Studies on heterologous expression of plant stress responsive genes in Human glioblastoma cell lines (U373), Cotton (*Gossypium hirsutum* L.) and Tomato (*Lycopersicum esculentum* L.)

Thesis submitted to the University of Hyderabad for the degree of

DOCTOR OF PHILOSOPHY

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Certificate

This is to certify that I, **K. Divya** have carried out the research work embodied in the present thesis entitled "Studies on heterologous expression of plant stress responsive genes in human glioblastoma cell lines (U373), cotton (*Gossypium hirsutum* L.) and tomato (*Lycopersicum esculentum* L.)", 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 Prof. P. B. Kirti, in the Department of Plant Sciences, School of Life Sciences, University of Hyderabad, and 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.

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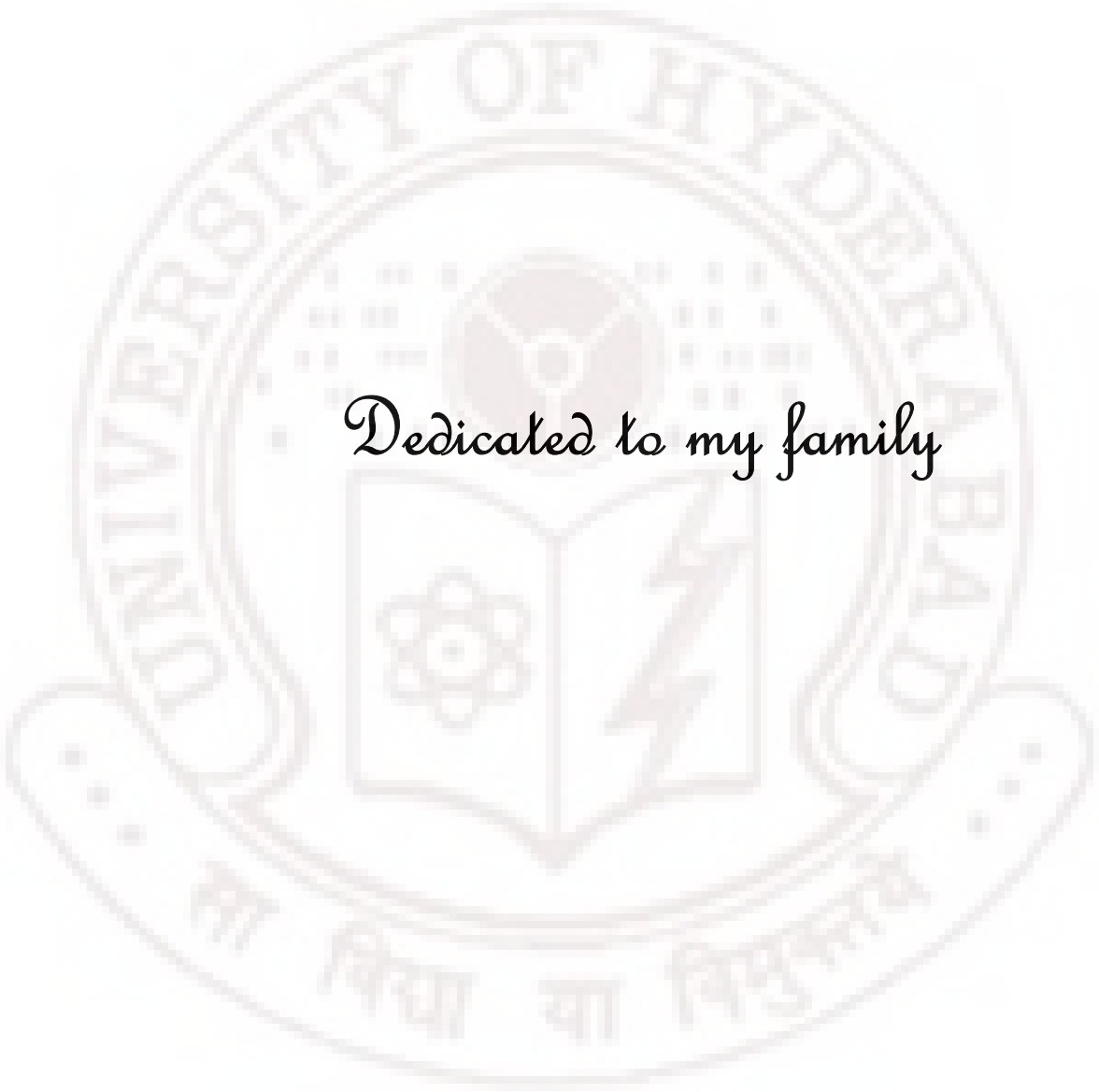

Dedicated to my family

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Divya

Abbreviations

µg	Microgram
µl	Microliter
µM	Micromolar
ABA	Abscisic acid
ATP	Adenosine triphosphate
BAP	Benzyl amino purine
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
bp	Base pairs
BSA	Bovine serum albumin
CaMV	Cauliflower mosaic virus
cDNA	Complementary DNA
CIAP	Calf intestinal alkaline phosphatase
cm	Centimeter
Cox-2	Cyclooxygenase-2
CTAB	Cetyl trimethyl ammonium bromide
d	Day
DAPI	4'-6-Diamidino-2-phenylindole
DEPC	Diethyl pyrocarbonate
DNA	Deoxy ribonucleic acid
dNTPs	Deoxy nucleotide triphosphates
DPA	Days post anthesis
EDTA	Ethylenediaminetetraacetic acid
ERK	Extracellular signal-regulated kinase
FITC	Fluorescein isothiocyanate
g	Gram
GA_3	Gibberellic acid
h	Hours
H_2DCFDA	2', 7'- dichlorodihydroflourescien diacetate

HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IAA	Indole Acetic Acid
IBA	Indole Butyric Acid
IKK	IκB kinase
INOS	Inducible nitric oxide synthase
IPTG	Isopropyl-β-D-thiogalactoside
IκB	Nuclear factor of kappa light chain gene enhancer in B-cells inhibitor
JNK	Jun N-terminal Kinase
Kb	Kilobases
KDa	Kilodalton
LB	Luria Bertani
M	Molar
MAPKs	Mitogen-activated protein kinase
MES	2-(N-Morpholino)-ethane sulfonic acid
min	Minutes
MJ	Methyl jasmonate
ml	Milliliter
MS	Murashige and Skoog
MTT	Dimethyl 2-thiazolyl 2, 5-diphenyl 2H-tetrazolium bromide
NAA	Naphthalene acetic acid
NBT	Nitroblue tetrazolium
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
ng	Nanogram
NN	Nitsch and Nitsch
LS	Linsmaier and Skoog
OD	Optical Density
ORF	Open reading frame
PCR	Polymerase chain reaction
PKC	Protein kinase C
RNA	Ribo nuceic acid
ROS	Reactive oxygen species

RPM	Revolutions per minute
SA	Salicylic acid
SDS	Sodium dodecyl sulfate
TDZ	Thidiazuron
TE	Tris.EDTA
Tris	Tris (hydroxymethyl) aminomethane
TRITC	Tetramethyl Rhodamine Isothiocyanate
U	Units
WT	Wild type

CONTENTS

Chapter1

Introduction

An increasing body of evidence signifies that several individual components of host-pathogen interactions are common, either theoretically or mechanistically, in the two major branches of the eukaryotes i.e. Plantae and Animalia. Both plants and animals are capable of recognizing self and non-self (Ausubel 2005). They have complex innate mechanisms to recognize and respond to pathogenic microorganisms attack (Menezes and Jared, 2002; Iriti and Faoro, 2007). There are about five million plant species, which is approximately five times the diversity seen in the animal kingdom. During the evolution, plants have developed various defense strategies in order to face invading pathogens. Although plants show an obvious passive nature due to their sedentary lifestyle, they have evolved an exclusive metabolic flexibility that permits them to employ effective defense strategies upon pathogen attack (Baker et al., 1997).

Two main strategies of defense responses have evolved in plants that are similar to innate and adaptive immunity seen in animals: inherent resistance and induced resistance systems (Agrios, 2005). To exemplify, in case of gene-for-gene to non-self control systems in plants, disease resistance (*R*) genes mediate the recognition of specific pathogen-derived components (products of the Avirulence [*Avr*] genes) in a fashion much similar to that the animal adaptive immune system is capable of identifying and respond towards foreign molecules (Taylor, 1998). So far, the molecules similar to immunoglobulin have not been found in plants, but at least 10 different families of *R* genes, producing pathogenesis-related proteins (PR-proteins) and including NBS-LRR sequences have been identified in at least 20 different plant species (Kitajima and Sato, 1999). The amino acid sequence of *N*, and *R* gene from tobacco, includes a domain that is related to Toll, which is a crucial regulator of disease resistance responses in Drosophila (Baker et al., 1997; Meister et al., 1997; Liu et al., 2002). Remarkably, the tobacco-virus-resistance gene that encodes N-protein is also similar to Toll in that it contains both a Toll signaling domain and an LRR domain (Whitham et al., 1994) suggesting that the immune-response system mediated by Toll represents an early and conserved host defense mechanism.

It is not only the pathogen recognition and signal transduction pathways that appear to be conserved between plants and animals but some of the defensive responses that are triggered when these pathways are activated are also similar in both lineages. For example, one of the earliest changes that can be detected following pathogen attack in plants and animals is a rapid increase in reactive oxygen species (Bauerle and Baltimore, 1996; Alvarez et al., 1998). The MAPK signaling pathways, which often include transmembrane receptors, MAPKKKs, MAPKKs, MAPKs and downstream transcription factors, are present in yeast, plants, invertebrates and vertebrates, and apparently emerged very early in evolution before the manifestation of multicellularity (Ausubel, 2005).

In addition, the recent evidences proved that the *R* gene-mediated PR protein expression and systemic pathogen resistance responses in *Arabidopsis* are activated via a signal transduction pathway that includes NPR1 (Non-expressor of pathogenesis related genes). NPR1 is a key regulatory protein in the activation of *PR* genes in systemic acquired resistance (SAR). This plant protein shows significant similarity to IκBα class of proteins, which regulates disease resistance responses in a range of animal species (Cao et al., 1997; Ryals et al., 1997). IκB is a class of inhibitory proteins, which bind to NF-κB and regulates its activation.

Nuclear factor kappa B (NF-κB) is a nuclear transcription factor that regulates expression of a large number of genes that are critical for the regulation of apoptosis, viral replication, tumorigenesis, inflammation, and various autoimmune diseases (Li and Verma 2002; Nishikori, 2005). The activation of NF-κB is thought to be a part of stress response as it is activated by a variety of stimuli that include growth factors, cytokines, lymphokines, UV, pharmacological agents, and stress. In its inactive form, NF-κB is sequestered in the cytoplasm, bound by members of the IκB family of inhibitor proteins, which include IκBα, IκBβ, IκBγ, and IκBε. This interaction blocks the ability of NF-κB to bind to DNA and results in the NF-κB complex being primarily localized in the cytoplasm due to with the masking of Nuclear localization Signal (NLS) with IκB proteins (Jacob and Harrison, 1998). The various stimuli that activate NF-κB

cause phosphorylation of IκB, which is followed by its ubiquitination and subsequent degradation. This results in the exposure of the nuclear localization signals (NLS) on NF-κB subunits and the subsequent translocation of the molecule to the nucleus. That is, the NF-κB/IκBα complex is continuously shuttling between the nucleus and the cytoplasm, but its rate of nuclear export exceeds its rate of import and thus the complex is generally cytoplasmic. In the nucleus, NF-κB binds with a consensus sequence (5'GGGACTTTCC-3') of various genes and thus activates their transcription.

