mold/ grey rot disease of castor leading to yield loss upto 80% in favourable conditions. Various approaches adopted for control of this disease in castor have not been successful. Also, limited genetic variability in the germplasm of castor with respect to the resistance/tolerance against grey mold necessitates the adoption of alternate strategies, such as biotechnological tools, for overcoming the huge losses inflicted by this fungus. However, the information available regarding the mechanism of infection by this fungus as well as the corresponding host gene(s) that could be involved in imparting tolerance against this fungus is very scanty. On the other hand, there is a wealth of information available with respect to regulated gene action that takes place during the interaction between *Botrytis cinerea* and its hosts, especially *Arabidopsis thaliana*. This understanding has led to the development of Botrytis tolerant transgenic lines in *Arabidopsis* by overexpressing the chosen gene(s) that are involved in sensing the infection, limiting the disease spread or those involved in the defense regulatory pathway. It was conjectured that if *Botrytis ricini* and *B. cinerea* are closely related phylogenetically, then the genes chosen and successful in genetic engineering strategies adopted to develop Botrytis tolerant transgenic lines in *Arabidopsis* could be used to develop suitable gene constructs for obtaining grey rot tolerant castor transgenic lines. With this background, studies on the genetic relatedness between *B. ricini* and *B. cinerea* was

undertaken in the present investigation, essentially following the procedure adopted by Staats et al., 2005. Fragments of three house-keeping genes, *G3PDH, HSP60* and *RPB2,* were isolated from the mycelium of *B.ricini,* cloned and sequenced. These sequences showed 95% similarity to the existing corresponding sequences of *Botrytis* and *Sclerotinia spp,* the latter also being a necrotroph. Based on this similarity, it could be opined that the strategies adopted in overcoming *B. cinerea* infection might work against *B. ricini* infection also and this formed the basis for designing the molecular strategies for imparting tolerance against *B.ricini* in castor. TREEBASE analysis of the sequences indicated *B. ricini* as monophyletic with the extant *Botrytis spp.* but as a unique descendant from the ancestral lineage, which perhaps is an indication of the unique single plant host range (castor, *Ricinus communis* L.) of *B. ricini*.

Expression of genes in the targeted location is also a challenge for the development of transgenic plants. Using promoters that allow the required spatial and temporal expression of the chosen genes is a prerequisite for the development of efficient and effective transgenics. Since *Botrytis ricini* basically infects inflorescence (Godfrey, 1923), floral specific promoters are the promoters of choice to develop the required gene constructs. Promoters of ACC synthases **(1-aminocyclopropane-1-carboxylate synthase)** from *Arabidopsis* have been shown to drive elevated expression levels in the inflorescence of *Arabidopsis* (Wang et al., 2005). Phylogenetic analysis of the *AtACS* gene family has shown that two distinctly related *AtACS* gene clusters exist in Arabidopsis (Ge *et al*., 2000; Yamagami *et al*., 2003). Regulation of *AtACS* gene expression has been investigated at the steady-state transcript level using RT-PCR and northern blot analysis (van der Straeten *et al*., 1992; Liang *et al*., 1992, 1996; Abel *et al*., 1995; Arteca and Arteca, 1997; Vahala *et al*., 1998; Miller *et al*., 1999; Smalle *et al*., 1999; Yamagami *et al*., 2003) as well as at the post-transcriptional level (Kim and Yang, 1992; Sanu *et al*., 1994; Li and

Mattoo, 1994; Chae *et al*., 2003). The expression profile of the promoters of *AtACS4, 5* and *7* was validated at the transcriptional level using the GUS reporter gene-aided histochemical and fluorimetric method in *Arabidopsis thaliana* (Wang *et al*., 2005) and this study has indicated that these promoters drive expression in all the floral parts. Based on this information it was concluded that the promoters of *AtACS4*, *5* and *7* could be used for developing gene cassettes with elevated expression level in the inflorescence tissues. But the expression pattern of these promoters have not been established in heterologous system. Hence, it was one of the objectives of the present study to establish the tissue specific elevated expression patterns of *AtACS4, 5* and *7* by using a reporter gene system in transgenic tobacco plants.

Several genes have been used to develop transgenic plants with increased ability to counter fungal pathogens albeit with small incremental advantage with respect to induced resistance (Glazebrook. 1999). Though it has been suggested that simultaneous expression of multiple genes that individually could confer only partial resistance may lead to better tolerance against fungal pathogens, there are not many such reports (Halpin. 2005). Therefore, in the present study the objective was to express multiple transgenes simultaneously to achieve better tolerance against Botrytis. From among the defense regulatory genes known to confer fungal disease tolerance, a set of genes were to be selected for developing the gene constructs under the present study. The main rationale for selecting a set of genes was that the chosen genes should be upstream key players in defense responses of plants so that once they are expressed in higher levels they would in turn switch on other downstream defense related genes enabling the plants to be more tolerant.

Plants have evolved the ability to recognize and respond to particular pathogen molecules, leading to rapid activation of defense responses. Deciphering the genes and pathways

involved in pathogen resistance has been mainly based on loss-of-function experiments. From a biotechnology point of view, however, it is important to obtain gain-of-function mutants (or transgenic plants), because the character of interest can easily be transferred to crop plants by transformation (Wilkinson *et al*., 1997). In addition, engineering the expression of transcription factors is a major objective in biotechnology, as modification of the expression of only one gene may drive the expression of all genes regulated downstream by the transcription factors. Integrating the knowledge of how these proteins function with the emerging understanding of other natural defense pathways will lead to an integrated approach towards engineering of novel and broad-spectrum defense mechanisms in crops. The combination of these defenses with the added protection provided by expression of potent antifungal proteins promises the future delivery to the grower of an effective arsenal to combat the most important microbial diseases limiting crop production today.

To develop gene constructs in the present study, *Arabidopsis thaliana BIK1* was considered, as it is known to be a crucial component of host response signaling required to activate the resistance responses to *Botrytis* infection in *Arabidopsis*. *BIK1* encodes a regulatory protein, specifically a protein kinase, predicted to be specific to Serine/Threonine residues, and that is similar to receptor like cytoplasmic protein kinases and would act early in the disease response pathway. And, *BIK1* signaling is required for the activation of cellular mechanisms involved in plant development and pathogen response pathways (Veronese *et al*., 2006). The function of *BIK1* as a disease resistance factor may be to regulate normal levels of SA required for resistance to necrotrophs. Upon infection, *BIK1* gets activated to trigger the *Botrytis* resistance response, including the regulation of optimal levels of SA synthesis. It was also studied earlier that in the absence of *BIK1*, this regulation is relieved and SA level increases,

which may lead to suppression of the mechanism required for necrotrophic resistance (Penninckx *et al*., 1996; Clarke *et al*., 1998; Gupta *et al*., 2000; Kunkel and Brooks, 2002; Spoel *et al*., 2003). And the loss of resistance to necrotrophic pathogens suggests that *BIK1* modulates the cross-talk between various defense signaling pathways. Localization of *BIK1* to the plasma membrane suggests that *BIK1* may act as an early component of the plant defense response, either directly in pathogen recognition or early in the signaling cascade and that *BIK1* might function through other components of the MAPK pathway. The kinase activity of *BIK1* may be required to transduce pathogen-derived signals to downstream molecules that are targeted for negative or positive regulation by *BIK1* depending on the pathogen. Based on these reports, it was decided that *BIK1* would be a candidate transgene for developing gene constructs to realize tolerance against Botrytis.

The major pathways involved in the defence against necrotrophic fungi are the jasmonic acid (JA), ethylene (ET) and salicylic acid (SA) pathways (Glazebrooke, 1999; Thomma *et al*., 2001). Loss of ethylene signaling by mutations of genes of the pathway, or activation of ET signaling by constitutive expression of one of its downstream components, *ERF1*, have opposite effects (susceptibility and resistance, respectively) in the response of *Arabidopsis* to *B.cinerea*, showing that ET is indeed essential to mount efficient defense against necrotrophic fungi (Marta Berrocal-Lobo *et al*., 2002). The cloning of *Ethylene-Response-Factor-1*, an early ET-response gene whose expression is regulated by *EIN3*, has been shown to regulate the expression of several pathogen responsive genes including *b-CHI* and *PDF1.2* (Solano *et al*., 1998). *ERF1* has been expressed as a transgene in many plants and it has been shown to confer not only fungal resistance but also tolerance against several abiotic stresses (this has been reviewed in chapter 2).

Thus, *ERF1* from *Arabidopsis* was considered as another candidate gene to develop constructs in the present study.

Hypersensitivity response, one of the effective armors in the plant weaponry against biotrophic fungi, turns the Achilles' heel when confronted by necrotrophic fungi. Necrotrophic fungi elicit hypersensitive response to kill the host tissue and therefore establishing infection. This could be tackled by impeding plant cell death through the expression of anti-apoptotic genes (Dickman *et al*., 2001). The *Arabidopsis thaliana* ethylene responsive element binding protein, *AtEBP*, acts as an anti-apoptotic gene. It has been demonstrated that *AtEBP* can bind the GCC-box in the promoter sequences *in vitro*. *PDF1.2* and other defensin genes that contain the GCC-box in their promoters are bound by *AtEBP* for the activation of downstream defense responses. Over-expression of *AtEBP* in tobacco transgenics using constructs possessing the GCC-box in the promoter element resulted in the upregulation of the respective reporter gene. Over-expression of *AtEBP* coordinately accumulated *PDF1.2* mRNA. *PDF1.2* is a typical downstream gene of the ethylene/jasmonic acid signaling (Penninckx *et al*., 1996, 1998). The GCC-box in the promoter of *PDF1.2* is known to be important for transcriptional regulation by methyl jasmonic acid (Brown *et al*., 2003). Thus, it is likely that *AtEBP* promotes transcription of *PDF1.2* through *in vivo* binding to the GCC-box. Previous studies had revealed that *AtEBP* confers resistance to Bax-induced cell death in tobacco plants and resistance to exogenous treatments such as H_2O_2 and heat in plant cells indicating its anti-apoptotic nature in maintaining the plants integrity. Therefore, it was hypothesized that if AtEBP1 gene is expressed in the inflorescence of castor it might help in conferring tolerance against Botrytis by reducing the cell death. Thus, *AtEBP1* was chosen as the third candidate gene for realizing Botrytis tolerance.

5.1. Development of gene constructs

Three constructs were developed for establishing the expression patterns of *AtACS4, 5* and *7* promoters. 1.2 kb-long DNA fragments flanking the 5`region of *AtACS4, 5* and *7* coding sequences were cloned upstream of promoterless *uidA* in the binary vector, pCAMBIA 1381Z, to carry out promoter analysis in the heterologous system, tobacco.

Three individual gene cassettes were developed using the three promoters *AtACS4, 5* and *7* and the three chosen genes *BIK1, ERF1* and *EBP1*, respectively. To understand the cumulative effect of expressing more than one gene simultaneously in the plant, one two-gene construct (*ERF1* and *BIK1*) and one three-gene construct (with all three genes) were developed independently by cloning two-gene cassettes and three-gene cassettes into the T-DNA. Each of the component sequences (promoters, genes and transcription terminators) were cloned individually in basal vectors, sequenced and only such clones which did not have any mutations in the sequences were selected. The component sequences were later cloned sequentially to realize the individual gene cassettes and the cassettes were completely sequenced to select only such clones which did not have any mutations.

The two individual gene cassettes consisting of *ERF1* and *BIK1* were then cloned within the same T-DNA to realize the two-gene cassette. The two gene cassettes were cloned in divergent orientation (with respect to directionality of transcription), deliberately, so that the promoters in opposite orientation would act as bidirectional dual promoter (BDDP). BDDP is known to increase the levels of expression of both the gene cassettes (Xiaomeng *et al*., 2009). Into this double gene cassette containing binary vector, the third gene cassette (*AtEBP1*) was cloned distal to *ERF1* in convergent orientation.

Thus, in all, eight gene constructs were developed in binary vectors and the confirmed vectors were mobilized into *Agrobacterium* for plant transformation. Schematic representation of the T-DNA region of the developed binary vectors is provided in Fig 5.1.