In plants systemic acquired resistance (SAR) is a plant immune response that is often induced after a local infection. SAR is suggested to be functionally analogous to inflammation in that normal resistance processes are potentiated after secondary pathogen encounter, leading to enhanced disease resistance (Gaffney et al., 1993; Cao et al., 1994; Delaney et al., 1994, 1995; Bi et al., 1995; Mauch-Mani and Slusarenko, 1996; Delaney, 1997). Furthermore, inactivation of the pathway (SAR and inflammation) leads to enhanced susceptibility to bacterial, viral, and fungal pathogens. Interestingly the effect of Salicylic acid (SA) is established in both the pathways: SAR signal transduction is activated in plants whereas it blocks the NF-κB pathway (Mauch-Mani Metraux, 1998).

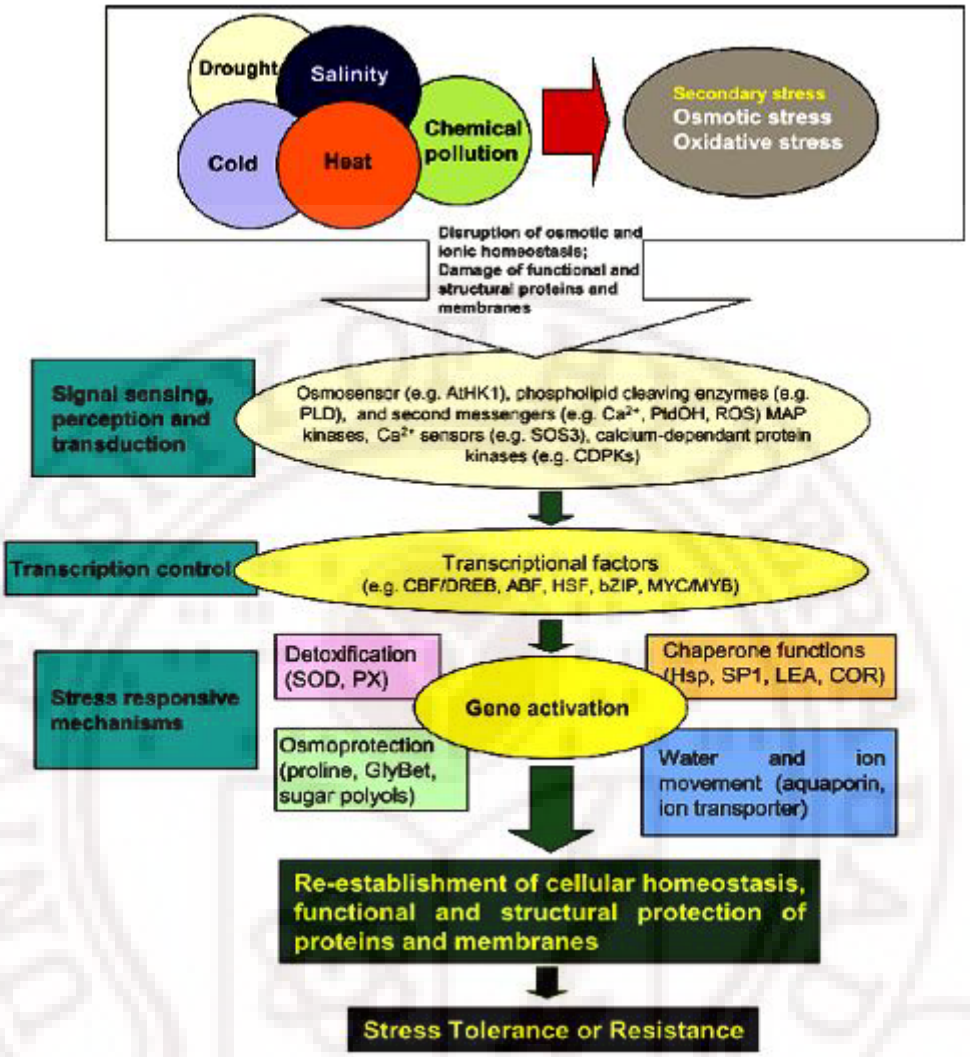
Bacterial infection of Drosophila activates a signal transduction cascade leading to the synthesis of a number of antifungal proteins such as cercropin 6, defensin, diptericin, and drosomycin (Ip et al., 1993; Lemaitre et al., 1996). This induction is dependent on the gene product of *dorsal* and *dif,* two NF- κB homologs, and is repressed by cactus, an IκB homolog in the fly. Mutants that have decreased synthesis of the antifungal and antibacterial proteins have dramatically lowered resistance to infection. Similarly, *nim* 7 mutants are blocked in pathogen-dependent induction of SAR gene expression, which includes the synthesis of a number of antimicrobial proteins such as β-1,3-glucanase and *PR-5,* and the mutants have significantly depressed pathogen resistance (Ryals et al., 1996). Thus, the Arabidopsis SAR pathway shows interesting functional parallels to the NF-κB/IκB regulation scheme in both mammals and flies.

Is plant SAR functionally similar to NF-κB/IκB signal transduction pathways in animals?

The NF-κB/IκB signal transduction pathways are conserved in both mammals and flies. Various stimuli lead to the activation of a signal transduction pathway because of the degradation of IκB or its homolog and the release of the NF-κB/ transcription factor to the nucleus to stimulate transcription (Baeuerle and Baltimore, 1996; Baldwin, 1996). Activation of the SAR pathway in Arabidopsis by SA or pathogen infection leads to enhanced SAR gene expression and resistance, in a way similar to that of NF-κB pathway in mammals and flies. In contrast to these organisms, mutations in the IκB component (i.e. NPRl), which should eliminate the inhibitor and cause nuclear localization of the transcription factor, result in inhibition of the SAR signal transduction pathway (Ryals et al., 1997). These findings suggest that SAR possesses interesting functional parallels with the NF-κB/IκB pathway in mammals and flies. Unlike the immune response in animals, which is specific to the inducing pathogen, SAR protects the plant against bacterial, fungal and viral infections.

SAR is one of the important mechanisms of stress responses in plants. The stresses can be biotic in the form of pests and diseases or abiotic that include drought, salinity, oxidative stresses, chemical toxicity, high and low temperatures.

Plant growth and productivity is greatly influenced by abiotic stress induced by salt, drought, extreme temperatures, nutrient imbalances exposure to ozone and heavy metal contamination etc. It has been estimated that two-thirds of the yield potential of major crops are routinely lost due to unfavorable environmental conditions. Plant responses to stresses lead to various biochemical and physiological changes such as accumulation of compatible solutes and activation of several detoxification enzymes. Abiotic stress is a complex phenomenon that involves activation of various genes that contribute plant to overcome the adverse effects (Wang et al., 2003). The concept of stress is intimately associated with that of stress tolerance, which is the plant's fitness to cope up with an unfavorable environment. The complex of common responses in plants towards stress is shown in Fig 1.1.



Wang et al., (2003) Planta, 218: 1-14.

Figure 1.1 The complexity of the plant response to abiotic stress. Primary stresses, such as drought, salinity, cold, heat and chemical pollution are often interconnected, and cause cellular damage and secondary stresses, such as osmotic and oxidative stress. The initial stress signals (e.g. osmotic and ionic effects, or temperature, membrane fluidity changes) trigger the downstream signaling processes and transcription controls, which activate stress-responsive mechanisms to re-establish homeostasis, and protect and repair damaged proteins and membranes. Inadequate response at one or several steps in the signaling and gene activation may ultimately result in irreversible changes of cellular homeostasis and destruction of functional and structural proteins and membranes, leading to cell death. Abbreviations: ABF, ABRE binding factor; AtHK1, *Arabidopsis thaliana* histidine kinase-1; bZIP, basic leucine zipper transcription factor; CBF/DREB, C-repeat-binding factor/ dehydration-responsive binding protein; CDPK, calcium-dependent protein kinase; COR, cold-responsive protein; Hsp, heat shock protein; LEA, late embryogenesis abundant; MAP, mitogen-activated protein; PLD, phospholipase D; PtdOH, phosphatidic acid; PX, peroxidase; ROS, reactive oxygen species; SOD, superoxide dismutase; SP1, stable protein 1.

Changes in gene expression are dependent upon duration and severity of stress, the genotype of stressed plant, the organ or tissue in which it is expressed, and stage development of the plant in its life cycle. The primary stresses such as drought, extreme temperatures, salinity and oxidative stresses lead to a chain of phenotypic, physiological, biochemical and molecular changes in plant (Wang et al., 2003). These stresses are often interlinked and are manifested mainly in the form of disruption of osmotic and ionic homeostasis and damage the structural and functional integrity of cell membranes (Zhu, 2001). Oxidative stress, which accompanies all the stress conditions, may lead to denaturation of structural and functional proteins. Soil salinity directly affects the agricultural productivity as it contributes to the seriously increased amounts of soluble salts in the rhizosphere, which limit water and nutrient uptake in plants resulting in physiological and metabolic disorders. It also interferes with plant growth as it leads to physiological drought and ion toxicity (Zhu, 2002).

According to surveys, it was estimated that more than 50% of the irrigated lands in arid and semiarid regions of the world are affected by salinization and millions of hectares of agricultural land is becoming uncultivable due to salinity hazards. The osmotic and oxidative stress is the consequence of drought, salinity and low temperature. Based on the presence of these general and very specific abiotic stress tolerance mechanisms, it is obvious to expect plants to have multiple stress perception and signal transduction pathways, which may cross-talk at various steps.

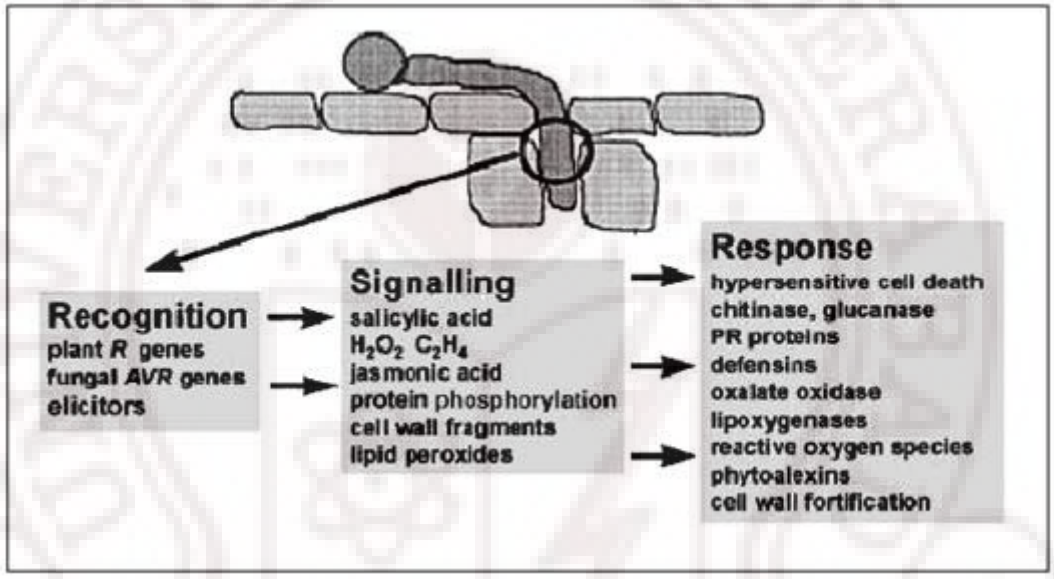
Genetic transformation offers powerful means to produce transgenic plants with stress responsible gene overexpression and better understanding of the mechanism of stress tolerance by studying the phenotypic and biochemical changes in response to abiotic stress treatments. Aiming at detoxification pathways, which are resultant of almost all the stress conditions is an appropriate way of developing plants with multiple stress tolerance for sustaining true field conditions with various stresses. Plant adaptation to enhanced tolerance towards stress includes the manipulation of genes involved in protection and maintenance of the structure and function of cellular components. Current genetic

transformation studies rely on transfer of one or more genes that are involved in signaling or regulatory pathways or that encode enzymes present in pathways leading to the synthesis of functional and structural protectants, such as osmolytes and antioxidants, or that encode stress-tolerance-conferring proteins. The efforts to improve plant abiotic stress tolerance by genetic engineering which have resulted in remarkable accomplishments were reviewed by Wang et al., 2003 (Table.1).

Table 1.1 Table enlists some of the genetically transformed plants with various stress responsive genes based on different responsive mechanisms.

Mechanism	Genes	Species	Reference
Transcription control	CBF1	*Arabidopsis thaliana*	Jaglo-Ottosen et al. 1998
	DREB1A	*A. thaliana*	Kasuga et al. 1999
	CBF3	*A. thaliana*	Gilmour et al. 2000
	CBFs	*Brassica napus*	Jaglo et al. 2001
	CBF1	*Lycopersicon esculentum*	Hsieh et al. 2002
	CBF4	*A. thaliana*	Haake et al. 2002
	AtMYC2 and AtMYB2	*A. thaliana*	Abe et al. 2003
	ABF3 or ABF4	*A. thaliana*	Kang et al. 2002
	HSF1 and HSF3	*A. thaliana*	JH Lee et al. 1995; Prändl et al. 1998
	HsfA1	*L. esculentum*	Mishra et al. 2002
	spl7	*Oryza sativa*	Yamanouchi et al. 2002
Compatible solute			
Proline	P5CS	*Nicotiana tabacum*	Kishor et al. 1995; Konstantinova et al. 2002; Hong et al. 2000
	ProDH	*A. thaliana*	Nanjo et al. 1999
Myo-inositol	IMT1	*N. tabacum*	Sheveleva et al. 1997
Sorbitol	*stpd1*	*N. tabacum*	Sheveleva et al. 1998
Antioxidants and	CuZn-SOD	*N. tabacum*	Gupta et al. 1993a, 1993b; Pitcher and Zilinskas 1996
detoxification	Mn-SOD or Fe-SOD	*Medicago sativa*, *N. tabacum*	McKersie et al. 1996, 1999, 2000; Van Camp et al. 1996
	GST and GPX	*N. tabacum*	Roxas et al. 1997
	*chy*B	*A. thaliana*	Davison et al. 2002
	Aldose-aldehyde reductase	*N. tabacum*	Oberschall et al. 2000
Ion transport	*AtNHX1*	*A. thaliana*	Apse et al. 1999
		B. napus	Zhang et al. 2001
		L. esculentum	Zhang and Blumwald 2001
	SOS1	*A. thaliana*	Shi et al. (2003)
	HAL1	*Cucurbita melo*	Bordas et al. 1997
			Rus et al. 2001
	AVP1	*A. thaliana*	Gaxiola et al. 2001
Hsps and molecular	Hsp17.7	*Daucus carota*	Malik et al. 1999
chaperones	Hsp21	*A. thaliana*	Härndahl et al. 1999
	AtHSP17.6A	*A. thaliana*	Sun et al. 2001
	DnaK1	*N. tabacum*	Sugino et al. 1999
	SP1	*Populus tremula*	Wang et al. 2003
LEA-type proteins	COR15a	*A. thaliana*	Artus et al. 1996; Steponkus et al. 1998; Jaglo-Ottosen et al. 1998
	HVA1	*O. sativa*	Xu et al. 1996
		Triticum aestivum	Sivamani et al. 2000
	WCS19	*A. thaliana*	Ndong et al. 2002

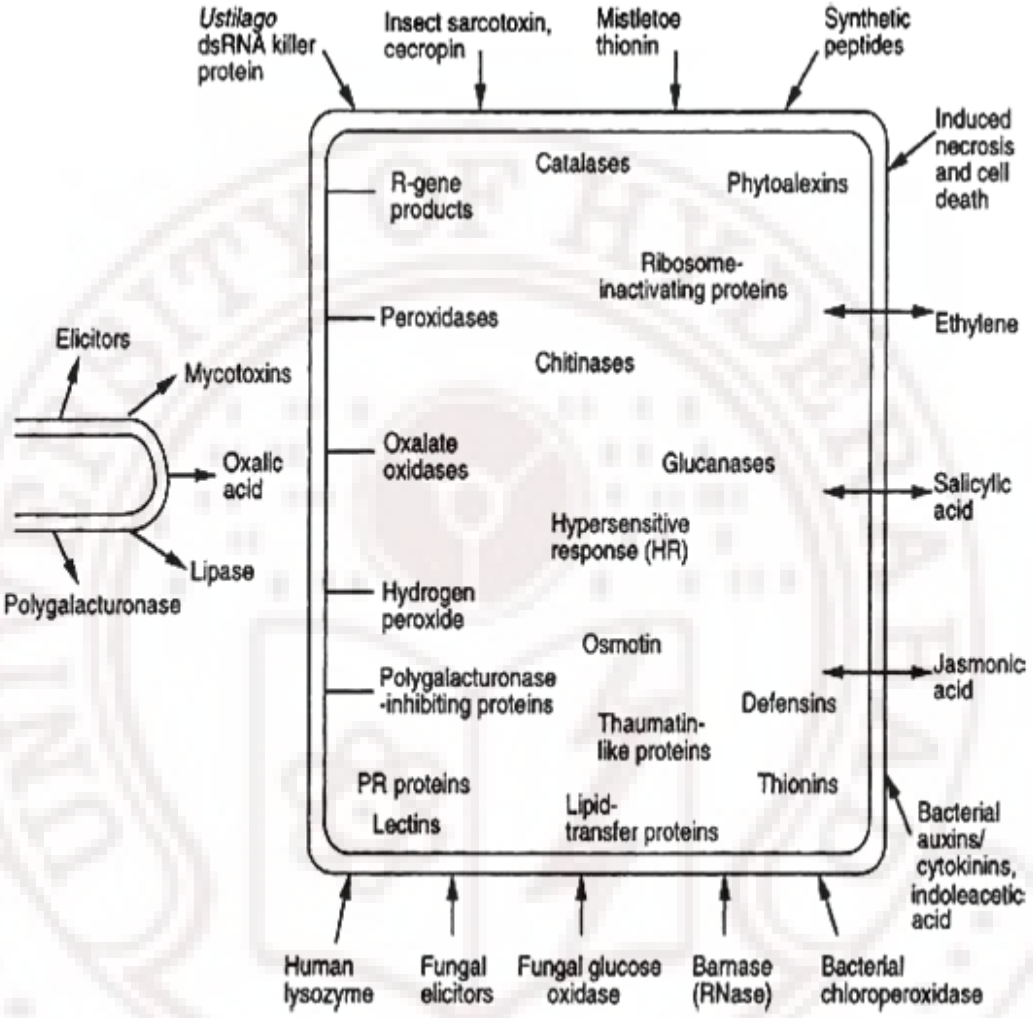
Plants are very attractive nutritional substrates for a wide range of parasites including fungi, bacteria, viruses, nematodes, insects and parasitic plants. Plants exhibit natural resistance to disease and attempted infection by pathogen attack or wounding induces a number of defenses, including phytoalexin synthesis, wall toughening through cell wall fortication by polyphenolic compounds, and deployment of lytic enzymes and other anti-microbial proteins (Dixon and Lamb, 1990) (Fig 1.2).



Evans and Greenland (1998) Pestic. Sci. 54: 353-359.

Figure 1.2 Defence responses evoked in a resistant plant following penetration of the leaf surface by an avirulent fungal pathogen.

Recent advances in the plant transgenic approaches seem to be successful in the production of disease resistant plants (Fig. 1.3).



Punja (2001) Can. J. Plant Pathol. 23: 216–235.

Figure 1.3 Transgenic plants with enhanced disease resistance have been engineered to express gene products to counterattack fungal virulence products (from hypha on left), enhanced expression of plant-derived gene products (inside of cell) or gene products from nonplant sources (outside of cell).

Lignification occurs as part of the stress response to oxidation stress, salt stress, or wounding, and is accompanied by a rise in peroxidase activity. Peroxidases are a large family of enzymes with very diverse functions. They often increase in response to stress and one of the principal roles of peroxidase appears

to be cellular protection from oxidative reactions imposed by various stresses. Anionic peroxidases are part of this multi-component defence system (Sherf et al., 1993; Zacheo et al., 1993). The anionic peroxidase associated with the pathogen infection and suberization was isolated and its expression induced to high levels by fungal pathogens, wounding or exposure to ABA or fungal elicitors (Robb et al., 1991).

These enzymes are involved in catalyzing the polymerization of cinnamoyl groups into lignin (Lagrimini et al., 1993), suberization of cell walls (Sherf et al., 1993), accumulation of phenolic polymers (Graham and Graham 1992) and the binding of extensin to cell wall (Brownleader et al., 1995). Anionic peroxidases are also differentially expressed in plant organs, and involved in normal growth and development (Repta and Jung 1995). Wall toughening in the defence reaction can include lignification and suberization, as well as insolubilization of cell wall structural proteins (Brisson et al., 1994). The first two processes involve synthesis of aromatic polymers that are covalently attached to carbohydrates of the cell wall and, or attached to cell wall proteins (Kolattukudy 1984). The deposition of these phenolic polymers as barriers on the walls of wound-healing or infected tissues may act to prevent entry by the pathogen. The polymerization of the phenols is catalyzed by a cell wall associated anionic peroxidase (Kolattukudy et al., 1992). A correlation between the wounding or pathogen induced expression and deposition of suberin have led to the proposed function of anionic peroxidases in cross-linking of suberin monomers leading to cell wall fortification and subsequent resistance to the wounding or pathogen induced damage(Sherf et al., 1993).