5.2. Validation of the developed gene constructs using tobacco as model system

The developed gene cassettes were deployed into tobacco using *Agrobacterium*-mediated leaf disc transformation. The plants obtained with each of the gene constructs were analysed for the presence and expression of the introduced gene(s) using standard procedures such as genomic PCR, dot-blot, Reverse transcriptase-PCR (RT-PCR), etc. Progenies of the confirmed transgenic plants carrying individual gene cassettes were also subjected to Botrytis bioassays to assess the disease resistance imparted.

5.3. Promoter analysis: Analysis of the c*is*-elements present in *AtACS4, 5* and *7* promoter sequences using various software clearly indicated that these promoters possessed the *cis*-elements necessary for driving the expression in flowers as well as under stress conditions. These results demonstrat that the promoter sequences chosen possess diverse functions with elevated expression in the inflorescence and induction through stress.

Transgenic plants that were confirmed for the presence of the intact gene cassettes showed GUS activity in all the floral parts as indicated by both Reverse-transcriptase PCR as well as histochemical analysis. However, prominent GUS activity was also noticed at the cut ends of the explants taken out for GUS analysis. These results are consistent with the previously reported RT-PCR and histochemical data for the expression pattern of these promoters (Wang *et al*., 2005). Stress conditions like mechanical wounding and insect bites induce ethylene production and subsequent *ACC synthase* expression (Yang and Hoffmann, 1984; van dar

Straeten *et al*., 1990; Li *et al*., 1992; Liu *et al*., 1993). Ethylene, in turn, is required for mediating wound responses (O`Donnell *et al*., 1996) and for a maximal expression of defense genes (Weiss and Bevan, 1991) in the presence of JA (Xu *et al*., 1994). Numerous ACC synthase genes have been identified to be wound –inducible (Ge *et al*., 2000). In the present study, it was found that *AtACS4, 5* and *7* promoters were also inducible by mechanical wounding.

5.4. Individual gene cassettes: Transgenic tobacco plants obtained with *BIK1* cassette were studied in both T_0 and T_1 generations. Expression of the *BIK1* was observed both in the vegetative tissues and with elevated levels of expression in the inflorescence, corresponding to the results of promoter analysis (discussed above). Similarly, the expression of 657bp *ERF1* gene driven by *AtACS*5 promoter in the tobacco transgenics was analysed in both the T_0 and T_1 progeny. Expression of the *ERF1* was observed both in the vegetative tissues and with elevated expression levels in the inflorescence which correlated to the expression pattern of *AtACS*5 promoter. T_0 and T_1 progeny analysis of transgenic tobacco carrying *AtEBP* driven by *AtACS7* promoter showed the expression of the gene at elevated levels in the inflorescence with concomitant basal levels of expression in the vegetative tissues. Transgenic tobacco plants carrying single-gene cassettes were subjected to pathological bioassays using the necrotrophic fungi *Botrytis cinerea* and *Alternaria alternata*. The excised leaves and flowers of the transgenic plants showed greater degree of tolerance against *Botrytis cinerea* infection when compared to untransformed tobacco plants. Infection analysis using *Alternaria alternata* showed similar tolerance when tested on excised leaves and flowers. This indicated that the expression of the transgenes at a basal level in the vegetative tissues and elevated expression in the inflorescence is sufficient to confer resistance to the necrotrophic fungi *B. cinerea* and *A. alternata*.

Transgenic tobacco plants showing higher degree of tolerance to the necrotrophic fungi were also tested for the expression of PR proteins. The three chosen genes, *BIK1*, *ERF1* and *EBP1,* are known to exert a positive effect on the defense/stress responses through regulating the expression of related PR genes that in turn is expected to enhance plant tolerance to various stresses. The hypothesis that the regulatory genes have positive effect of defense response was supported by the results obtained in the present study. RT-PCR experiments indicated that the expression of the three transgenes activated the expression of GCC box-containing genes such as, *Prb-1b (PR-1), GLA (PR-2), CHN50 (PR-3)* and *Osmotin (PR-5)* under normal growth conditions, and subsequently resulted in enhanced innate resistance of host plants against pathogen infection. The PR proteins were shown to be up-regulated at least by two fold in the transgenic tobacco plants. These results indicated that the disease tolerance observed in the transgenic plants expressing *BIK1, ERF1* and *AtEBP* might be due to induced expression of the downstream PR genes. The obtained results are in line with the results obtained by several other workers (Zhang *et al*. 2004). Thus, the results obtained further strengthens the hypothesis that if defense regulatory genes are expressed they may confer better disease tolerance against Botrytis as compared to expressing the PR protein encoding genes individually.

Tobacco transformation was not successful in generating transgenic tobacco plants with both double and triple gene transgenic plants in spite of repeated attempts. To rule out the possibility that the cells transformed with double or triple gene cassette vectors were getting killed during the process of transformation itself, PCR analysis of the small shoots obtained with the triple gene cassette was preformed and the results confirmed the presence of all three cassettes in these plants. Therefore, though not investigated further, it could be conjectured that

the elongation of the regenerated shoots could have been inhibited due to the deleterious interactions between the transgenes, *BIK1* and *ERF1*, in the double cassette vector and among the transgenes, (*BIK1, ERF1* and *AtEBP),* in the triple three-gene cassette in the plant system.

5.5. Genetic transformation of castor

Castor is extremely recalcitrant to *in vitro* regeneration. Most of the early studies on regeneration using vegetative tissues as explants have proven to be either inefficient or difficult to reproduce (Reddy *et al*., 1987; Reddy and Bahadur, 1989; Sarvesh *et al*., 1992). Then, researchers started to focus on meristematic tissues to improve regeneration efficiency (Molina and Schobert, 1995; Lakshmi and Bahadur, 1997; Sujatha and Reddy, 1998). Sujatha and Reddy (1998) reported the first reliable protocol using embryonic tips and shoot apex. However, all the shoots were derived from the preexisting meristem, and adventitious shoot formation was not observed. When applied in *Agrobacterium*-mediated transformation, this protocol resulted in a low rate of putative transformant recovery (0.08%, one putative transformant in 1,200 embryonic tips used; Sujatha and Sailaja, 2005). The first success at stable transformation of meristematic tissues of castor through *Agrobacterium*-mediated transformation has been reported (Sujatha and Sailaja 2005). Subsequently, transgenic castor resistant to castor semilooper through deployment of Cry1Ab gene has been developed (Malathi *et al*. 2006). McKeon *et al*. (2003) also developed a meristem-based transformation system that involved *Agrobacterium*-mediated transformation using vacuum infiltration of flower buds, but the efficiency was also very low.

To obtain the number of transgenic castor plants needed to screen for the expression of three trangenes in castor plant, a meristem-based transformation was carried out along with the non-meristem-based transformation of castor by *in planta* methods (Kumar *et al*., 2011). The later protocol was an improved alternative to the previous meristem-based methods that resulted

in very low level of putative transformant recovery when applied in *Agrobacterium*-mediated transformation (Sujatha and Sailaja, 2005). Despite its generally high-regeneration efficiency, meristem-based transformation often presents limitations, such as 1) low rate of transformants, 2) chimerism of primary transformants, and 3) escape of regenerants from selection conditions (reviewed in Sticklen and Oraby, 2005). Recently, protocols for adventitious shoot regeneration and somatic organogenesis have been reported (Ahn *et al*. 2007; Ganesh Kumari *et al*. 2008). However, the efficiency of these regeneration systems for transformation of castor needs to be established before exploiting them for transformation of castor with agronomically desirable genes.

As meristem-based system for transformation has been used in the present study, there is a possibility of sectorial chimeras. Unless the progeny plants of these T_0 plants are analyzed it would not be possible to ascertain the inheritance of the transgenes.

In planta transformation strategy that involves *Agrobacterium*-mediated transformation of apical meristem or the meristems of axillary buds has been successfully standardized for maize (Chumakov *et al*. 2006), wheat (Supartana *et al*. 2006), rice (Supartana *et al*. 2005), buckwheat (Kojima *et al*. 2000), kenaf (Kojima *et al*. 2004), soybean (Chee *et al*. 1989), and mulberry (Ping *et al*. 2003). This technique is advantageous because it does not involve regeneration procedures and therefore the tissue culture-induced somaclonal variations are avoided. In the present study, the amenability of *in planta* transformation protocol was also attempted as per the protocol described by Kumar *et al*., 2011.

In the present study, meristem-based protocol (Sujatha *et al*., 2005) was followed to realize castor transgenics . Seeds of the cultivar DCS-9 (Jyoti) were used in all experiments. This genotype was selected as it is cultivated as a variety under rainfed conditions and also used as pollen parent for a hybrid (DCH-177). All the experiments were carried out with *Agrobacterium tumefaciens* strain LBA4404 harbouring triple gene cassette vector.

Owing to the poor amenability of castor transformation and regeneration, only the multi gene cassette vector, LBA4404:: pCAMBIA 1300 AEB, containing the three gene cassettes (*ERF1*, *BIK1* and *AtEBP*) in a single T-DNA was tried in castor. Responding embryo axes showed shoot proliferation during the first cycle of selection. The explants produced white excessive flaky callus on hygromycin selection medium. On medium with hygromycin for selection, the explants turned brown at the base with green proliferating shoots. On hygromycin selection medium, shoot proliferation was concomitant with base expansion. With increasing selection pressure, untransformed shoots turned brown while transformed sectors remained dark green with proliferation. After three cycles of increasing hygromycin selection, actively growing green shoot clusters were separated carefully from the surrounding brown/bleached tissues and transferred to shoot multiplication medium devoid of the selection agent. Following withdrawal of selection pressure, shoot proliferation was vigorous in explants recovered from hygromycin selection medium. However, in the subsequent subculture cycle, differences in shoot growth and proliferation were not significant and the elongation could not be achieved. From over 20 thousand embryos used for co-cultivation none of them could survive beyond third cycle of selection. This low frequency (0.01%) is a major limiting factor in realizing more transgenic castor plants. Also, the protocol employed in the present study is a very long duration one and about 12 months are needed to get sufficiently elongated shoots ready for transfer to the rooting

medium. This long duration of tissue culture makes the regenerating shoots more liable for contaminations due to frequent sub-culturing. Thus, many shoots were also lost during the course of the investigation.

However, based on the experiments carried out to transform castor using the triple gene cassette, the reasons for failure to get the transgenic castor plants could not be ascertained. This is because of the confounding of the effects of the inherent limitations of the transformation protocols that yield very low frequency of transformants in castor and the deleterious effects on regeneration observed with triple gene cassette even in tobacco transformation. The transformation experiments were carried out with only triple gene cassette with the hunch that the multi-gene vector would confer better tolerance in castor against *Botrytis ricini*. As mentioned above, the meristem based transformation was attempted first and only later the in planta method was attempted. Meristem based transformation experiments were carried out immediately after the triple gene cassette was assembled but due to the long duration of the protocol, almost one year had to be spent before concluding that the transgenic plants had not been realized in castor. But in tobacco, the experiments were carried out initially with the single gene cassettes to assess their utility and only after the failure to get plants in castor with the triple gene cassette, experiments were carried out in tobacco to find out whether the negative results in castor were due to the multi-gene cassette *per se* or due to inefficient protocol. However, to conclude this additional experiments are needed and this will form future line of work.

In conclusion, the study was undertaken to develop strategies for realizing transgenic castor plants with resistance to *Botrytis ricini*. It was hypothesized that if multiple genes that individually impart partial resistance are expressed in the plant simultaneously it would provide sufficient tolerance against *Botrytis.* Gene constructs were developed using three inflorescence

expressed promoters and three genes known to impart resistance against necrotrophic fungi. Also, two/three individual gene cassettes were cloned within a T-DNA to get multi-gene constructs. To validate the gene constructs developed, tobacco transgenic plants were developed. T_0 and T_1 generation plants were analyzed at molecular level for the presence and expression of the introduced gene cassettes. The putative tobacco transgenic plants were studied for their tolerance against *Botrytis cinerea* and *Alternaria alternata* and was found that these cassettes do impart substantial tolerance against infection. RT-PCR analysis confirmed that the introduced gene cassettes up-regulated the expression PR proteins and this could be the mechanism behind the tolerance imparted by these transgenes in the transgenic plants. Attempts to develop transgenic castor plants using meristem based transformation method in castor did not yield any transgenic castor plants. Therefore, *in planta* transformation of castor was attempted and the T_1 plants obtained will be analyzed for transgenicity.