Chapter 2

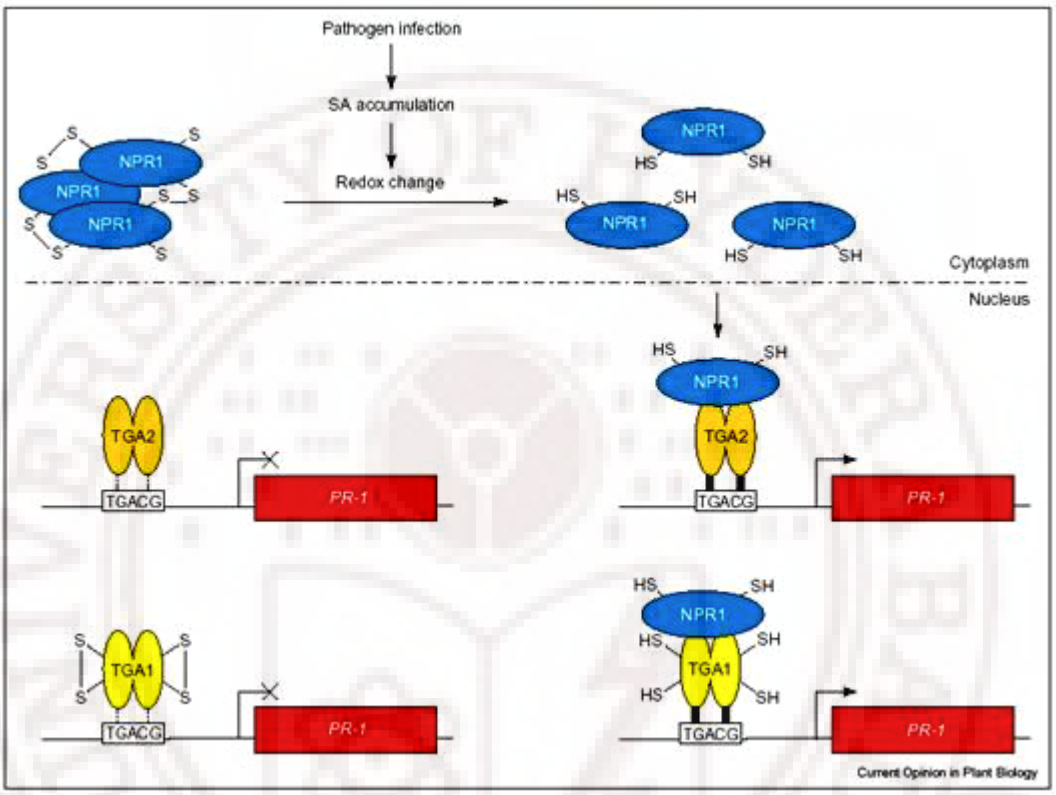
Review of literature

2.1 Systemic Acquired Resistance

All organisms including plants possess active defense mechanisms against pathogen attack. Induced resistance is a state of enhanced defensive capacity developed by a plant when appropriately stimulated. Induced resistance is not the creation of resistance where there is none, but the activation of latent resistance mechanisms that are expressed upon subsequent, so-called "challenge" inoculation with a pathogen. Induced resistance occurs naturally as a result of limited infection by a pathogen, particularly when the plant develops a hypersensitive reaction, and can also be triggered by certain chemicals, nonpathogens, avirulent forms of pathogens, incompatible races of pathogens, or by virulent pathogens under circumstances where infection is delayed due to environmental conditions. Generally, induced resistance is systemic, because the defensive capacity is increased not only in the primary infected plant parts, but also in non-infected, spatially separated tissues. Because of this systemic character, induced resistance is commonly referred to as systemic acquired resistance (SAR) (Sticher et al., 1997). However, induced resistance is not always expressed systemically: Localized acquired resistance (LAR) occurs when only those tissues exposed to the primary invader become more resistant. SAR and LAR are similar in that they are effective against various types of pathogens.

SAR is characterized by an accumulation of salicylic acid (SA) and pathogenesis-related proteins (PRs). Accumulation of SA occurs both locally and, at minor levels, systemically, associated with the development of SAR (Metraux 2001). Both pathogen- and SA-induced resistance are associated with the induction of several families of PRs. Induction of PRs is invariably linked to necrotizing infections giving rise to SAR, and has been taken as a marker of the induced state. The association of PRs with SAR suggests an important contribution of these proteins to the increased defensive capacity of induced tissues. As a consequence, plants are able to mount a systemic response that confers an increased, long-lasting, and broad-spectrum resistance to subsequent pathogen attacks for the whole plant (Durrant and Dong 2004). The plant SAR shows interesting functional parallels with the NF-κB/IκB regulatory pathway in

mammals and flies. Several attempts to genetically dissect the SAR pathway downstream of SA signal resulted in the identification of numerous alleles of a single gene designated *NPR1*, *NIM1* or *SAL1* acting as a key regulator (Fig 2.1).



Pieterse and Van loon (2004) Curr. Opi. in Plant Biol. 7: 456-464.

Figure 2.1 Model illustrating the role of SA-mediated redox changes, NPR1, and TGA transcription factors in SAR-related gene expression. In non-induced cells, oxidized NPR1 forms inactive oligomers that remain in the cytosol. Binding of TGAs to the cognate SA-responsive promoter elements (TGACG) (indicated by dotted lines) is not sufficient to activate the expression of *PR-1* genes. Upon infection by a necrotizing pathogen, SA accumulates and plant cells attain a more reducing environment, possibly because of the accumulation of antioxidants. Under these conditions, NPR1 is reduced from an inactive oligomeric complex to an active monomeric state through the reduction of intermolecular disulfide bonds. Monomeric NPR1 is then translocated into the nucleus where it interacts with TGAs, such as TGA2. The binding of NPR1 to TGAs stimulates the DNA-binding activity of these transcription factors to the cognate *cis* element (represented by black boxes), resulting in the activation of *PR-1* gene expression. In non-induced cells, TGAs that do not interact with NPR1 in yeast two-hybrid assays, such as TGA1, are oxidized and form intramolecular disulfide bridges that prevent interaction with NPR1. Upon accumulation of SA *in planta*, the change in redox status reduces the disulfide bonds in these TGAs, resulting in a conformational change that allows interaction with NPR1.

2.2 NPR1 is a SA mediated transcriptional regulator in SAR

NPR1 encodes a protein containing an ankyrin repeat domain and a BTB/POZ (broad-complex, tramtrack, and bric-a-brac/poxvirus, zinc finger) domain both of which are involved in protein-protein interactions (http://smart.embl-heidelberg.de/). NPR1 acts as a transcription regulator which activates *PR* gene expression (Makhtar et al., 2009). NPR1 localizes to the nucleus via a functional nuclear localization signal (NLS) and its nuclear localization is the prerequisite for the activation of *PR* gene expression (Kinkema 2000).

Overexpression of *NPR1* in Arabidopsis leads to improved disease resistance to both bacterial and oomycete pathogens in a dose-dependent manner (Cao et al., 1998). Similarly, overexpression of *AtNPR1* in rice resulted in enhanced resistance to pathogen *Xanthomonas oryzae* pv. *oryzae* and constitutive activation of defense responses (Chern et al., 2001; 2005), indicating the presence of a similar defense pathway in rice. Although transgenic *Arabidopsis* plants overexpressing *NPR1* acquired enhanced sensitivity to SA and BTH (Freidrich et al., 2001), they did not display detrimental morphological changes and do not have elevated *PR* gene expression until activated by inducers or by infection of pathogens (Cao et al., 1998). However, in rice, overexpression of *Arabidopsis NPR1* potentiates a BTH- and low-light-environment-induced lesion mimic or cell death (LMD) phenotype (Fitzgerald et al., 2004). In addition to SA, jasmonic acid (JA) and ethylene are well studied signals that regulate distinct defense pathways. Cross talk between SA- and JA-mediated pathways has been well documented (Dong 2004; Glazebrook 2001). The function of *NPR1* also is essential for JA- and ethylene-regulated, SA-independent induced systemic resistance (ISR) (Pieterse et al., 1998). NPR1 also appears to modulate the cross talk between SA- and JA dependent pathways; the antagonistic effect of SA on JA signaling requires NPR1, but not nuclear localization of the NPR1 protein (Spoel et al., 2003). It has been demonstrated that proteasome-mediated degradation of NPR1 in the nucleus promotes efficient expression of defense response genes following

infection and prevents spurious activation of defensive responses in the absence of infection (Spoel et al. 2009).

Recently *Brassica juncea NPR1* (*BjNPR1*) has been cloned (Gargi et al 2006). Its amino acid sequence revealed the presence of ankyrin repeats, broad complex tramtrack and bric-a-brac/pox virus and zinc finger domains when analyzed by SMART program (http://smart.embl-heidelberg.de/).

2.2.1 BTB/POZ Domain

The BTB domain is a protein-protein interaction module consisting of approximately 120 amino acids that is found in over 600 different proteins in organisms ranging from yeast to humans. The domain was first identified as a conserved sequence element in the developmentally regulated *Drosophila* proteins Broad-complex, Tramtrack and Bric-a-brac. The BTB domain, also known as the POZ (poxvirus and zinc finger) domain, is regularly found at the N-termini of numerous zinc finger transcription factors as well as Shaw-type potassium channels. Experimental studies have strongly connected the BTB domain in the regulation of gene expression through the local control of chromatin conformation. In several cases, the BTB domain has been revealed to mediate protein oligomerization, which subsequently prevents high affinity DNA binding. Both homotypic and heterotypic protein-protein interactions have been observed because the BTB domain can form dimers in addition to mediating interactions with non-BTB domain containing proteins (Bardwell and Treisman 1994).

2.2.2 Ankyrin repeats

Proteins containing relatively short, tandemly repeating amino acid sequences have received an enormous deal of consideration in recent times. Biochemical and structural characterization revealed that these repeats act as building blocks that stack side by side, forming the essential architecture of a modular, precise protein-binding interface (Mosavi et al., 2004). Among the most abundant repeat motifs are the ankyrin repeats, which do not recognize any

specific aminoacid sequence or structure. Instead they form an elongated surface of varying size depending on the number of repeats to facilitate binding of other proteins. The ankyrin repeat, a 33-residue sequence motif was first identified in yeast cell cycle regulator Swi6/Cdc10 and the *Drosophila* signaling protein Notch (Breeden and Nasmyth 1987). Subsequently, ankyrin repeats have been found in many proteins participating in a wide range of functions.

Ankyrin repeat proteins are present in all three super kingdoms including bacteria, archaea, and eukarya, as well as in a number of viral genomes. However, a phylogenetic breakdown of the organisms that contain ankyrin repeats indicates that the majority are found in eukaryotes. The number of ankyrin repeats found in a single protein varies greatly. Analysis of the SMART and PFAM databases show that the number of repeats per protein ranges from 1 to 33, with the majority of proteins containing 6 or fewer repeats. The PFAM database suggests 3 as the most popular number of repeats. Ankyrin repeats have been observed to exist by themselves as a single domain protein or in conjunction with other domains in the same protein. Each repeat folds into two antiparallel α-helices followed by a β-hairpin or a long loop. Consecutive repeats stack together to form an L-shaped domain, which resembles a cupped hand with the β-hairpins representing the fingers and the helices as the palm. The overall shape of a typical ankyrin repeat domain is slightly curved, and this feature is particularly apparent in the structure of proteins that contain a large number of these repeats. A search of the PUBMED database with the term “ankyrin repeat proteins” yielded 766 publications (June 2009). We observed each of these reports for particulars of protein–protein interacns mediated by ankyrin repeat domains as detected by yeast two-hybrid, co-immunoprecipitation, *in vitro* binding assays and GST pull-down experiments. These proteins and their corresponding complexes are essential for many cellular processes such as cell fate determination, endocytosis, transcription regulation, and cell cycle control. They participate in cell–cell signaling, cytoskeleton integrity, transcription and cell–cycle regulation, inflammatory response, tumorigenesis, development, and various transport phenomena. Of the ankyrin repeat proteins that have been characterized, a unifying trait is that they typically

function in mediating specific protein–protein interactions. The ankyrin repeat is found in a number of biologically important proteins. The family of INK4 tumor suppressors, p15, p16, p18, and p19, as well as 53BP2, a regulator of the tumor suppressor p53, all contains ankyrin repeats. The signaling protein Notch, which is involved in many cell-fate decisions during development, has seven ankyrin repeats. NF-κB, a transcription factor that regulates inflammatory response is inhibited by IκB, which contains seven ankyrin repeats.

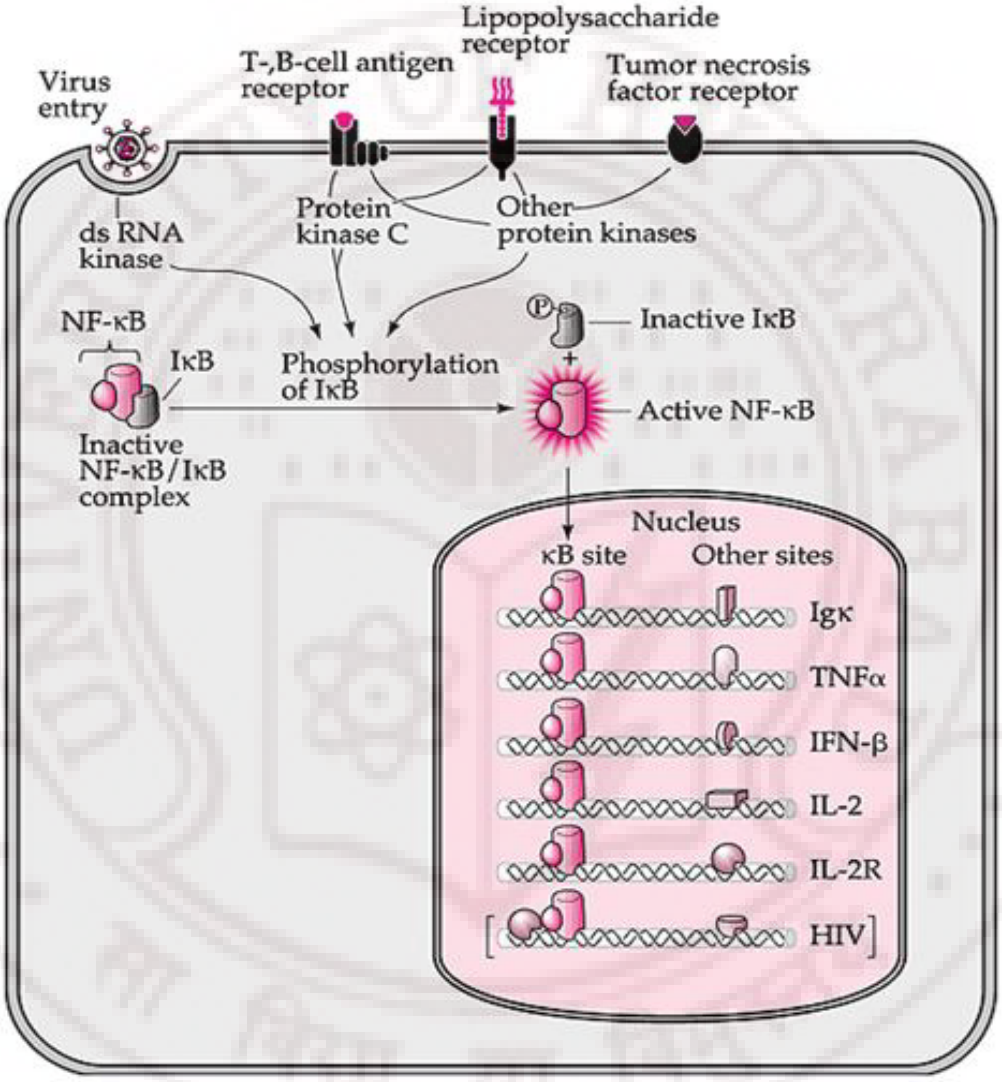
2.3 NF-κB/ IκB pathway

Nuclear factor-κB (NF-κB) was first described as a transcription factor in B cells that binds to the enhancer element controlling immunoglobulin kappa light chain expression. Since its discovery in 1986, NF-κB and its role in inflammatory responses, immune reactions, and tumorigenesis has been extensively studied. In mammalian cells, the NF-κB/Rel family contains five members: RelA (p65), c-Rel, RelB, NF-κB1 (p50; p105), and NF-κB2 (p52; p100). These proteins possess a structurally conserved 300 amino acid sequence called the *REL* region, which contains the dimerization, nuclear localization, and DNA-binding domains. Three of the family members, RelA, c-Rel, and RelB, have a transactivation domain at the C-terminus. NF-κB1/p105 and NF-κB2/p100 are the inactive precursors of the p50 and p52 proteins, respectively; in an unstimulated state, these proteins are localized to the cytoplasm.

2.3.1 NF-κB/ IκB nuclear localization

NF-κB is expressed in the cytoplasm of virtually all cell types, where its activity is controlled by a family of regulatory proteins, called inhibitors of NF-κB (IκB) comprising IκBα, IκBβ, IκBε, and Bcl-3, members of the IκB family, commonly possess 5 to 7 ankyrin repeats, which are 33 amino acid sequences that mediate binding to NF-κB dimers. The unprocessed NF-κB1/p105 and NF-κB2/p100 proteins also contain ankyrin repeats at their C-termini, which cause them to be included in this inhibitory family. IκB proteins appear to inhibit NF-κB activity by masking their nuclear localization sequence (NLS), located just at

the C-terminal end of the Rel homology region (RHR) in each of the NF-κB subunits (Zabel et al., 1993). Recent observations, however, have indicated that both IκBα and IκBε shuttle between the nucleus and cytoplasm within NFκB- IκB complexes ; these complexes are capable of displacing NF-κB from target DNA sites and transporting it back to the cytoplasm (Birback et al., 2002) (Fig. 2.2).



Glibert (2006). Dev. Biol, 8th edition.

Figure 2.2 Regulation of NF-κB by IκB. IκB binds to the larger subunit of NF-κB and prevents the complex from entering the nucleus. IκB can be phosphorylated by several kinases that are activated by replicating viruses, antigens, lipopolysaccharides, or tumor necrosis factor a. The phosphorylation of IκB releases NF-κB which can then enter the nucleus and bind to those promoter and enhancer sites it recognizes. Such genes include those encoding the kappa immunoglobulin light chain, tumor necrosis factor, interleukin 2, and the receptor for interleukin 2. The human immunodeficiency virus also has sites for NF-κB binding.

The expression of IκB proteins is regulated by NF-κB; this feedback regulation is believed to contribute to the rapid shut down of NF-κB signaling. IκBβ expression, however, is not regulated by NF-κB. Instead, IκBβ is constitutively retained in the cytoplasm, indicating that it is not involved in the autoregulatory loop terminating NF-κB signaling.

The most important families of proteins that contain several ankyrin repeats in their structure are presented in Table 2.1.

Table 2.1 Families of proteins with ankyrin repeats revealed in animals, plants, and bacteria.

Organisms	Protein	The number of repeats	Functions
Animals	IkB	6–7	Regulator of transcription
	BCL-3	7	Oncoprotein, regulator of transcription
	BARD1	3	Inhibitor of polyadenylation
	INK4	3, 4, 5	Tumor suppressors, cell cycle regulators
	RNase L	9	Ribonuclease
	Mbp1	4	Transcription factor (late-G@1 phase of cell cycle)
	Tvl-1	4	Adapter of signal transduction
	RFXANK	4	Regulator of transcription
	53BP2	4	Tumor suppressor
	TRPC	1, 3, 4	Cation channels
	Notch (Drosophila)	7	Determination of cells, participation in early development (embryogenesis)
Plants	NPR1/NIM	4	Regulator of transcription
	cpSRP43	4	Signal transduction in chloroplasts
Yeast	Swi4	4	Transcription factor
	Swi6	4	Transcription factor
Alpha-proteobac-teria	ankA	11	Initiator of infection in eukaryotes
	ankB		Provides localization of catalase

Voronin and Kiseleva (2008) Cell and Tissue Boil. 2: 1-12.

2.3.2 NPR1 shows sequence similarity with mammalian IκB proteins

As seen for NF-κB, each IκB family member has both distinct and redundant actions. NF-κB activation is tightly regulated by signals that degrade IκB. In NF-κB signaling pathway, IκB proteins are phosphorylated by an activated IκB kinase (IKK) complex at specific sites equivalent to Ser32 and Ser36 of IκBα. Phosphorylation triggers

polyubiquitination at sites equivalent to Lys21 and Lys22 of IκBα and degradation by the 26S proteasome, releasing free NF-κB dimers (Karin and Ben-Neriah 2000). Previous studies with *Arabidopsis thaliana NPR1* (*AtNPR1*) revealed sequence similarity with mammalian IκB proteins (Ryals et al., 1997). Recently *Brassica juncea NPR1* was isolated and cloned (Gargi et al., 2006), also showed striking structural similarity with animal IκB proteins.

2.4 Objectives of the study for chapters 4

Based on the strong evidences suggesting the similarity of BjNPR1 with animal IκB class of proteins, it is tempting to study the consequences of *BjNPR1* transfection in mammalian cell lines. As NF-κB/IκB pathway is a potent signaling pathway which is constitutively activated in almost all the cancers, stable transfection and subsequent studies in human glioma cell lines were framed as follows:

1. Cloning NPR1 in mammalian expression vectors pcDNA3.0. Development of stably transfected lines and confirmation through northern blotting, western blotting and Immunofluorescence.
2. Checking the p65 and p50 levels in nucleus and cytosol of control and transfected cell lines.
3. Checking the binding of BjNPR1 with p65 and p50 with co-immunoprecipitation.
4. Studying the expression levels of NF-κB target genes and MAPkinases.
5. Performing cell proliferation assays in control and transfected cell lines.
6. Checking caspase activity in control and transfected cell lines.

In plants NPR1 is a key regulator in SA mediated systemic acquired resistance, a main operating system against pathogen encounter or biotic stress. The BjNPR1 was overexpressed in cotton, the most important fiber yielding plant often severely damaged by various pathogens such as fungi and insects.

2.5 The objectives framed for the study in chapter 5:

1. Optimization of efficient direct regeneration protocol using hypocotyl explants in cotton.
2. Development of transgenic cotton plants with Agrobacterium mediated transformation using pCAMBIA2300 binary vector.
3. Confirmation of transgene integration and expression using Southern, northern and western hybridization.
4. Detection of BjNPR1 protein in different parts of the transgenic plants by Immunoflourescence.
5. To study the expression levels of *PR* genes in transgenic plants.
6. Evaluation of transgenic plants for resistance to different pathogenic fungi.