Based on the results obtained in the present study, the future line of work includes further studies on the improved tolerance against necrotrophs imparted when more than one gene cassette is deployed in the plants, to elucidate the problem associated with multi-gene cassette with respect to its effect on regeneration and transformation, to obtain transgenic plants in castor using single gene cassettes or concoction of the single gene cassettes.

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Appendices

***Botrytis ricini* G3PDH**:
CLUSTAL 2.1 multiple sequence alignment

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B.fukeliana          -ATCGGAGCTCCCGCAGATATCAAGGACCCGAGCTAATTTATGTTTTGCACAGGCATACA 59
B.cinerea-pseudo     ----------------------------------------------GCAGGCATACA 11
B.porri              ----------------------------------------------ATAGGCATACA 11
B.ricini             TATCGGACCGCCCGCAGATATCACGGACCCGAGCTAATTTGTATTCTTTACAGGCATACA 60
A.ricini             TATCGGACCGCCCGCAGATATCACGGACCCGAGCTAATTTGTATTCTTTACAGGCATACA 60
                                                         *********

B.fukeliana          TGTTGAAGTATGATTCCACCCACGGTCAATTCAAGGGTGACATCAAGGTCCTTGCCGATG 119
B.cinerea-pseudo     TGTTGAAGTATGATTCTACCCACGGCCAATTCAAGGGTGATATCAAGGTCCTTGCCGATG 71
B.porri              TGTTGAAGTATGATTCCACCCACGGTCAATTCAAGGGTGATATCAAGGTCCTTTCCGATG 71
B.ricini             TGTTGAAGTATGATTCTACCCACGGCCAATTCAAGGGTGACGTCAAGGTCCTTCCCGACG 120
A.ricini             TGTTGAAGTATGATTCTACCCACGGCCAATTCAAGGGTGACGTCAAGGTCCTTCCCGACG 120
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B.fukeliana          GATTGGAGGTCAATGGCAAGAAGGTCAAGTTCTACACCGAGAGAGACCCAGCCAACATCC 179
B.cinerea-pseudo     GATTGGAGGTCAATGGCAAGAAGGTCAAGTTCTACACTGAGAGAGACCCAGCCAACATCC 131
B.porri              GATTGGAGGTCAATGGCAAGAAGGTCAAGTTCTACACCGAGAGAGACCCAGCCAACATCC 131
B.ricini             GATTGGAGGTCAATGGCAAGAAGGTCAAGTTCTACACCGAGAGAGACCCAGCCAACATCC 180
A.ricini             GATTGGAGGTCAATGGCAAGAAGGTCAAGTTCTACACCGAGAGAGACCCAGCCAACATCC 180
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B.fukeliana          CATGGGCTGAGTCTGAGGCATACTACGTCGTCGAGTCCACCGGTGTTTTCACCACCACCG 239
B.cinerea-pseudo     CATGGGCTGAGTCTGAGGCATACTACGTCGTCGAGTCCACCGGTGTTTTCACCACCACCG 191
B.porri              CATGGGCTGAGTCTGAGGCATACTACGTTGTCGAGTCCACCGGTGTTTTCACCACCACCG 191
B.ricini             CATGGGCTGAGTCTGAGGCATACTACGTTGTCGAGTCTACCGGTGTTTTCACCACCACCG 240
A.ricini             CATGGGCTGAGTCTGAGGCATACTACGTTGTCGAGTCTACCGGTGTTTTCACCACCACCG 240
                     ************************** ******** ********************

B.fukeliana          AGAAGGCCAAGGCACATTTGAAGGGTGGTGCCAAGAAGGTTGTTATCTCTGCTCCTTCTG 299
B.cinerea-pseudo     AGAAGGCCAAGGCACATTTGAAGGGTGGTGCCAAGAAGGTTGTTATCTCTGCTCCTTCTG 251
B.porri              AGAAGGCCAAGGCACATTTGAAGGGTGGTGCCAAGAAGGTTGTTATCTCTGCTCCTTCTG 251
B.ricini             AGAAGGCCAAGGCACATTTGAAGGGTGGTGCCAAGAAGGTTGTCATCTCTGCTCCTTCTG 300
A.ricini             AGAAGGCCAAGGCACATTTGAAGGGTGGTGCCAAGAAGGTTGTCATCTCTGCTCCTTCTG 300
                     ****************************************** ****************

B.fukeliana          CCGATGCCCCAATGTACGTTATGGGTGTCAACAACGAGACCTACACTGGTGATGTTGATG 359
B.cinerea-pseudo     CCGATGCCCCAATGTACGTTATGGGTGTCAACAACGAGACCTACAAGGGTGATGTTGATG 311
B.porri              CCGATGCCCCAATGTACGTTATGGGTGTCAACAACGAGACCTACAAGGGTGATGTTGATG 311
B.ricini             CCGATGCCCCAATGTACGTTATGGGTGTCAACAACGAGACCTACAAGGGTGATGTTGATG 360
A.ricini             CCGATGCCCCAATGTACGTTATGGGTGTCAACAACGAGACCTACAAGGGTGATGTTGATG 360
                     ******************************************  **************

B.fukeliana          TTATCTCCAACGCCTCTTGCACAACCAACTGCTTGGCTCCTCTCGCCAAGGTCATCAACG 419
B.cinerea-pseudo     TTATCTCCAACGCCTCTTGCACAACCAACTGCTTGGCTCCTCTCGCCAAGGTCATCAACG 371
B.porri              TTATCTCCAACGCCTCTTGCACAACCAACTGCTTGGCTCCTCTCGCCAAGGTCATCAACG 371
B.ricini             TTATCTCCAACGCCTCTTGCACAACCAACTGCTTGGCTCCTCTCGCCAAGGTCATCAACG 420
A.ricini             TTATCTCCAACGCCTCTTGCACAACCAACTGCTTGGCTCCTCTCGCCAAGGTCATCAACG 420
                     ************************************************************

B.fukeliana          ATGAGTTCACCATCATTGAAGGTTTGATGACCACCATCCACTCCTACACCGCTACCCAAA 479
B.cinerea-pseudo     ATGAGTTCACCATCATTGAAGGTTTGATGACCACCATCCACTCCTACACCGCCACCCAAA 431
B.porri              ATGAGTTCACCATCATTGAAGGTTTGATGACCACCATCCACTCCTACACCGCCACCCAAA 431
B.ricini             ATGAGTTCACCATCATTGAAGGTTTGATGACCACCATCCACTCCTACACCGCCACCCAAA 480
A.ricini             ATGAGTTCACCATCATTGAAGGTTTGATGACCACCATCCACTCCTACACCGCCACCCAAA 480
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B.fukeliana          AGACCGTTGATGGTCCATCCGCTAAGGATTGGCGTGGAGGACGTACCGCTGCTCAAAACA 539
B.cinerea-pseudo     AGACCGTTGATGGTCCATCCGCTAAGGATTGGCGTGGAGGACGTACCGCTGCTCAAAACA 491
B.porri              AGACCGTCGATGGTCCATCCGCTAAGGATTGGCGTGGAGGACGTACCGCTGCTCAAAACA 491
B.ricini             AGACCGTCGACGGTCCATCCGCTAAGGATTGGCGTGGAGGACGTACCGCTGCTCAAAACA 540
A.ricini             AGACCGTCGACGGTCCATCCGCTAAGGATTGGCGTGGAGGACGTACCGCTGCTCAAAACA 540
                     ******* ** ********************************************

B.fukeliana          TCATCCCATCGAGCACCGGTGCTGCCAAGGCTGTCGGAAAGGTCATCCCAGTCCTTAACG 599
B.cinerea-pseudo     TCATCCCATCGAGCACCGGTGCTGCCAAGGCTGTCGGAAAGGTCATCCCAGAGCTTAACG 551
B.porri              TCATCCCATCGAGCACCGGTGCTGCCAAGGCCGTCGGAAAGGTCATCCCAGAGCTTAACG 551
B.ricini             TCATCCCATCGAGCACCGGTGCCGCCAAGGCTGTCGGAAAGGTCATTCCAGAGCTTAACG 600
A.ricini             TCATCCCATCGAGCACCGGTGCCGCCAAGGCTGTCGGAAAGGTCATTCCAGAGCTTAACG 600
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B.fukeliana          GCAAACTCACCGGAATGTCCATGCGTGTTCCAACTGCCAACGTCTCAGTTGTTGACTTGA 659
B.cinerea-pseudo     GCAAACTCACCGGAATGTCCATGCGTGTTCCAACTGCCAACGTCTCAGTTGTTGACTTGA 611
B.porri              GCAAACTCACCGGAATGTCCATGCGTGTTCCAACTGCCAACGTCTCAGTTGTTGACTTGA 611
B.ricini             G-AAGCTCACCGGAATGTCTATGCGTGTTCCAACTGCCAACGTCACAGTTGTTGACTTGA 659
A.ricini             GCAAGCTCACCGGAATGTCTATGCGTGTTCCAACTGCCAACGTCTCAGTTGTTGACTTGA 660
                     * ** ************** ************************ ***************

B.fukeliana          CTGTCCGCATTGAGAAGGGTGCTTCTTACGATGAGATCAAGGCCGTCATCAAGAAGGCTG 719
B.cinerea-pseudo     CTGTCCGCATTGAGAAGGGTGCTTCTTATGATGAGATTAAGGCCGTCATCAAGAAGGCTG 671
B.porri              CTGTCCGCATTGAGAAGGGTGCTTCTTATGATGAGATCAAGGCCGTCATCAAGAAGGCTG 671
B.ricini             CTGTCCGTCTTGAGAAGGGTGCTTCTTACGATGAGATCAAGGCCGTCATCAAGAAGGCTG 719
A.ricini             CTGTCCGTCTTGAGAAGGGTGCTTCTTACGATGAGATCAAGGCCGTCATCAAGAAGGCTG 720
                     *******  ******************* ******** **********************

B.fukeliana          CTGATGGTCCTCTCAAGGGTAAGTTACTCCATT-ACTCTTTCTTCGG-CTCTAATTT-GC 776
B.cinerea-pseudo     CCGATGGTCCTCTCAAGGGTAAGTCACTCTATC-ACTCTTTCTTCGG-CTCTAATTT-AC 728
B.porri              CTGATGGTCCTCTCAAGGGTAAGTTACTCTATT-AATCTTTCTTCCA-TTCTAATTT-AC 728
B.ricini             CTGATGGTCCCCTCAAGGGTAAGTAATTCCATTTAATCTTACTTAGGGTTCCAATTTTAC 779
A.ricini             CTGATGGTCCCCTCAAGGGTAAGTAATTCCATTTAATCTTACTTAGGGTTCCAATTTTAC 780
                     * ******** ************* * ** **  * **** ***     ** *****  *

B.fukeliana          TAATCGTAACACAGGCATATTGGCTTACACTGAGGACGATGTTGTCTCCACTGACATGAA 836
B.cinerea-pseudo     TAATCGTAACACAGGCATTTTGGCTTACACTGAGGACGATGTTGTCTCCACTGACATGAA 788
B.porri              TAATCGTAATATAGGCATATTGGCTTACACTGAGGACGATGTTGTCTCCACTGACATGAA 788
B.ricini             TAATCGTCACATAGGCATATTGGCTTACACTGAAGACGAGGTTGTCTCCACTGACATGAA 839
A.ricini             TAATCGTCACATAGGCATATTGGCTTACACTGAAGACGAGGTTGTCTCCACTGACATGAA 840
                     ******* * * ****** ************** ***** ********************

B.fukeliana          CGGTGACAACCACTCCTCCATCTTCGATGCCAAGGCTGGTATCTCCCTCAACGCAAACTT 896
B.cinerea-pseudo     CGGTGACAACCACTCCTCCATCTTCGATGCTAAGGCTG---------------------- 826
B.porri              CGGTGACAACCACTCCTCCATCTTCGATGCCAAGGCTG---------------------- 826
B.ricini             CGGTGACAACCACTCTTCCATCTTCGATGCCAAGGCTGGTATCTCCCTCAACGCAAACTT 899
A.ricini             CGGTGACAACCACTCTTCCATCTTCGATGCCAAGGCTGGTATCTCCCTCAACGCAAACTT 900
                     *************** ************** *******

B.fukeliana          CGTCAAGTTGGTTT-CCTGGTACGACAACGAGTGGGGT 933
B.cinerea-pseudo     ---------------------------------------
B.porri              ---------------------------------------
B.ricini             CGTCAAGTTGGTTTTCATGGTACGACAACGAGTGGGGT 937
A.ricini             CGTCAAGTTGGTTT-CATGGTACGACAACGAGTGGGGT 937
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***Botrytis ricini* HSP60:**

CLUSTAL 2.1 multiple sequence alignment

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B.fukeliana       GATACGACATGGATATCTGGTGATTGTAAACTGATCATGTTTTTAATTTAGGAGCTCAAA 60
B.cinerea         ------------------------------------------------------------
Botryis           ------------------------------------------------------------
B.ricini          -ATATGAGATATATATCTGGCGGTCATATACTAACTATG-CTATTATTTAGGAGCTCAAA 58
A.ricini          -ATATGAGATATATATCTGGCGGTCATATACTAACTATG-CTATTATTTAGGAGCTCAAA 58


B.fukeliana       TTCGGTGTTGAGGGCAGAGCAGCTCTTCTTGCTGGTGTTGAGACTTTGGCAAAAGCTGTT 120
B.cinerea         ----------------------CTCTTCTTGCTGGTGTTGAGACTTTGGCAAAAGCTGTT 38
Botryis           ----------------------CTCTTCTTGCTGGTGTTGAGACTTTGGCAAAAGCTGTT 38
B.ricini          TTCGGTGTGGAGGGTAGAGCAGCTCTTCTCGCTGGTGTTGAGACTTTGGCAAAGGCTGTT 118
A.ricini          TTCGGTGTTGAGGGTAGAGCAGCTCTTCTCGCTGGTGTTGAGACTTTGGCAAAGGCTGTT 118
                                  ******* ************************.******


B.fukeliana       GCTACAACCTTGGGTCCCAAAGGCCGAAATGTTCTTATTGAGTCAGCATATGGCTCCCCA 180
B.cinerea         GCTACAACCTTGGGTCCCAAAGGCCGAAATGTTCTTATTGAGTCAGCATATGGCTCCCCA 98
Botryis           GCCACAACCTTAGGTCCCAAAGGCCGAAATGTTCTTATTGAGTCAGCATATGGCTCCCCA 98
B.ricini          GCCACAACCTTAGGTCCTAAAGGTCGAAATGTTCTTATTGAGTCAGCATATGGCTCCCCA 178
A.ricini          GCCACAACCTTAGGTCCTAAAGGTCGAAATGTTCTTATTGAGTCAGCATATGGCTCCCCA 178
                  ** ********.***** ***** ************************************


B.fukeliana       AAGATCACTAAAGGTTTGCAAAACTCCCGGCTACCTAGTTTCAAGATTCTAATTATTGGT 240
B.cinerea         AAGATCACTAAAGGTTTGCAAAACTCCCGGCTACCTAGTTTCAAGATTCTAATTATTGGT 158
Botryis           AAGATTACTAAAGGTTTGCGAACTCCCCGGTTACCTAGTTGTAAAATTCTAATCGTTGGT 158
B.ricini          AAGATCACTAAAGGTTTGCGAAACTTCACGTTACCTTGTTGTAAAATTCTAATTGTTGTT 238
A.ricini          AAGATCACTAAAGGTTTGCGAAACTTCACGTTACCTTGTTGTAAAATTCTAATTGTTGGT 238
                  ***** *************.**.   *. * *****:***  **.******** .*** *


B.fukeliana       GAATAGATGGTGTAACCGTTGCCAGAGCTATTTCCCTCAAGGACAAATTCGAGAACCTCG 300
B.cinerea         GAATAGATGGTGTAACCGTTGCCAGAGCTATTTCCCTCAAGGACAAATTCGAGAACCTCG 218
Botryis           GAATAGATGGTGTAACTGTTGCCAGAGCTATTTCCCTCAAGGACAAATTCGAGAACCTCG 218
B.ricini          GAATAGATGGTGTAACTGTGGCCAGAGCCATTTCCCTCAAGGACAAATACGAGAACCTCG 298
A.ricini          GAATAGATGGTGTAACTGTGGCCAGAGCCATTTCCCTCAAGGACAAATTCGAGAACCTCG 298
                  ***************** ** ******** ********************:***********


B.fukeliana       GTGCTAGACTTATCCAAGATGTTGCCTCGAAAACCAACGAGACCGCTGGTGATGGAACCA 360
B.cinerea         GTGCTAGACTTATCCAAGATGTTGCCTCGAAAACCAACGAGACCGCTGGTGATGGAACCA 278
Botryis           GTGCTAGACTCATCCAAGATGTTGCCTCGAAAACCAACGAGACCGCTGGTGATGGAACCA 278
B.ricini          GTGCTAGACTTATCCAAGATGTCGCCTCGAAAACCAACGAGACCGCTGGTGATGGAACCA 358
A.ricini          GTGCTAGACTTATCCAAGATGTTGCCTCGAAAACCAACGAGACCGCTGGTGATGGAACCA 358
                  ********** *********** ************************************


B.fukeliana       CAACCGCTACTGTCCTTGCTAAATCTATTTTCTCCGAGACCGTAAAGAACGTCGCCGCAG 420
B.cinerea         CAACCGCTACTGTCCTTGCTAAATCTATTTTCTCCGAGACCGTAAAGAACGTCGCCGCAG 338
Botryis           CAACTGCTACTGTCCTTGCTAAATCTATCTTCTCCGAGACCGTAAAGAACGTCGCCGCAG 338
B.ricini          CATCTGCTACTGTCCTTGCTAAATCTATTTTCTCCGAGACCGTCAAGAACGTCGCCGCTG 418
A.ricini          CATCTGCTACTGTCCTTGCTAAATCTATTTTCTCCGAGACCGTCAAGAACGTCGCCGCAG 418
                  **:* ********************** **************.**************:*


B.fukeliana       GATGCAACCCAATGGACTTGCGCAGAGGTACCCAAG-CTGCCGTGGAGGCCGTTGTTGAG 479
B.cinerea         GATGCAACCCAATGGACTTGCGCAGAGGTACCCAAG-CTGCCGTGGAGGCCGTTGTTGAG 397
Botryis           GATGCAACCCAATGGACTTGCGCAGAGGAACCCAAG-CCGCCGTGGAGGCCGTTGTTGAG 397
B.ricini          GTTGCAACCCAATGGACTTGCGCAGAGGTACACAGGGCCGCCGTG-AGGCCGTTG-TGAA 476
A.ricini          GTTGCAACCCAATGGACTTGCGCAGAGGTACACAGG-CCGCCGTGGAGGCCGTTGTTGAA 477
                  *:*************************:**.**.* * ****** ********* ***.
```

```
B.fukeliana     TTTTTGCAAAAGAACAAGCGTGATATCACAACAAGCGAGGAAATCGCACAAGTTGCGACT 539
B.cinerea       TTTTTGCAAAAGAACAAGCGTGATATCACAACAAGCGAGGAAATCGCACAAGTTGCGACT 457
Botryis         TTTTTGCAAAAGAACAAGCGTGATATCACAACCAGCGAGGAAATCGCACAAGTTGCGACT 457
B.ricini        TTCTG--CAAAGAACAAGCGTGATATCACAACCAGTGAGGAAATCGCACAG-TTGC-ACT 532
A.ricini        TTCTTGCAAAAGAACAAGCGTGATATCACAACCAGTGAGGAAATCGCACAAGTTGCGACT 537
                ** *   .************************.** **************. **** ***

B.fukeliana     ATCAGTGCAAACGGTGATACCCACATCGGAAAATTGATTGCCAACGCTATGGAAAAGGTT 599
B.cinerea       ATCAGTGCAAACGGTGATACCCACATCGGAAAATTGATTGCCAACGCTATGGAAAAGGTT 517
Botryis         ATCAGTGCAAACGGTGATACCCACATCGGAAAGTTGATTGCCAACGCTATGGAGAAGGTT 517
B.ricini        ATCAGTGCAAACGGTGATACACACGTCGGAAAGTTGATTGCCAACGCTATGGAGAGGGTT 592
A.ricini        ATCAGTGCAAACGGTGATACACACGTCGGAAAGTTGATTGCCAACGCTATGGAGAGGGTC 597
                **********************.***.*******.**********************.*.***

B.fukeliana     GGAAAGGAAGGTGTTATCACAGTTAAGGAAGGAAAGACCATGGAGGACGAACTCGATATT 659
B.cinerea       GGAAAGGAAGGTGTTATCACAGTTAAGGAAGGAAAGACCATGGAGGACGAACTCGATATT 577
Botryis         GGAAAGGAAGGTGTCATCACAGTCAAGGAGGGAAAGACGATGGAGGATGAACTCGATATT 577
B.ricini        GGAAAGGAAGGTGTCATCACAGTCAAGGAAGGAAAGACCATGGAGGACGAACTGGACATC 652
A.ricini        GGAAAGGAAGGTGTCATCACAGTCAAGGAAGGAAAGACCATGGAGGACGAACTGGACATC 657
                ************** ******** ******.******** ******** ***** ** **

B.fukeliana     ACCGAGGGAATGAGATTTGACCGCGGTTATGTTTCCCCATACTTCATCACCGATACCAAG 719
B.cinerea       ACCGAGGGAATGAGATTTGACCGCGGTTATGTTTCCCCATACTTCATCACCGATACCAAG 637
Botryis         ACCGAGGGAATGAGATTTGACCGCGGTTATGTCTCCCCATACTTCATCACCGATACCAAG 637
B.ricini        ACTGAGGGAATGAAATTTGACCGCGGTTATGTCTCTCCATACTTTATCACCGATACCAAG 712
A.ricini        ACTGAGGGAATGAAATTTGACCGCGGTTATGTCTCTCCATACTTTATCACCGATACCAAG 717
                ** **********.****************** ** ******** ***************

B.fukeliana     TCGCAAAAGGTGGAATTCGAGAAGCCATTGATTCTCCTTTCTGAGAAGAAGATTTCAAAC 779
B.cinerea       TCGCAAAAGGTGGAATTCGAGAAGCCATTGATTCTCCTTTCTGAGAAGAAGATTTCAAAC 697
Botryis         TCGCAAAAGGTCGAATTCGAGAAGCCATTGATTCTCCTTTCTGAGAAGAAGATCTCAAAC 697
B.ricini        TCGCAAAAAGTGGAATTCGAGAAGCCATTGATTCTTCTTTCCGAGAAGAAGATCTCAAAC 772
A.ricini        TCGCAAAAAGTGGAATTCGAGAAGCCATTGATTCTTCTTTCCGAGAAGAAGATCTCAAAC 777
                ********.** ********************** ***** *********** ******

B.fukeliana     GTCCAAGATATTATCCCAGCACTTGAGGCGTCTACTCAACTTCGTCGTCCTTTGGTCATC 839
B.cinerea       GTCCAAGATATTATCCCAGCACTTGAGGCGTCTACTCAACTTCGTCGTCCTTTGGTCATC 757
Botryis         GTCCAAGATATCATCCCAGCACTTGAGGCATCTACTCAACTTCGCCGTCCTTTGGTCATC 757
B.ricini        GTCCAAGATATCATCCCAGCACTTGAGGCTTCTACTCAACTTCGTCGTCCTTTGGTCATC 832
A.ricini        GTCCAAGATATCATCCCAGCACTTGAGGCTTCTACTCAACTTCGTCGTCCTTTGGTCATC 837
                *********** ********** ********** ********** ***************