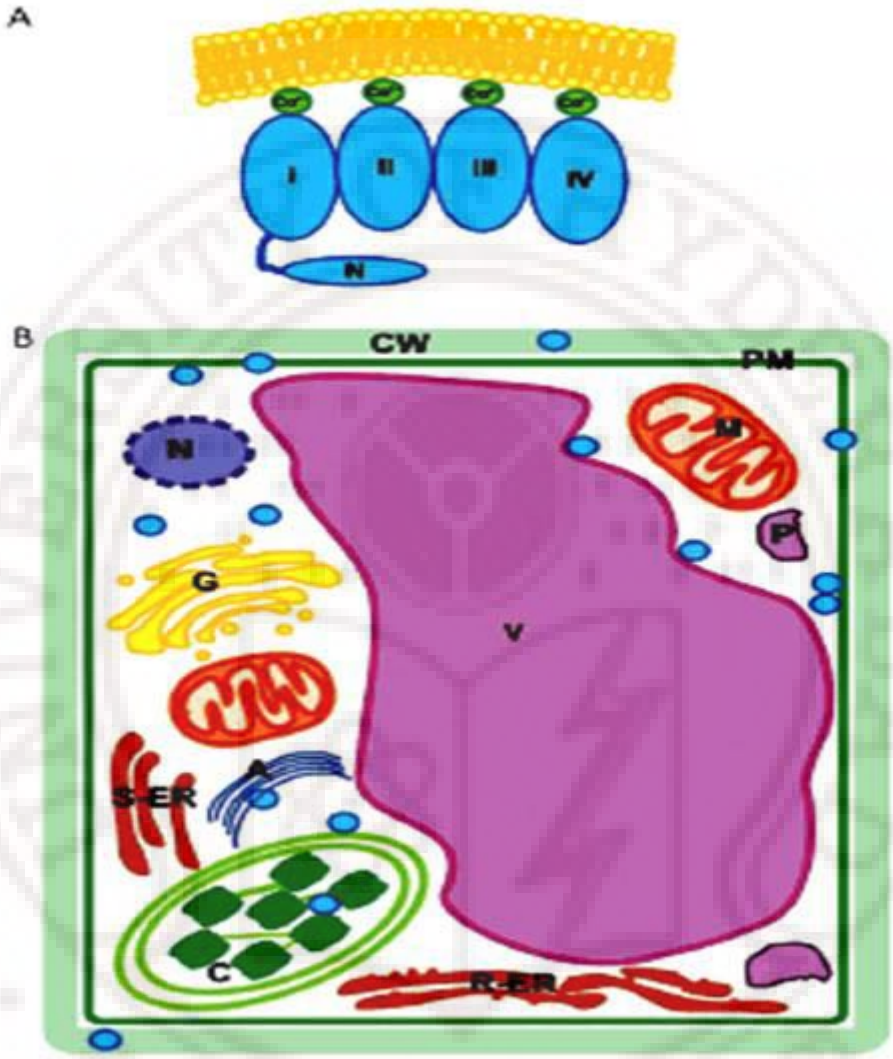
2.6 Abiotic stress in plants

The signal perception of abiotic stresses and signal transduction to switch on adaptive responses are crucial steps in determining the responses, survival and reproduction of plants when exposed to adverse environments. Plants have stress-specific adaptive responses as well as responses, which protect them from more than one environmental stress. There are several stress perception and signaling pathways, some of which are specific, but others may cross-talk at various points. Recently progress has been made in identifying the mechanism of signaling pathways involved in salt, drought and cold stresses. The products of these genes many participate in the generation of regulatory molecules such as plant hormones, abscisic acid (ABA), ethylene and salicylic acid (SA). These regulatory molecules modulate secondary messengers such as calcium (Ca^{2+}) initiating the protein phosphorylation cascade that finally targets protein directly involved in cellular protection or the transcriptional factor controlling specific sets of stress-regulated genes. In plant cells, Ca^{2+} serves as a second messenger during abiotic stress signaling (Knight, 2000; Knight and Knight, 2001). Calcium is a major point of signaling cross-talk because it can be elicited by several abiotic as well as developmental, hormonal and biotic stress signals (Chinnuswamy et al., 2003). The increase in calcium levels during abiotic stress is perceived by

calcium-binding proteins. Genetic analyses has defined the Salt-Overly-Sensitive (SOS) pathway, in which a salt induced calcium signal is probably sensed by the calcium binding protein SOS3, which then activates the protein kinase SOS2. The SOS3-SOS2 kinase complex regulates the expression and activity of ion transporters such as SOS1 to re-establish cellular ionic homeostasis under salinity (Zhu 2002). Both ABA dependent and independent signaling pathways appear to be involved in osmotic stress tolerance. Components of mitogen-activated protein kinase (MAPK) cascades may act as converging points of multiple abiotic as well as biotic stress signaling pathways. Besides SOS3 family calcium sensing, Calcium-dependent protein kinases (CDPKs) have been implicated in signaling pathways in response to stresses such as drought, wounding and cold. Transgenic analysis studies of stress-responsive gene expression and characterization of CDPK activation suggest that some stress induced Ca^{2+} signals are perceived and transduced by CDPKs. (Chinnuswamy et al., 2003). Annexins belong to the class of Ca^{2+} binding proteins that participate more directly in Ca^{2+} signaling in response to various stress conditions (Fig. 2.3).

The name annexin is derived from a greek word “annex” which means ‘bring/hold together’ and it describes the principal property of all most all annexins, ie., the binding to and possibly holding together of certain biological structures, in particular membranes (Gerke and Moss, 2002). The name ‘annexins’ received diverse and unrelated names referring to their biochemical properties. These included synexin 9 (for granule aggregating protein), chromobindins (proteins binding to chromaffin granules), calcimedins (proteins mediating Ca^{2+}, phospholipid and actin). Studies dealing with biochemical properties of these proteins, cloning and cDNA sequencing analyses identified key biochemical properties as a well gene structure and sequence features. The concept of a novel

multigene family arising from by gene duplication was developed and the common name annexin was introduced to represent these proteins.



Mortimer et al., (2008) J. of Exp. Bot. 59: 533-544.

Figure 2.3 Annexin structure and localization. (A) Animal annexin (blue) membrane association in the presence of Ca^{2+} ions (green). Each of the four conserved annexin domains (I–IV) is predicted to bind a single Ca^{2+} ion, forming a slightly concave disc. In plants, typically only the first and fourth repeated domains have the characteristic endonexin sequence. The N-terminal region (N) is the site of secondary modification and in plants is only 10 amino acids long. (B) Subcellular localization of plant annexin proteins (blue circles) in an idealized plant cell. The localization depends on many factors, including plant species, tissue type, and $[Ca^{2+}]$cyt, as described in the text. A, actin; C, chloroplast; CW, cell wall; G, Golgi; M, mitochondria; N, nucleus; P, peroxisome; PM, plasma membrane; R-ER, rough endoplasmic reticulum; S-ER, smooth endoplasmic reticulum; V, vacuole.

By definition, an annexin protein has to fulfill two major conditions. First, it must be capable of binding in a Ca^{2+} dependent manner to negatively charged phospholipids. Second, it has to possess the conserved structural element called annexin repeat, a fragment of about 70 amino acid residues (Gerke and Moss, 2002). They belong to a multigene family of Ca^{2+}-, phospholipid-and cytoskeleton binding proteins with representatives in animals, plants and fungi. They may exist in a soluble or membrane-bound state and, in plant cells; they may contribute up to 0.1% of the total protein. The identity of annexins is derived from a conserved C-terminal domain, the 'annexin core'. This is composed of four similar sequences (or eight in the case of mammalian annexin VI) of approximately 70 amino acids, each forming a compact Q-helical domain comprising a region termed the 'endonexin fold', which contains the G-X-G-T-{38}-(D/E) motif that binds calcium (White et al., 2002).

2.7 Discovery of Plant annexins

Over 200 unique annexin sequences have been described in >65 species covering plants, fungi, protists, higher vertebrates, and recently a prokaryote (reviewed by Gerke and Moss, 2002; Hofmann, 2004; Moss and Morgan, 2004). Little is known about the plant annexins, which are phylogenetically distinct from the extensively studied animal annexins. Plant annexins are both ubiquitous and abundantly found in plants generally representing at least 0.1% of the total protein (Delmer et al., 1997). Research on plant annexins started about 15 years ago with their identification in tomato based on amino acid sequence identity, antibody cross-reactivity and functional similarity with mammalian annexins (Boustead et al., 1989). They were identified in the plants such as corn and lily (Blackbourn et al., 1991; 1992). Subsequently, annexins were also identified from pea (Clark et al., 1992), cotton fibers (Andrawis et al., 1993), celery (Seals et al., 1994), pepper (Hoshino et al., 1995, Proust et al., 1996, Hofmann et al., 2000), rhizoids of fern (Clark et al., 1995), alfalfa (Kovacs et al., 1998), tobacco (Proust et al., 1999), wheat (Breton et al., 2000), mimosa (Hoshino et al., 2004) and *Arabidopsis* (Lee et al., 2004; Clark et al., 2005). Furthermore, annexin-like proteins have been

found to be associated with the vacuoles in celery (Seals et al., 1994) and tobacco (Seals et al., 1997) and termed VCaB42 due to its apparent molecular weight of 42 KDa. However, it seems that these proteins are homologues of Anx23 (Ca38) from *Capsicum annuum* (bell pepper) and Anx (Le35) from *Lycopersicon esculentum* (tomato) (Seals et al., 1997).

2.8 Annexin gene expression

Annexins are encoded by twelve genes in vertebrates and by eight in higher plants. Their physiological significance is underlined by two facts: the number of the annexin genes seems to grow during evolution and in some cell types they comprise up to 2% of total protein (Bandorowicz-Pikula 2007). They were first discovered in animal cells, where there are at least thirteen distinct members of the annexin family (Raynal and Pollard 1994) where as in plants, southern analysis in Arabidopsis (Gidrol et al., 1996), maize (Battey et al., 1996), bell pepper (Proust et al., 1996), tobacco (Proust et al., 1999) have all indicated that annexin gene family is comparatively simple and possess at least two different annexins. Also partial or complete cDNA clones of annexins have been identified in alfalfa (Pirck et al., 1994), soybean (Shi et al., 1995), strawberry (Wilkinson et al., 1995), cotton (Potikha and Delmer, 1997). The complete genomic sequencing of *Arabidopsis thaliana* and *Oryza sativa japonica* revealed that there are eight and ten different annexin cDNAs in *Arabidopsis* and rice respectively (Clark et al., 2001, Cantero et al., 2006). The primary structure of plant annexins is characterized by the tetrad repeat of 70 amino acid sequence and within which is present an endonexin sequence as in case of mammalian and non-vertebrate metazoan annexins, which is usually referred as GX-G-T-{38}-(D/E) motif that binds calcium. Within the family of plant annexins, the endonexin sequence is only conserved within the first and fourth domains with the presence of type-II calcium-binding sites (acidic residues). The animal annexins have highly variable N-terminal regions, both in length and amino acid composition. These N-terminal regions are responsible for the distinct functions of annexins, as they contain the major sites for phosphorylation, proteolysis or cellular

interactions with other proteins (Raynal and Pollard, 1994). In case of plant annexins, a very short N-terminal region preceding the first structural repeat was observed. The variation in functions among plant annexins would have to be due to the variation found within their structural repeats (Clark et al., 2001, Cantero et al., 2006).