B.fukeliana     ATTGCTGAAGATATCGATGGAGAAGCTCTCGCAGTATGCATTCTTAACAAGCTCCGTGGT 899
B.cinerea       ATTGCTGAAGATATCGATGGAGAAGCTCTCGCAGTATGCATTCTTAACAAGCTCCGTGGT 817
Botryis         ATTGCTGAAGATATCGATGGAGAAGCTCTCGCTGTATGCATTCTCAACAAGCTCCGTGGT 817
B.ricini        ATTGCTGAAGATATCGATGGAGAAGCTCTCGCTGTATGCATTCTTAACAAGCTCCGTGGT 892
A.ricini        ATTGCTGAAGATATCGATGGAGAAGCTCTCGCTGTATGCATTCTTAACAAGCTCCGTGGT 897
                *********************************:*********** ***************

B.fukeliana     CAACTCCAAGTTGCCGCTGTCAAGGCCCCCGG-TTTCGGTGATAACCGAAAGTCC-ATCC 957
B.cinerea       CAACTCCAAGTTGCCGCTGTCAAGGCCCCCGG-TTTCGGTGATAACCGAAAGTCC-ATCC 875
Botryis         CAACTCCAAGTTGCCGCTGTCAAGGCCCCCGG-TTTCGGTGATAACCGAAAGTCT-ATCC 875
B.ricini        CAACTCCAAGTTGCCGCTGTCAAGGCGCCCGG-CTTTGGTGACAACCGAAAGTCCCATTC 951
A.ricini        CAACTCCAAGTTGCCGCTGTCAAGGCGCCCGG-CTTTGGTGACAACCGAAAGTCCATTCT 956
                ************************** *****  ** ***** ***********  :*

B.fukeliana     TCGGCGATCTCGGTATCTTGACCAATGCTACCGTCTTCACTGACGAGCTTGATCTCAAGC 1017
B.cinerea       TCGGCGATCTCGGTATCTTGACCAATGCTACCGTCTTCACT------------------- 916
Botryis         TCGGCGATCTCGGTATCCTGACCAATGCTACCGTCTTCACT------------------- 916
B.ricini        TTG-CGATCTCGGCATTTTGACCAATGCCACCGTCTTCACTGATGAGCT-GATCTCAAGC 1009
A.ricini        TGG-CGATCTCGGCATTTTGACCAATGCCACCGTCTTCACTGATGAGCTTGATCTCAAGC 1015
                * * ********* **  ********** ************
```

```
B.fukeliana      TCGAGAAGGCTACCGCAGACATGCTCGGTACCACTGGATCCATC 1061
B.cinerea        ---------------------------------------------
Botryis          ---------------------------------------------
B.ricini         TCGAGAAGGCCACTGCAGACAG-CTCGGTACCACTGGATCCATC 1052
A.ricini         TCGAGAAGGCCACTGCAGACATGCTCGGTACCACTGGATCCATC 1059
```

***Botrytis ricini* RPB2:**

CLUSTAL 2.1 multiple sequence alignment

```
B.fukeliana      --------AAATTAG--CCTTGAATGAATAA-----ATTCATTGA-ACTTAAA-GCTTAC 43
B.cinerea        ------------------------------------------------------------
B.ricini         --------ACATTAG--CCATGAATGAATAA-----ATTCATTGG-ACTTAAAAGCTTAC 44
A.ricini         TTGTTCAGAAATCTATTCCGTAGATTGACAACAGATGTGTACAGATACTTGCAACGTTGC 60


B.fukeliana      CTGATTGTGATCCGGGAAGGGAATAATGCTT---GCGC-AGATACCCAA-GATCATACTT 98
B.cinerea        ---------------------------------GCGC-AGATACCCAA-GATCATACTT 24
B.ricini         CTGATTGTGATCTGGGAAGGGAATAATGCTT---GCGC-AGATACCCAA-GATCATACTT 99
A.ricini         GTGGAAAACAACCGAGAGTTCAATTTGACTTTGGGTGTGAAATCCACAACAATCACGAAC 120
                                                 * *  * ** * ***  ****


B.fukeliana      GGA-TGAA------TTTCACAATGAGT-----------CCAGACATGAGCAGTTGGATTG 140
B.cinerea        GGA-TGAA------TTTCACAATGAGT-----------CCAGACATGAGCAGTTGGATTG 66
B.ricini         GGA-TGAA------TTTCACAATGGGT-----------CCAGACATGGGCAGTCAGATTG 141
A.ricini         GGTCTGAAATATTCTTTGGCCACAGGTAACTGGGGTGATCAGAAGAAGGCCGCAAGTTCT 180
                 **  ****      ***  * *   **           ****     ** *   * *


B.fukeliana      ATAGGTGCCTTAACACGCTTGTTCAA--------------ATCACCACTTTC--GTCAG 183
B.cinerea        ATAGGTGCCTTAACACGCTTGTTCAA--------------ATCACCACTTTC--GTCAG 109
B.ricini         ATAGGCGCCTTGACACGCTTGTTCAA--------------ATCACCACTTTC--GTCAG 184
A.ricini         ACCGCTGGTGTATCGCAAGTGTTGAACAGATATACCTTTGCATCAACACTTTCCCATTTG 240
                 *  *  *   *  * *   **** **              **** *******   *  *


B.fukeliana      GACGAATTTGGTA-ACCAGCCTGAAGCTGTCGGGAGATGTCCAGATCTTCAGGTGT-CAT 241
B.cinerea        GACGAATTTGGTA-ACCAGCCTGAAGCTGTCGGGAGATGTCCAGATCTTCAGGTGT-CAT 167
B.ricini         GACGAATCTGATA-ACCAGCCTGAAGCTGTCGAGAGATGTCCAAATCTTCAGGAGT-CAT 242
A.ricini         CGCCGAACCAATACACCCATTGGACGTGATGGAAAGATCGCCAAACCTAGACAACTACAT 300
                   *  *     ** ***     ** *   * *  ****  *** * **  *    * ***


B.fukeliana      TGTAATCATGACAGTCTCTTCTTCCTCGGCATCCAGGTACTCAA---CCACACC-GTCAT 297
B.cinerea        TGTAATCATGACAGTCTCTTCTTCCTCGGCATCCAGGTACTCAA---CCACACC-GTCAT 223
B.ricini         GGTAATCATGACAGTCTCTTCTTCCTCAGCGTCCAGGTATTCAA---CCACGCC-ATCGT 298
A.ricini         AATACTCACTGGGGCTTGGTCTGCC-CGGCAG--AGACACCCGAAGGTCAAGCTTGTGGT 357
                   ** ***     *  *  *** ** * **    **  *  * *    **  *   *  *


B.fukeliana      TAATCAAACCTTGGAATCCATAGTATCCGTTTCTTACTTTCTCATCCTTAT-CCATGTTT 356
B.cinerea        TAATCAAACCTTGGAATCCATAGTATCCGTTTCTTACTTTCTCATCCTTAT-CCATGTTT 282
B.ricini         TGATCAAACCTTGGAATCCATAGTATCCGTGTCTTACTTTGTCATCCTTAT-CCATATTT 357
A.ricini         TTGGTAAA-----GAACT--TGGCTCTGATGTGTTACGTTACAGTCGGTACGCCAAGTGA 410
                 *    ***     ***    * *      * * **** **    **  **  ***  *


B.fukeliana      GCTGGTAGC--AACTGATCATCCTCCAGAC------------GGCGAATGTGATCCTTG 401
B.cinerea        GCTGGTAGC--AATTGATCATCCTCCAGAC------------GGCGAATGTGATCCTTG 327
B.ricini         GCTGGTAGC--AATTGATCATCTTCCAGAC------------GGCGAATATGATCCTTG 402
A.ricini         TCCAATCGTCGAATTCATGATTCAACGAAATATGGAAGTGTTGGAGGAGTATGAACCACT 470
                  *   * *   ** * ** **    *  *              *  ** * *** **


B.fukeliana      TTCAACACCAAGTT--------ACCTTTGTTTGCGCTGTCAGGATCATTGTCAATAACCA 453
B.cinerea        TTCAACACCAAGTT--------ACCTTTGTTTGCGCTGTCAGGATCATTGTCAATAACCA 379
B.ricini         TTCAATACCAAGTT--------ACCTTTGTTAGCGCTGTCAGGATCATTGTCGATGACCA 454
A.ricini         CCGAGCACCTAATGCAACAAAGGTTTTCGTCAATGGTGTTTGG------GTTGGTATTCA 524
                    *  *** * *           ** **    * ***  **      **   *   **


B.fukeliana      ATAGAGGTC----TGCACAC------------TCGTCCTGCATCTGTGAA----AATCT 492
B.cinerea        ATAGAGGTC----TGCACAC------------TCGTCCTGCATCTGTGAA----AATCT 418
B.ricini         ATAAAGGTCGCACTGTCCATGGTAAATCGAACTTCGTCACG-ATTTCTGAATGGTGAACT 513
A.ricini         -TCGAGATC---CTGCTCAT------------TTGGTCAAATGTGTACAAG-----ATCT 563
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                 *  ** **    **  **             * ***     * *   *      * **

B.fukeliana      T----GAATTC------TCTAT--------CACGAA-TTTCTCGAATAAGTGAAACTTCA 533
B.cinerea        T----GAATTC------TCTAT--------CACGAA-TTTCTCGAATAAGTGAAACTTCA 459
B.ricini         TCAGAGAGATCAGTGGATCTACGAGATCTGTACACA-TTGACCAAATGAGCAGGATCTGA 572
A.ricini         TCGTAGATCACACTTGATCTCT--------CATGAAGTCTCACTCATTCGAGAAA-TTCG 614
                 *    **   *      ***           *   * *    *  **  *    *  *

B.fukeliana      TGAGA--GA--TCAAG--TGTGATCTACG-AAGAT--------AT-TGGGGGCTCGGAGT 577
B.cinerea        TGAGA--GA--TCAAG--TGTGATCTACG-AAGAT--------AT-TGGGGGCTCGGAGT 503
B.ricini         TGATACCAA--CCCAA--CACCATTGACG-AAAACCTTGTTGCAT-TAGGTGCTCGGAGT 626
A.ricini         TGACAGAGAGTTCAAGATTTTCACCGATGCAGGAC------GAGTGTGCAGACCTTTATT 668
                 *** *   *   * *       *   * * * *  *          * *     *    * *

B.fukeliana      GGTTCAT--AC-----TCCTC-CAATACTTCCA----TATTTCG---TTGAATCATGA-- 620
B.cinerea        GGTTCAT--AC-----TCCTC-CAATACTTCCA----TATTTCG---TTGAATCATGA-- 546
B.ricini         GGT-CAT--AC-----TCCTC-CAACACTTCCA----TATTTCG---TTGAATCATGA-- 668
A.ricini         GGT-CATCGACAATGATCCTGACAGCGCTAACAAAGGTAACTTGGTATTGAACAAGGATC 727
                 *** ***  **     ****  **   **  **    **  * *   *****  * **

B.fukeliana      --ACTCAACGATTGGA------TCACTTGGCGTACCGACTGTAACG--------TAACAC 664
B.cinerea        --ACTCAACGATTGGA------TCACTTGGCGTACCGACTGTAACG--------TAACAC 590
B.ricini         --ATTCGACGATTGGA------TCACTTGGCGTACCGACTG--ACG--------TAACAC 710
A.ricini         ATATTCGCCGTCTGGAAGATGATCAATTGC--TACCAGCAAATATGGATAAGGATGACAA 785
                   * **  **  ****      *** ***   ****  *    * *        * ***

B.fukeliana      ATCAGAGC-CAAATTCTTAACCAAACCACAAGCTTGTCCTTCG--GGCGTCTCTG----- 716
B.cinerea        ATCAGAGC-CAAATTCTTAACCAAACCACAAGCTTGTCCTTCG--GGCGTCTCTG----- 642
B.ricini         ATCAA--C-CAAGTCTT--ACCAAACCACA-GCTTGACCTTCG--GGTGTCTCTG----- 757
A.ricini         AGTAAGACACGGATACT--ATGGATTCCAAGGTTTGATCAACGATGGCGTGGTTGAATAC 843
                 *  *   * *   *  *  *   *  *  * * *** *  **  ** **   **

B.fukeliana      CCGGAC--------AGACCAAGCCCCAATGGGTATTATGCAGCTGTCTAGGTTTGGCGAT 768
B.cinerea        CCGGAC--------AGACCAAGCCCCAATGGGTATTATGCAGCTGTCTAGGTTTGGCGAT 694
B.ricini         CCGGGC--------AGACCAAGCCCCAGTGAGTATTATGTAGTTGTCTAGGTTTGGCGAT 809
A.ricini         CTGGACGCTGAGGAAGAAGAGACTGTCATGATTACCATG-ACTCCTGAAGATTTGGACAT 902
                 * ** *        ***  *  *     **  **  *** *    *  ** *****  **

B.fukeliana      CTTTCCATCACGTCCAATGGGTGTATTGGTTCGGCGCAAATGAGAAAGTGTGGATGCAAA 828
B.cinerea        CTTTCCATCACGTCCGATGGGTGTATTGGTTCGGCGCAAATGAGAAAGTGTGGATGCAAA 754
B.ricini         CTTTCCATCACGTCCAATGGGTGTATTGGTTCGGCGCAAATGGGAAAGTGTTGATGCAAA 869
A.ricini         CTCTCGACAGCTTCAGGCTGGT-TATCAGATTCGTCCTGACG--AAAGTGGTGATTTGAA 959
                 ** ** *   * **     *** ***  * *  *  *  * *  ******  ***   **

B.fukeliana      --GGTATATC----TGTTCAACACTTGAGACACTCCGGCGGTAGAAC---TTGCTGCCTT 879
B.cinerea        --GGTATATC----TGTTCAACACTTGAGACACTCCGGCGGTAGAAC---TTGCTGCCTT 805
B.ricini         --GGTATATC----TGTTCAACACTTGCGATACACCAGCGGTAGAAC---TTGCGGCCTT 920
A.ricini         CAAGCGTGTCAAGGCGCCTATCAATC-TGAC-TGCCCATGTCTGGACCCATTGTGAAATT 1017
                    *  * **     *   * ** *   **    **   *   * **   ***     **

B.fukeliana      CTTCTGG---TCACCCCAGTTACCTGTGGCCAAAGAATATTTCAGACC--GTTGGTGATT 934
B.cinerea        CTTCTGG---TCACCCCAGTTACCTGTGGCCAAAGAATATTTCAGACC--GTTGGTGATT 860
B.ricini         CTTCTGA---TCACCCCAGTTACCTGTGGCCAAAGAATATTTCAGACC--GTTCGTGATT 975
A.ricini         CATCCAAGTATGATCTTGGGTATCTGCG--CAAGCATTATTCCCTTCCCAGATCACAATC 1075
                 * **      * * *   * ** *** *  ***  * **** *   **  * *    **

B.fukeliana      GTTGTTGATTTCACACCCAAAGTCAAATTAA---ACTCTCGGTTGTTTTCCACGCAACGT 991
B.cinerea        GTTGTTGATTTCACACCCAAAGTCAAATTAA---ACTCTCGGTTGTTTTCCACGCAACGT 917
B.ricini         GTTGTGGATTTCACACCCAAAGTCAAATTGA---ACTCTCGGTTGTTTTCCACGCAACGT 1032
A.ricini         AGGTAAGCTTTTAAGTCCAATG---AATTTATTCATTCATGGCTAATGT-----TATTGC 1127
                       * *** *   **** *   **** *   * **  ** * * *      *  *
```

```
B.fukeliana      TGCAAGTATCTGTACACGTCTGTTGTCAATCTGCGGAACAGATTTCTGAACAACTTTGCA 1051
B.cinerea        TGCAAGTATCTGTACACGTCTGTTGTCAATCTGCGGAA---------------------- 955
B.ricini         TGCAAGTATCTGTACACATCTGTTGTCAATCTACGGAATAGATTTCTGAACAACTTTGCG 1092
A.ricini         TACAAA-GTCTCCTCGTAACTGTTATCAATCTGC----------TATGGGTAA----GCA 1172
                 * ***   ***   *    ***** ******* *

B.fukeliana      AGTAAAGGTCCAGCCAAATCCAAACGCTTCTTTCCGAAATGATCTCGATCATC 1104
B.cinerea        ------------------------------------------------------
B.ricini         AGCAAAGGTCCAGCCAAATCCAAACGCTTCTTTCCGAAATGATCACGATCATC 1145
A.ricini         AGCTATGGG---------------------------------------------- 1181
```

Phylogenetic analysis of *Botrytis ricini*, GenBank accession numbers

1.6.1.Botrytis ricini glyceraldehyde-3-phosphate dehydrogenase (G3PDH) gene, partial sequence

GenBank: GQ860998.1
LOCUS GQ860998 997 bp DNA linear PLN 19-OCT-2009
DEFINITION : Botrytis ricini glyceraldehyde-3-phosphate dehydrogenase (G3PDH) gene, partial sequence.
ACCESSION GQ860998
VERSION GQ860998.1 GI:261286635
KEYWORDS .
SOURCE Botrytis ricini
ORGANISM Botrytis ricini, Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina; Leotiomycetes; Helotiales; Sclerotiniaceae; mitosporic, Sclerotiniaceae; Botrytis.
REFERENCE 1 (bases 1 to 997)
AUTHORS Durga,B.K.B. and Dinesh,K.V.
TITLE Molecular phylogenetic relationship of *Botrytis ricini* with other Botrytis spp. using house keeping gene sequences
JOURNAL J Oilseeds Res 26, 190-191 (2009)
REFERENCE 2 (bases 1 to 997)
AUTHORS Durga,B.K.B. and Dinesh,K.V.
TITLE Direct Submission
JOURNAL Submitted (06-AUG-2009) Biotechnology, Directorate of Oilseeds Research, Rajendranagar, Hyderabad, Andhra Pradesh 500030, India
FEATURES Location/Qualifiers
source 1..997
/organism="Botrytis ricini"
/mol_type="genomic DNA"
/db_xref="taxon:680430"
/PCR_primers="fwd_seq: attgacatcgtcgctgtcaacga, rev_seq:

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                accccactcgttgtcgtacca"
     gene       <1..>997
                /gene="G3PDH"
                /note="glyceraldehyde-3-phosphate dehydrogenase; coding
                region not determined"
ORIGIN
        1 attgacatcg tcgctgtcaa cgacccttтc atcgagactg aatatgccgt aagttcccgc
       61 tatcggaccg cccgcagata tcacggaccc gagctaattt gtattcttta caggcataca
      121 tgttgaagta tgattctacc cacggccaat tcaagggtga cgtcaaggtc cttcccgacg
      181 gattggaggt caatggcaag aaggtcaagt tctacaccga gagagaccca gccaacatcc
      241 catgggctga gtctgaggca tactacgttg tcgagtctac cggtgttttc accaccaccg
      301 agaaggccaa ggcacatttg aagggtggtg ccaagaaggt tgtcatctct gctccttctg
      361 ccgatgcccc aatgtacgtt atgggtgtca acaacgagac ctacaagggt gatgttgatg
      421 ttatctccaa cgcctcttgc acaaccaact gcttggctcc tctcgccaag gtcatcaacg
      481 atgagttcac catcattgaa ggtttgatga ccaccatcca ctcctacacc gccacccaaa
      541 agaccgtcga cggtccatcc gctaaggatt ggcgtggagg acgtaccgct gctcaaaaca
      601 tcatcccatc gagcaccggt gccgccaagg ctgtcggaaa ggtcattcca gagcttaacg
      661 gaagctcacc ggaatgtcta tgcgtgttcc aactgccaac gtcacagttg ttgacttgac
      721 tgtccgtctt gagaagggtg cttcttacga tgagatcaag gccgtcatca agaaggctgc
      781 tgatggtccc ctcaagggta agtaattcca tttaatctta cttagggttc caattttact
      841 aatcgtcaca taggcatatt ggcttacact gaagacgagg ttgtctccac tgacatgaac
      901 ggtgacaacc actcttccat cttcgatgcc aaggctggta tctccctcaa cgcaaacttc
      961 gtcaagttgg ttttcatggt acgacaacga gtggggt
//
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1.6.2.Botrytis ricini heat shock protein 60 (HSP60) gene, partial sequence

GenBank: GQ860996.1 LOCUS GQ860996 1112 bp DNA linear PLN 19-OCT-2009
DEFINITION Botrytis ricini heat shock protein 60 (HSP60) gene, partial sequence.
ACCESSION GQ860996
VERSION GQ860996.1 GI:261286633
SOURCE Botrytis ricini
ORGANISM Botrytis ricini Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina; Leotiomycetes; Helotiales; Sclerotiniaceae; mitosporic Sclerotiniaceae; Botrytis.
REFERENCE 1 (bases 1 to 1112)
AUTHORS Durga,B.K.B. and Dinesh,K.V.
TITLE Molecular phylogenetic relationship of Botrytis ricini with other Botrytis spp. using house keeping gene sequences
JOURNAL J Oilseeds Res 26, 190-191 (2009)
REFERENCE 2 (bases 1 to 1112)
AUTHORS Durga,B.K.B. and Dinesh,K.V.
TITLE Direct Submission
JOURNAL Submitted (02-SEP-2009) Biotechnology, Directorate of Oilseeds Research, Rajendranagar, Hyderabad, Andhra Pradesh 500030, India

FEATURES Location/Qualifiers
source 1..1112
/organism="Botrytis ricini"
/mol_type="genomic DNA"
/db_xref="taxon:680430"
/PCR_primers="fwd_seq: caacaattgagatttgccca, rev_seq: gatggatccagtggtaccgagc"
gene <1..>1112
/gene="HSP60"
/note="heat shock protein 60; coding region not determined"
ORIGIN
1 caacaattga gatttgccca caaggtatgt tgacttagct ttagttcatg tcactcttag
61 atatgagata tatatctggc ggtcatatac taactatgct attatttagg agctcaaatt
121 cggtgtggag ggtagagcag ctcttctcgc tggtgttgag actttggcaa aggctgttgc
181 cacaacctta ggtcctaaag gtcgaaatgt tcttattgag tcagcatatg gctccccaaa
241 gatcactaaa ggtttgcgaa acttcacgtt accttgttgt aaaattctaa ttgttgttga
301 atagatggtg taactgtggc cagagccatt tccctcaagg acaaatacga gaacctcggt
361 gctagactta tccaagatgt cgcctcgaaa accaacgaga ccgctggtga tggaaccaca
421 tctgctactg tccttgctaa atctattttc tccgagaccg tcaagaacgt cgccgctggt
481 tgcaacccaa tggacttgcg cagaggtaca cagggccgcc gtgaggccgt tgtgaattct
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661 aggtgtcatc acagtcaagg aaggaaagac catggaggac gaactggaca tcactgaggg
721 aatgaaattt gaccgcggtt atgtctctcc atactttatc accgatacca agtcgcaaaa

781 agtggaattc gagaagccat tgattcttct ttccgagaag aagatctcaa acgtccaaga
841 tatcatccca gcacttgagg cttctactca acttcgtcgt cctttggtca tcattgctga
901 agatatcgat ggagaagctc tcgctgtatg cattcttaac aagctccgtg gtcaactcca
961 agttgccgct gtcaaggcgc ccggctttgg tgacaaccga aagtcccatt cttgcgatct
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1081 actgcagaca gctcggtacc actggatcca tc
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1.6.3.Botrytis ricini RNA polymerase II binding protein (RPB2) gene, partial sequence

GenBank: GQ860997.1 LOCUS GQ860997 1205 bp DNA linear PLN 19-OCT-2009
DEFINITION Botrytis ricini RNA polymerase II binding protein (RPB2) gene, partial sequence.
ACCESSION GQ860997 VERSION GQ860997.1 GI:261286634
SOURCE Botrytis ricini
ORGANISM Botrytis ricini Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina;
Leotiomycetes; Helotiales; Sclerotiniaceae; mitosporic Sclerotiniaceae; Botrytis.
REFERENCE 1 (bases 1 to 1205)
AUTHORS Durga,B.K.B. and Dinesh,K.V.
TITLE Molecular phylogenetic relationship of Botrytis ricini with other Botrytis spp. using house keeping gene sequences
JOURNAL J Oilseeds Res 26, 190-191 (2009)
REFERENCE 2 (bases 1 to 1205)
AUTHORS Durga,B.K.B. and Dinesh,K.V.
TITLE Direct Submission
JOURNAL Submitted (02-SEP-2009) Biotechnology, Directorate of Oilseeds Research,
Rajendranagar, Hyderabad, Andhra Pradesh 500030, India
FEATURES Location/Qualifiers
source 1..1205
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/PCR_primers="fwd_seq: cccatagcttgcttacccatag, rev_seq:
gatgatcgtgatcatttcgg"
gene complement(<1..>1205)
/gene="RPB2"
/note="RNA polymerase II binding protein; coding region
not determined"
ORIGIN
1 cccatagctt gcttacccat agcagattga taacagttac gaggagactt tgtagcaata
61 acattagcca tgaatgaata aattcattgg acttaaaagc ttacctgatt gtgatctggg
121 aagggaataa tgcttgcgca gatacccaag atcatacttg gatgaatttc acaatgggtc
181 cagacatggg cagtcagatt gataggcgcc ttgacacgct tgttcaaatc accactttcg
241 tcaggacgaa tctgataacc agcctgaagc tgtcgagaga tgtccaaatc ttcaggagtc
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361 atcaaacctt ggaatccata gtatccgtgt cttactttgt catccttatc catatttgct
421 ggtagcaatt gatcatcttc cagacggcga atatgatcct tgttcaatac caagttacct

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1021 agaccgttcg tgattgttgt ggatttcaca cccaaagtca aattgaactc tcggttgttt
1081 tccacgcaac gttgcaagta tctgtacaca tctgttgtca atctacggaa tagatttctg
1141 aacaactttg cgagcaaagg tccagccaaa tccaaacgct tctttccgaa atgatcacga
1201 tcatc
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Promoter PLACE Analysis :

AtACS4 Promoter :

Web Signal Scan Program

Database searched: PLACE

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AtACS5 promoter :

Web Signal Scan Program

Database searched: PLACE

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AtACS7 Promoter :

Web Signal Scan Program

Database searched: PLACE

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form], 1218 bases, 905BB2C4 checksum.
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gagaagaagtttgtgtaaagaataaggaagattcgattgtggaaaaatac
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tatccatttacactttat
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CLUSTAL 2.0.10 multiple sequence alignment of ACS4, 5 and 7 promoter sequences