Plant annexin gene expression is influenced by tissue/cell-specific, developmental controls or upon induction by a variety of environmental signals. Genome sequencing has revealed seven annexin genes in Arabidopsis (with an eighth evident; Cantero et al., 2006) and nine in rice (Moss and Morgan, 2004). Annexins are expressed throughout the body and lifespan of the plant; embryo (Yu et al., 2005), seedlings (Clark et al., 1992, 2001; Proust et al., 1996; Cantero et al., 2006), roots and tubers (Gidrol et al., 1996; Carroll et al., 1998; Niebel et al., 1998; Kovacs et al., 1998; Lim et al., 1998; Clark et al., 2001, 2005a, b; Bassani et al., 2004; Hoshino et al., 2004; Bauw et al., 2006; Cantero et al., 2006), stems, hypocotyls, and coleoptiles (Blackbourn et al., 1991; Thonat et al., 1997; Kovacs et al., 1998; Hoshino et al., 2004; Cantero et al., 2006), cotyledons and leaves (Kovacs et al., 1998; Santoni et al., 1998; Hofmann et al., 2000; Seigneurin-Berny et al., 2000; Hoshino et al., 2004; Cantero et al., 2006), inflorescence (Blackbourn et al., 1992; Kovacs et al., 1998), and fruit (Wilkinson et al., 1995; Proust et al., 1996; Hofmann et al., 2000). In addition to expression in the vasculature (Clark et al., 2001), annexin proteins have been found in phloem sap (Barnes et al., 2004; Giavalisco et al., 2006). Proteomic studies now show that plant and oomycete annexins exist in the cell wall as well as the cytoplasm (Kwon et al., 2005; Meijer et al., 2006). To date, plant studies have focused on annexin structure and *in vitro* protein function.

The alfalfa annexin has been reported to be associated with the nucleolus, where as the pea annexins were found consistently spread throughout the nucleus. Thus, plant annexins can interact with DNA, primer recognition proteins, microfilaments, as well as nuclear envelope and its proteins. This suggests that the plant annexins might play a role in transcription, replication and nuclear membrane processes (Hofmann, 2004). Similar to alfalfa and pea annexins,

mammalian annexins such as A1, A5 and A11 have also been found to have cytoplasmic and nuclear localization. The annexins A1, A5, the alfalfa and pea annexins do not possess a nuclear localization signal; it is only annexin A11, which carries an N-terminal tail necessary and sufficient for its nuclear localization (Hofmann, 2004).

Gene expression of annexins was found to be induced by various abiotic stress factors. They also respond to drought in alfalfa (Kovacs et al., 1998) or in Arabidopsis (Cantero et al., 2006). The regulation of expression by northern blot analysis was also found upon induction with osmotic stress inducers such as NaCl, Mannitol and PEG in alfalfa cells (Kovacs et al., 1998), exposure of Arabidopsis seedlings to NaCl (Cantero et al., 2006) or its induction by probing with anti-AnnAt1 antibody in Arabidopsis (Lee et al., 2004) or in wheat by anti-HIS-ANXLT1 antibody (Breton et al., 2000). In Arabidopsis, annexin expression was also shown to be regulated by Salicylic acid, an inducer of systemic acquired resistance (SAR), (Gidrol et al., 1996). They are also induced by low temperatures in wheat membranes (Breton et al., 2000), low or high temperatures to seedlings of Arabidopsis (Cantero et al., 2006) or by mechanical stimulation as in *Bryonia dioica* (Thonat et al., 1997) by using western blot studies. Similar to mammalian annexins (A1, A5 and A6), plant annexins have been implicated in oxidative stress response (Rhee et al., 2000, Kush and Sabapathy, 2001; Sacre and Moss, 2002). The mRNA expression was increased when the tissues were exposed to H_2O_2 in Arabidopsis (Gidrol et al., 1996) or in *Medicago sativa* (Kovacs et al., 1998). An annexin from Arabidopsis, AnnAt1 was able to rescue *Escherichia coli* ΔoxyR mutants from oxidative stress and also protects mammalian cells from H_2O_2 stress by decreasing the production of superoxide radicals (Gidrol et al., 1996, Kush and Sabapathy, 2001). The overexpression of AtAnn1 in tumour cells also protected them from TNF induced apoptosis (Janicke et al., 1998). Transgenic Arabidopsis plants overexpressed with AtAnn1 showed enhanced tolerance to pH induced stress (Gorecka et al, 2007). The expression of *MtAnn1* gene in *Medicago truncatula* was shown to be transcriptionally activated in roots

in response to nod factors and during symbiotic association with *Sinorhizobium meliloti* (Niebel et al., 1998, 2002).

2.9 Structural properties of plant annexins

2.9.1 Tertiary structure of plant annexins

The first three-dimensional structure of a plant annexin was determined by x-ray diffraction of a crystallized bell pepper annexin, Anx24 (Ca32). This structure confirms the presence of characteristic annexin fold known from the other members of this protein. The protein core is built by four domains, each comprising five Q-helices, among these, four of them arranged parallel and anti-parallel to each other (helices A, B, D and E) and the fifth (helix C) lying almost perpendicular to the four-helix bundle (Hofmann et al., 2000). The crystal structure of annexin from *Gossypium hirsutum*, AnnGh1 was also resolved and revealed a highly conserved annexin fold (Hofmann et al., 2003).

2.9.2 Membrane binding loops

The high level of conservation in primary and tertiary structures indicates an important role in membrane binding. It is likely that the AB and DE loops on the convex surface will serve as membrane binding loops in plant annexins, as was previously observed in mammalian annexins. The shapes of these two plant annexins are split and they contain several clefts and grooves which are not observed in their mammalian counterparts. Both the plant annexins share hydrophobic and positively charged residues stalking out of the convex surface of the core. Apart from the loops IIIDE and IVDE (Table 2.2), all other membrane binding loops carry conserved residues, which might either interact with the phospholipid head group or the glycerol backbone region (Hofmann, 2004).

Table 2.2 Surface-exposed residues (Hofmann et al., 2004).

Location	Anx (Gh1)	Anx24 (Ca32)	AnxA5
IAB	Trp32	Trp35	Leu31
IDE	Lys68	Lys71	-
IIAB	Lys102	Lys105	Lys101
IIAB	Arg103	Arg106	-
IIAB	Trp104	Trp107	-
IIDE	His144	Tyr147	Gly143
IIDE	His145	His148	Asp144
IIIAB	Lys187	Lys190	Trp187
IIIAB	Tyr189	Tyr192	-
IIIDE	Lys230	Gly231	Arg227
IVAB	Arg261	Arg262	Lys260
IVAB	Arg262	Arg263	Ala262

Surface-exposed residues of plant annexins putatively important for membrane binding according to structural alignment. Residues of annexin A5 are shown for comparison.

2.9.3 Calcium binding

Membrane binding of mammalian annexins requires calcium coordination in the canonical binding sites and it was observed from the crystal structure of AnxA5 in complex with phospholipids and calcium (Swairjo et al., 1995). But co-crystallization of plant annexins in their calcium bound forms was found to be difficult. With respect to primary structure, canonical calcium binding can only be anticipated in the first and possibly fourth domain (Battey et al., 1996). Structural comparison with annexin A5 indicated that the IAB loop in Anx24 (Ca32) seems to be the most likely calcium binding site among all the four AB loops, although no loop conformation suitable for binding has been observed (Hofmann and Huber, 2003). The crystal structure of AnnGh1 shows a conformation of the IAB loop perfectly suited for coordinating a calcium ion. It is evident that plant annexins bind calcium in a ternary complex with phospholipids and the most

likely candidates for the calcium binding sites are the IAB loops of domain I and IV and not in the IAB loops of domains II and III. Crystallographic studies have indicated that certain animal annexins have a hydrophilic pore, which could act as a channel for Ca^{2+} ions. The bell pepper annexin was also shown to have Ca^{2+} channel activity and this activity was higher than that of any of the animal annexins tested (Hofmann et al., 2000). Similarly, wheat annexin accumulates in the plasma membrane in response to cold treatment and may be acting as Ca^{2+} channel (Breton et al., 2000).

2.9.4 Oligomerization

Some mammalian annexins have been shown to self-associate in solution and exist in monomer-dimer equilibrium in a calcium-dependent fashion. Hoshino et al. (1995) first reported on the oligomerization state of plant annexins described for an annexin from *Capsicum annum*, Anx (Ca35), purified from a natural source in the membrane bound state and they observed the formation of homodimers during crosslinking of Anx (Ca35) binding to phosphotidyl inositol vesicles. Recently, the oligomerization state of four more recombinant plant annexins, such as Anx23 (Ca38), Anx24 (Ca32), AnnGh1 and AnnGh2 has been demonstrated by means of gel filtration and equilibrium sedimentation. The proteins Anx23 (Ca38), Anx24 (Ca32) and AnnGh1 exist in monomer-trimer equilibrium in solution (Hofmann et al., 2002). For AnnGh2, only a small fraction of the protein was found to be trimeric and mainly exists in monomer-dimer equilibrium. Unlike in mammalian annexins, calcium is not required for the formation of these oligomers. In the case of cotton annexins, the presence of calcium causes the dissolution of the oligomers by the reduction of trimeric or dimeric oligomers. According to gel filtration results, the trimer interactions seem to be a balanced mix of hydrophobic and polar interactions (Hofmann et al., 2002; Hofmann, 2004). An annexin from Arabidopsis, AnnAt1 also has shown the formation of oligomers, which is induced by H_2O_2 and can be prevented by the addition of reducing agents implying the sensitivity of AnnAt1 to the redox state (Gorecka et al., 2005).

2.9.5 Structural basis for oxidative stress response

AnnGh1 possesses four cysteine residues, two of which (Cys116 and Cys243) belong to helices IIB and IIIE respectively. In the crystal structure, these Cys116 and Cys243 were positioned adjacent to each other and exist in reduced (thiol) forms even without the addition of the reducing agent, although the formation of a disulfide bridge is sterically possible. Furthermore, the side chain of Met112 is positioned in close vicinity to both the cysteine residues and, therefore, establishes a triangular sulfur cluster with 3S-2H topology, if both cysteine side chains exist in their protonated forms. The cluster is located in the lower part of the annexin core in module II/III and is accessible only from the hydrophilic cleft between both molecules. The cluster is likely to be involved in redox reactions and might constitute the molecular basis of oxidative stress response by annexins.

2.10 Biochemical properties of plant annexins

2.10.1 Phosphodiesterase activity

Plant annexins not only bind purine nucleotides but also hydrolyse them. ATP and GTP binding feature is most common in animal annexins (Bandorowicz-Pikula et al., 2003). In contrast to animal annexins, nucleotide binding and hydrolysis may depend on Walker A motif (GXXXXGKT/S) and a GTP-binding motif of GTPase superfamily proteins (DXXG). Maize annexin, p68 was first found to be associated with ATPase activity (Mc Clung et al., 1994). Similar ATPase activity was also observed with tomato annexins that were not affected by F-actin binding, but could be inhibited by the specific Ca^{2+}-dependent interaction of the annexins with phospholipids suggesting a role for the proteins in mediating Ca^{2+}-dependent events involving interactions of the cytoskeleton and cellular membranes (Calvert et al., 1996; Lim et al. (1998) have generated two mutant forms by site-directed mutagenesis of the acidic residues in the DE loops of repeats-I and IV and suggested the repeat-IV plays an important role in Ca^{2+}-dependent phospholipid binding compared to the repeat-I. The recombinant cotton

fiber annexin displayed higher GTPase activity than ATPase activity and was dependent on Mg^{2+} while Ca^{2+} was inhibitory. They also reported that GTP-binding site was mapped onto carboxy-terminal of the fourth domain by domain-deletion mutants of the annexin (Shin and Brown, 1999). Mg^{2+}-dependent ATPase activity has also been found for Anx24 (Ca32) from bell pepper and was similar to the one reported for cotton fiber annexin. Annexins from maize, tomato and cotton have different requirements for catalyzing the same reaction, reflecting the diverse roles of plant annexins.