```
AtACS5          ------TTAATTATTAAAAAAGTACTTCAAAAA-AGTGG-ATAT-ACGATTTTGAAATGA 51
AtACS7          --------TTTTCTTAGAGCTTCGAACCTGACA-CGTGACACGTGACAGCTCTAAAAACA 51
AtACS4          CAAATCTTCCCTCATATTATATAGCCATTGATATCATAGAGGATGTGAGTTTTAACTTAA 60
                             *  **               * *   *      *      * * *    *

AtACS5          TAACGATTTCCTGACGTCAGAAAAAATCCTATAACAAACATTAATGTTTTTTT-TTGGTT 110
AtACS7          CAATCGGAGAGAGAGA--GGGAGAGAGGTTGAGAGAAAGAGAAGTGTGTTGGAGTTAAAC 109
AtACS4          TATTTACCCGTTTGAAACTAGCTATTTACTTAAATATGAATTATAATCTAGT--TTAACT 118
                 *                    *     *   * *   *  *   * *     **

AtACS5          AGTTAAGAGACAATAATATGTTACATCTACCAAAGAATATTCCAGTTAAGTTAGTTAATA 170
AtACS7          ATTTGTGTG--AATGAAGGAGTATATATAGCAGACGTGACCAAGAGGAAAGTCTTTGACA 167
AtACS4          ACCAAAAAACATCATATGGGGACAAGAAAAAGTAATAAAACGTATGGAAAATTTTGTAGAT 178
                *           **        *    *    *    *        **  *   *

AtACS5          GTTGGAGTCAAACTTAA-ACGAACAACGTCGCCTACAAATCTGGGG---GTATAAGTTAT 226
AtACS7          AGT----TCAATTTTAATATTAGTTTACTTACATACAGAGAAGAAGTTTGTGTAAAGAAT 223
AtACS4          GTT----ATAAATGGATAATTATTCAAGTGATAATCTATCACTTTG---ATCTTATCTCT 231
                  *      **    *  *  *      *      *         *    * * *    *

AtACS5          AACCAACGGCC-ACCAACATGGGTTTTGATATCGTCCACCTG-ATTTTTATGTTAATATA 284
AtACS7          AAGGAAGATTCGATTGTGGAAAAATACGGTGTCATTGACCTGCAAATGGATGAATAGATA 283
AtACS4          TTATCCAATTTAATTACTTTGTCTCTAAGTGATTTGCTTCCA-AAATCTAAGTGTAGTCT 290
                             *                 *    *    *   *  *  * *   *

AtACS5          ACTCAATTTGATTGATTAAATATTATGCTCTTATACTAATCATTCTAGGT---CCAAAAC 341
AtACS7          AGAGTGTTTGGCTATTTTTAT-TTTTGTTTTTATCTTGAAACTTCTCGGTGGTTCATCTT 342
```

```
AtACS4          ATCCTATTTCTATCTTATCCTATCATATAATCTTCTATATATATGTGAGT---CCGATGT 347
                *     ***   *  *    * *  *    *  *    *    * *  **    *

AtACS5          TGAATCACTCATTGCACATCATTTTTATGATTTTTATTTTGAAAATCTCATGTTTCAAGA 401
AtACS7          TAGGAGATCGCCAGTTCTATAAAACACTGATTATCAATTTTTAAACGTAATGTTGT---T 399
AtACS4          TGTAAAGCGTACGAGAGAGAGTAATGAAGAGTGA-AGTGTTATATTGTTCTCTCGTCCAC 406
                *                        ** *   * * *   *   *  * *

AtACS5          AATATTTGTGCATTCTACTAGTAGCTTA-TTTTAAATAAAGTTT-CGAGTTTATATAATA 459
AtACS7          TAGACTTTAGTCTGCTAAAAATAGTTTAGTTTCTATTATTGTGTACGAGTTTAAATGTGT 459
AtACS4          TTCCACTCTCTCTTTTATCTCTTACTTACTTCTTCGTAAGATC----ATTACATATAATA 462
                      *     *  **    *   *** **     **   *     * *  * **

AtACS5          AACAATAT-GTTTATTTCTTTGA---TTTGTATACATTTTAAAAAAAAAAACAGGATGAA 515
AtACS7          TTTAATGTTGTTTGTTTTTGGAAAACTTTCTAAATATTTTGGTTTAGTCGGCTGCTACGA 519
AtACS4          AATAATATTATTTATGTTTGTGT---------TATATTTAATAACAGTAAAAAGTTTTAA 513
                   *** *  *** * * *              * ****      *       *     *

AtACS5          GATATATACAACTAAAGCTCAATTCAACTAGTAGGAAAATTGTATTCTA-CAAAAAATTT 574
AtACS7          TTTAAATCCAA--AAGGGTCACTAATGCGTTTTAGATAATAGTCTCGTAATTAATAATCC 577
AtACS4          AACGT-TGAAAAAATTAGCCGACATAGAATACAAAAGAGGGTTAGCATCGGGGGAGAAAC 572
                      *  **  *     *                  * *    *    *       *

AtACS5          GTGTTTATGTTTTTATTATGATAACCATCCAAACTCATTATTTCACCCATGATCAAAGTA 634
AtACS7          TTCACTAATACAATATTGTTACACAGAAAAAAAGATTCTCGTTTACCTATTTT-----TC 632
AtACS4          GTGG--ACCAACATGATACACCCTCCAAAATAGTCCCCAAGTTGAAACATTGACATGTTT 630
                 *    *      *  *         *    *         ** *   **        *



AtACS5          ACTATATCAATATATCTGCAACAATATCTGACTCAACATTTATTTTTACATAAACTGCGG 694
AtACS7          ACTAT-TTGATATTCTTACAGAATGGTCACTAAGAAAACTGTAAAGTACTTTTGTTTTTT 691
AtACS4          CGCTTTTTCTTTTCTGTGTA-TACTTTTTTTTTCTGTGGGTCACATTATTTAATATTTGT 689
                    * *   * *   *  *  *   *                        **  *    *

AtACS5          ACCAACACAATTATGCTAAACTATCATCATTTATTCTATTGGTATTGGAGATCAGTCTAC 754
AtACS7          CTTAGAG--ACCATAACAAAAGGTAGTTACTTTTTTTCTTTGA---GAATACAAATTTAC 746
AtACS4          ATACAAGCAGCTATTTTACATGGAGATTTCCTGTCGGTATAGC----GTCCTCATTTCTC 745
                             **   * *      *    * *    * *          * *   *

AtACS5          GTCAGTGAATACCGCATAATTCACATGAAACTAACTCCAAAACCCTTTA-ACCAAAAAAA 813
AtACS7          GTAAACAAAGAGT-CATCAGTACTAGTATGTCGTTTTTAACTACATACA-GTTTCTATAT 804
AtACS4          CATCGCTTCCACT-TTTTTCCTATACTAATTTGATCTAATTAATTCATATGTCAAAACAT 804
                          *     *       *  *          *       *      * *

AtACS5          AAAAAAAACTAATTACAACTCCGGTTAACCTGTCGATAATTACAAGACTGAAAAC--ATC 871
AtACS7          AAATTATACGCGT--CTGTTTTACATTATGTGT-GTTAAATATAATAATAGGAGCTGGAA 861
AtACS4          TAAGAAAATGAAA--CTCGTAATTCATACTTGA-ATTTAATAGATTAATTAAAATGCTAT 861
                 **  * *       *   *       *  **    * * ** *  * *   *

AtACS5          CTAGTCAGTAAAAAACATATTTGTATGTATCCGATCCCACCAAATAAATAAGTTGTTGTC 931
AtACS7          TAAGTAACTAATCAAATTTATTGGGTATACTAAACTCCATAGATGAAAGAAATGA--GGA 919
AtACS4          TTATTGGCAAAATAAACTCGGTTTATAT-CTAAATTTTAGAATCACTAAAACTTTTTGCC 920
                  * *    **  **  *   *   * *     *    *       * ** *    *

AtACS5          AATGATAGAGGAAGAGAAAACGAAAAAAAATTGCGGACCAAGACATTTGGGAC----TAC 987
AtACS7          AACAACTAAATTAAATGTTCTGATTCGATGTTTTAAGCAAAATCAAAGATCACCTTTTGC 979
```

```
AtACS4          CAAAAAAAAATAAAAATAAATCACTAAAA---CAAAAAACAATCAAAAGAAAACCCATGT 977
                 *  *   *   * *       *   *            *  **      *    *

AtACS5          TAG-AGTTTAGGGAGCATAAATGGT--CCTATTTGACTTACCCTCTACCGTATAAATAA- 1043
AtACS7          CAA-AATCAATTTCGGACAACTGTTAGCTTATGTCTTCTTCTTCCTTCTCTCTTGTTGAT 1038
AtACS4          TGGTAAATCGGATAATGAAAATAAT---TAGAATCCCCGTCCTTTGTGTATTTTGGCGTA 1034
                    *              ** *  *       *      *        * *

AtACS5          ATGTATTTTAGCCGTATAAATGTATACATACGTAGATGCATAGGTATACAGGATTACTTT 1103
AtACS7          ACTTATTTTTTCTACTTAA---CATTTTTTAATTTATTCATTTATTTCCA---TAACTTT 1092
AtACS4          GCATG---AAACTATATAA---TAAACATGCATTCATTCTTAGACTTCTCGTAGCTTATC 1088
                   *       *    ***    *    *   *  ** * *     *           *

AtACS5          AG--ACGATTTGATGTCGGTATGACATTACATTTGACT-CCC-CTAGCTCCACTTGTCTC 1159
AtACS7          AGTTATGATCTATAGCCCAAAT-ACGGTGGTTGAAATT-CCGATTATATGGATTTGGCAT 1150
AtACS4          AACAACAACGCGCTCGATCTCTCTCAGCCTGTCTGACAACTCTTTCTCTAGTTCTAGAGT 1148
                *   *  *              *  *      *   *   *    *   *     *

AtACS5          TGTTCATTTTTCTT-GTATGTCATTTCCTTGTTTTCCAATTTTACGTTTACCACCCTATT 1218
AtACS7          CATTAAAAAAATGA-GAAATTTACATTATACGAGAGGAGTGGGTGGGTGGCTATCCATTT 1209
AtACS4          TTTCAATTTATTGTTGAGCCTTTTATTAAAAAAAAAAAAACAAGAACAAAAGAAATGGTT 1208
                  *  *          *    *    *           *                *    **

AtACS5          TTATTTTGTTAATATTTTC 1237
AtACS7          ACACTTTAT---------- 1218
AtACS4          CAA---------------- 1211
                  *
```

***Cis*-element analsis of AtAACS4 promoter :**

Factor	Seq	Species	Source
AGL3	aaaacatcaTATGGgac	Arabidopsis	TRANSFAC
AG	actaCCAAAaacatcata	Arabidopsis	TRANSFAC
ANT	caTCGGGgagaaa	Arabidopsis	TRANSFAC
Athb-1	ctcatATTATatag	Arabidopsis	TRANSFAC
ATHB-5	tAATAAaac	Arabidopsis	TRANSFAC
ATHB-9	cattgatATCATagaggat	Arabidopsis	TRANSFAC
CDC5	ctcTCAGCctg	Arabidopsis	TRANSFAC
PIF3	gggagaaACGTGgaccaa	Arabidopsis	TRANSFAC
RAV1	ccgaTGTTGtaa	Arabidopsis	TRANSFAC
Dof1	ttgTAAAGcgt	Maize	TRANSFAC
Dof2	ttgtAAAGCgt	Maize	TRANSFAC
Dof3	ttgtAAAGCgt	Maize	TRANSFAC
O2	cgagagagagtAATGA	Maize	TRANSFAC
PBF	aacAAAAGaaa	Maize	TRANSFAC
P	aaCTACCaa	Maize	TRANSFAC
MYB.Ph3	taaactCGGTTta	Petunia	TRANSFAC
TEIL	catTACAT	Tobacco	TRANSFAC
-10PEHVPSBD	AGAATA	Barley	PLACE
-300ELEMENT	ATTTTACA	wheat	PLACE

ABRELATERD1	ACGTG	Arabidopsis	PLACE
ABRERATCAL	AACGTGG	Arabidopsis	PLACE
ACGTATERD1	ACGT	Arabidopsis	PLACE
AMYBOX2	ATGGATA	barley/rice/wheat	PLACE
ANAERO2CONSENSUS	AGCAGC	maize/Arabidopsis/pea/barley/rice	PLACE
AP1	CCAAAAA	Arabidopsis	AGRIS
ARFAT	TGTCTC	Arabidopsis/Soybean/rice	PLACE
ARF	TGTCTC	Arabidopsis	AGRIS
ARR10	TATAATCT	Arabidopsis	JASPER
ARR1AT	AATCA	Arabidopsis	PLACE
BIHD1OS	TGACA	rice	PLACE
BOXIINTPATPB	ATAGAA	tobacco	PLACE
Bellringer	ACTAATTT	Arabidopsis	AGRIS
C1MOTIFZMBZ2	TAGTTTA	maize	PLACE
C8GCARGAT	CTTAAATATG	Arabidopsis	PLACE
CAATBOX1	CAAT	pea	PLACE
CAREOSREP1	CAACTC	rice	PLACE
CARGCW8GAT	CAATTTATTG	Arabidopsis	PLACE
CATATGGMSAUR	CATATG	soybean	PLACE
CBFHV	GCCGAC	barley	PLACE
CCAATBOX1	ATTGG	Soybean	PLACE
CIACADIANLELHC	CAAAAACATC	tomato	PLACE
Core	TAAT	Arabidopsis	AGRIS
DOFCOREZM	CTTT	maize	PLACE
DRE2COREZMRAB17	GTCGGT	maize	PLACE
DRECRTCOREAT	GCCGAC	rice/maize/sunflower	PLACE
Dof2	AAAGCG	Maize	JASPER
Dof3	CACTTT	Maize	JASPER
EBOXBNNAPA	CATATG	rape	PLACE
GATABOX	GATA	petunia/Arabidopsis/rice	PLACE
GATABOX	TATC	petunia/Arabidopsis/rice	PLACE
GT1CONSENSUS	GATAAT	pea/oat/rice/tobacco/Arabidopsis	PLACE
GT1GMSCAM4	TTTTTC	soybean	PLACE
HMG-1	GAACAAAAG	Pea	JASPER
HMG-IY	TATTAAAAAAAAAAAA	Pea	JASPER
IBOXCORENT	TCTTATC	tobacco	PLACE
IBOX	CTTATC	tomato/Arabidopsis	PLACE
INRNTPSADB	TTCATTCT	tobacco	PLACE
LTREATLTI78	TGTCGGT	Arabidopsis/barley	PLACE
LTRECOREATCOR15	CCGAC	Arabidopsis/rape	PLACE
MNB1A	AAAGA	Maize	JASPER
MYB.ph3	AAACAATCA	Petunia	JASPER

MYB4	AACTACC	Arabidopsis	AGRIS
MYBCOREATCYCB1	CCGTT	Arabidopsis	PLACE
MYBCORE	TAACAG	Arabidopsis/petunia	PLACE
MYBCORE	TAACAG	Arabidopsis/petunia	PLACE
MYBST1	GGATA	potato	PLACE
MYCCONSENSUSAT	CATATG	Arabidopsis	PLACE
NODCON2GM	CTCTT	soybean	PLACE
PALBOXAPC	CCGTCC	parsley	PLACE
PBF	AAAGT	Maize	JASPER
POLASIG3	ATTATT	maize	PLACE
POLLEN1LELAT52	AGAAA	tomato	PLACE
PYRIMIDINEBOXHVEPB1	TTTTTTCC	barley	PLACE
PYRIMIDINEBOXOSRAMY1A	CCTTTT	rice/barley	PLACE
RAV1-A	TGTTG	Arabidopsis	AGRIS
RAV1AAT	CAACA	Arabidopsis	PLACE
TATABOX2	TATAAAT	pea/tobacco/bean	PLACE
TBOXATGAPB	ACTTTG	Arabidopsis	PLACE
TGBOXATPIN2	AACGTG	tomato/Arabidopsis	PLACE
WBOXNTERF3	GGTCA	tobacco	PLACE
WRKY71OS	TGAC	rice/parsley	PLACE
id1	TTTTTTTTTCTG	Maize	JASPER

***Cis*-element analsis of AtAACS5 promoter :**

Factor	Seq	Species	Source
AG	atgtttttttTTTGGttag	Arabidopsis	TRANSFAC
Athb-1	gacaATAATatgtt	Arabidopsis	TRANSFAC
ATHB-5	taaTTATTa	Arabidopsis	TRANSFAC
ATHB-9	ttttgaaATGATaacgatt	Arabidopsis	TRANSFAC
RAV1	cacCAACAtggg	Arabidopsis	TRANSFAC
Dof1	ttaCTTTAgac	Maize	TRANSFAC
Dof2	aactAAAGCtc	Maize	TRANSFAC
Dof3	aactAAAGCtc	Maize	TRANSFAC
O2	tacgattttgaAATGA	Maize	TRANSFAC
PBF	caaAAAAGtgg	Maize	TRANSFAC
P	atCTACCaa	Maize	TRANSFAC
MYB.Ph3	aaAACTAattaca	Petunia	TRANSFAC
bZIP910	ccTGACGtcaga	Snapdragon	TRANSFAC
ACGTABOX	TACGTA	plantrice	PLACE
ACGTABOX	TACGTA	plantrice	PLACE

ACGTATERD1	ACGT	Arabidopsis	PLACE
ACGTATERD1	ACGT	Arabidopsis	PLACE
ACGTATERD1	ACGT	Arabidopsis	PLACE
ACGTATERD1	ACGT	Arabidopsis	PLACE
ACGTATERD1	ACGT	Arabidopsis	PLACE
ACGTATERD1	ACGT	Arabidopsis	PLACE
ACGTATERD1	ACGT	Arabidopsis	PLACE
ACGTATERD1	ACGT	Arabidopsis	PLACE
ACGTATERD1	ACGT	Arabidopsis	PLACE
ACGTATERD1	ACGT	Arabidopsis	PLACE
AMYBOX1	TTTGTTA	barley/rice/wheat	PLACE
AMYBOX1	TTTGTTA	barley/rice/wheat	PLACE
AMYBOX1	TTTGTTA	barley/rice/wheat	PLACE
AMYBOX1	TAACAAA	barley/rice/wheat	PLACE
AMYBOX1	TAACAAA	barley/rice/wheat	PLACE
ASF1MOTIFCAMV	CGTCA	tobacco/Arabidopsis	PLACE
AtMYB2	TGGTTAG	Arabidopsis	AGRIS
C8GCARGAT	CAATAATATG	Arabidopsis	PLACE
Core	TAAT	Arabidopsis	AGRIS
DOFCOREZM	AAAG	maize	PLACE
MYCCONSENSUSAT	CATTTG	Arabidopsis	PLACE
NODCON2GM	AAGAG	soybean	PLACE
POLLEN1LELAT52	AGAAA	tomato	PLACE
bZIP910	ACGTCAG	Snapdragon	JASPER
id1	CAAAAAAAAAAA	Maize	JASPER

***Cis*-element analsis of AtAACS7 promoter :**

Factor	Seq	Species	Source
AGL3	gagaCCATAacaaaaggt	Arabidopsis	TRANSFAC
AG	ttatatggaTTTGGcatc	Arabidopsis	TRANSFAC
Athb-1	cactgATTATcaat	Arabidopsis	TRANSFAC
GAmyb	ggtGGTTG	Barley	TRANSFAC
Dof1	gtgTAAAGaat	Maize	TRANSFAC
MYB.Ph3	taatatTAGTTta	Petunia	TRANSFAC
AMYBOX2	TATCCAT	barley/rice/wheat	PLACE
ANAERO1CONSENSUS	TTTGTTT	maize/Arabidopsis/pea/barley/rice	PLACE
AP1	TTTTTGG	Arabidopsis	AGRIS
Agamous	CCAAATACGGT	Arabidopsis	JASPER

CAATBOX1	ATTG	pea	PLACE
CACGTGMOTIF	CACGTG	tomato/Arabidopsis/snapdragon/wheat	PLACE
CARGCW8GAT	CTAAAAATAG	Arabidopsis	PLACE
CBFHV	GTCGGC	barley	PLACE
CCAATBOX1	ATTGG	Soybean	PLACE
CGCGBOXAT	ACGCGT	Arabidopsis	PLACE
CGCGBOXAT	ACGCGT	Arabidopsis	PLACE
CIACADIANLELHC	GATTCGATTG	tomato	PLACE
CPBCSPOR	TATTAG	cucumber	PLACE
DOFCOREZM	CTTT	maize	PLACE
DPBFCOREDCDC3	ACACGTG	carrot/Arabidopsis	PLACE
Dof3	TACTTT	Maize	JASPER
EBOXBNNAPA	CATTTG	rape	PLACE
MYCCONSENSUSAT	CACGTG	Arabidopsis	PLACE
OSE2ROOTNODULE	AAGAG	bean/Medicago/soybean/Sesbania	PLACE
PBF	ACTTT	Maize	JASPER
POLLEN1LELAT52	AGAAA	tomato	PLACE
TATABOX5	TTATTT	pea	PLACE
TATABOXOSPAL	TTAAATA	rice	PLACE
TATCCAOSAMY	TATCCA	rice	PLACE
WRKY71OS	GTCA	rice/parsley	PLACE