2.10.2 Peroxidase activity

Peroxidase activity of both recombinant *AnnAt1* and *AnnAt1* purified from Arabidopsis was demonstrated (Gidrol et al., 1996; Gorecka et al., 2005). The peroxidase activity of AnnAt1 is suggested to be due to a region of the first annexin domain in the N-terminal region (with a conserved histidine residue; His40) which has a strong similarity to the ~30 amino acid heme-binding motif of plant peroxidases (Clark et al., 2001; Gorecka et al., 2005). Further, it was also able to protect mammalian cells from oxidative stress (Kush and Sabapathy, 2001). The crystal structure of cotton annexin, AnnGh1 revealed the presence of two adjacent cysteines, in combination with a nearby methionine forming an S3 cluster. Even though it has not been completely demonstrated, it was suggested that it may have a role in transfer of electrons to an oxidizing molecule (Hofmann et al., 2003). *In silico* analysis of the AnnAt1 primary structure revealed sulfur cluster similar to that present in cotton AnnGh1 (Hofmann et al., 2003), suggesting that *AnnAt1* could play a role in oxidative stress response. The mutation by site-directed mutagenesis from His40 to Ala40 reduced this enzymatic activity. The activity as recombinant *AnnAt1* purified from *E. coli* is low when compared to that purified from *Nicotiana benthamiana*, the activity of which is reduced by dephosphorylation, suggesting that peroxidase activity depends on posttranslational modifications. The peroxidase activity of annexins is based on the presence of the conserved His40 residue in the first annexin repeat which has similarity to heme binding domain of plant peroxidases (Clark et al.,

2001; Gorecka et al., 2005; Jami et al., 2008; Laohavisit et al., 2009). A recent study by Jami et al., (2008) demonstrated the peroxidase activity recombinant *Brassica juncea* AnnBj1. The *in vitro* peroxidase of Zea Mays annexin and its calcium channel activity modulating the cytosolic calucium influx was also proved (Laohavisit et al., 2009).

2.10.3 Signal transduction

Breton et al., (2000) identified four annexin proteins in wheat based on the cross-reactivity with an antibody directed against Anx (Lt1) from *Lavatera thuringiaca*. Whilst proteins p34 and p36 were found to soluble and most abundant, and bind phospholipid membranes in a Ca^{2+}-dependent manner whereas, the proteins with molecular masses of 22.5 and 39 KDa were intrinsic proteins found associated within the microsomal fraction and are Ca^{2+}-independent. These two intrinsic proteins accumulate upon exposure of the plant to low temperatures; however the accumulation was not correlated to freezing tolerance. It may represent a general purpose to low temperature stresses, usually associated with cold acclimatization. Based on these observations, they concluded that these intrinsic membrane annexins might play a role as sensors or transducers of the calcium signal triggered by in the early stage of low temperature response. Recent studies suggested that AnnAt1 from Arabidopsis translocated from the cytosol to the membrane with potential turnover of this protein upon salt stress induction. This process is blocked by EGTA treatment, implying that AnnAt1 functions in stress response and tightly regulated by Ca^{2+}. T-DNA insertional mutants of annAt1 and its isoform annAt4 displayed hypersensitivity to NaCl, osmotic stress and abscisic acid (ABA) during germination and early seedling growth, suggesting that AnnAt1 and AnnAt4 play important roles in osmotic stress and ABA signaling in a Ca^{2+}-dependent manner (Lee et al., 2004). But the exact stress signaling processes is unclear. They speculated that AnnAt1 senses the Ca^{2+} signal elicited by ABA and stress treatments, and transmits it to downstream signaling pathways via mechanisms of protein degradation and translocation to the membrane.

2.10.4 Actin binding

Certain plant annexins can bind F-actin in vitro and tomato annexins bind to actin in a calcium- and pH-dependent manner, and the binding is specific to F-actin, not G-actin. This F-actin binding did not affect phosphodiesterase activity (Calvert et al., 1996). Based on the sequence information of tomato annexins, the presence of potential IRI motif has been speculated for this F-actin binding (Lim et al., 1998). Similarly, plant annexins containing this motif should have actin binding properties. Hoshino et al. (2004) showed that mimosa annexin binds to F-actin in the presence of calcium and also forms bundles of F-actin in the presence of calcium *in vitro*.

2.10.5 Exocytosis

Exocytosis is the final event in the secretory pathway and requires the fusion of secretory vesicle membrane with the plasma membrane. These vesicles originate from the Golgi to the plasma membrane in order to secrete polysaccharides. Carroll et al. (1998) examined protoplasts from maize root cap cells for exocytosis using cell capacitance measurements, where they observed an increase in exocytosis activity when the calcium concentration was slightly elevated above the resting level and also when the concentration of maize annexin, p35 was raised. The annexin effect could be reversed by the addition of anti-p35 antibodies and GTP. Similarly, lily (*Lilium longiflorum*) and Arabidopsis annexins are also believed to be involved in Golgi-mediated secretion of polysaccharides (Blackbourn et al., 1992; Clark et al., 2001; 2005).

2.10.6 Callose synthase activity

Higher plants contain glycosyltransferases in plasma membranes which catalyze the synthesis of 1,3-β-D-glucan (Callose) and 1,4- β-D-glucan (cellulose) from UDP-glucose. These enzymes are of central importance in normal development, since they are necessary for the biosynthesis of the cell wall and, particularly for the plant response to mechanical wounding, environmental

stresses and during pathogen attack (Hofmann, 2004). These enzymes require both Ca^{2+} and a β-glucoside for activity, and are normally latent and become activated upon elevation of intracellular calcium. Andrawis et al., (1993) have observed that cotton fiber annexins and callose synthase co-localize on the plasma membrane with an inhibitory activity on glucan synthesis when added to an assay using partially purified cotton fiber callose synthase. A recent study also showed an interaction between annexins and callose synthase in Arabidopsis (Verma and Hong, 2001). Based on the co-localization of cotton fiber annexins with glucan synthase, inhibitory effect on callose synthase and the putative Redox activity of the sulfur cluster of Anx (Gh1), a speculation can be made about the role of annexin in the Redox-dependent activation mechanism of cellulose synthase (CesA), which presents three possibilities (Hofmann et al., 2003).

1. Anx (Gh1) reduces (excessive) H_2O_2 to H_2O and acts as a housekeeping protein.
2. Anx (Gh1) reduces intramolecular disulfide bonds, which would rescue inactive CesA protein for rosette formation; or
3. Anx (Gh1) reduces the intermolecular disulfide bonds of CesA, which leads to monomerization and thus inhibition of glucan synthesis.

To date, plant studies have focused on annexin structure and *in vitro* protein function. Such studies have revealed a capacity for annexins to be multifunctional and point towards possible *in vivo* roles.

2.11 Cotton

Cotton is the primary source of natural fiber and its seeds yield different products of commercial importance such as edible oil, hulls, linters and seed meal. It has been estimated that cotton contributes over $20-30 billion to the world's agricultural economy and up to one billion people depend directly or indirectly on this crop for their livelihood (International cotton advisory committee, 2008). This crop is highly susceptible to most of the abiotic and biotic stresses causing considerable loss to the fiber quality and yield. In particular salt stress causes severe damage to the vegetative growth, fiber yield and quality of

cotton (Ashraf 2002). Although, traditional cotton breeding is a dynamic process, various incompatibility barriers still limit available germplasm usage. Genetic engineering studies in cotton for modifying agronomic traits through *Agrobacterium*-mediated transformation is a promising approach in the development of disease resistant and stress tolerant transgenic cotton plants. Based on the previous studies with *Brassica juncea* annexin which confers multiple stress tolerance when oxerexpressed in tobacco plants we attempted to develop stress tolerant transgenic plants by framing the following objectives:

2.12 The objectives framed for the study of chapter 6

1. Development of transgenic cotton plants expressing *Brassica juncea* annexin through *Agrobacterium* mediated transformation using pCAMBIA2300.
2. Molecular confirmation of transgenic plants by PCR, Southern and northern analyses.
3. Bioassays including various abiotic stress treatments with WT and transgenic plants at seedling and matured stages.
4. Determination of fiber quality dimensions in WT and transgenic plants after salt stress.

2.13 Tomato

Tomato (*Lycopersicum esculentum*) is the world's most important vegetable crop. It is a rich source of minerals, vitamins and organic acids, essential amino acids and dietary fibers. It is rich in vitamin A and C, also contains minerals like iron and phosphorus. Tomato contains lycopene and β-carotene pigments. This crop is susceptible to various fungal, bacterial, viral, insect and nematode diseases. In particular the economically important part of the plant, tomato fruit is susceptible to a variety of bacterial and fungal pathogens in the ripened stage. Resistance to these multiple diseases is an important trait, which requires a lot of resources and efforts when traditional breeding approaches

are considered. Exploitation of the genetic transformation technology in the development of transgenic plants with enhanced resistance towards different pathogens is increasing (Brunetti et al., 1997; Kaniewski et al., 1999; Lee et al., 2002; Li and Steffens 2002; Whitham et al., 1996; Tabaeizadeh et al., 1999; Tai et al., 1999 Dowd et al., 1999).

2.14 Peroxidases in plants

Peroxidases constitute a diverse family of heme-containing enzymes believed to function in a variety of normal and stress-related physiological processes of plants. Peroxidases have been implicated in numerous biochemical processes such as lignification (Lagrimini et al., 1987), suberization (Espelie and Kolattukudy, 1985; Espelie et al., 1986), cross linking of hydroxyproline-rich wall proteins and feruloylated polysaccharides (Smith and O'Brian, 1979; Fry, 1986), both oxidation and polymerization of soluble phenolics (Strivastava and van Huystee, 1977), the formation of hydrogen peroxide (Mader et al., 1980), Chlorophyll degradation and senescence (Yamauchi and Watada, 1991), and auxin degradation (Jinnman and Lang, 1965).

2.15 Tomato Anionic peroxidase

The highly anionic peroxidases of tomato (*tap1*) appear to be related to pathogenesis. Although the basal levels of transcripts of these genes are very low in the root, stem, leaf, and red fruit tissues of healthy tomato plants, expression of these genes is induced to high levels in tissues responding to challenge by fungal pathogens, wounding, or exposure to either ABA or fungal elicitor preparations (Roberts and Kolattukudy, 1989; Mohan and Kolattukudy, 1990; Robb et al., 1991). When near-isogenic resitant and susceptible lines of tomato were used, it was demonstrated that in petioles and cell cultures from the resistant plants exposed to *Verticillium alboatrum* conida or cell-free elicitor preparations, *tap* gene expression was rapidly induced to high levels. The tissues from susceptible tomato variety showed both delayed and reduced accumulation of *tap1* transcripts. It was suggested that the *tap1* activity plays a crucial role in the

plant's overall defense towards pathogens. It was shown that *tap1* is constitutively expressed in mature green fruits but it is not detected in red ripened fruits of tomato (Sherf and Kolattukudy 1993). The dimished *tap* activity in ripened fruits of tomato is in corroboration with their vulnerability to various fungal pathogens when compared to green fruits. A correlation has previously been established between the temporal and spatial expression of wound, fungal elicitor, and ABA inducible anionic peroxidase activities with the onset of suberization in potato and tomato tissues (Espelie and Kolattukudy, 1985; Espelie et al., 1986; Roberts et al., 1988; Robb et al., 1991). Deposition of suberin occurs on the cell wall of tissues responding to pathogen ingress or wound trauma (Espelie et al., 1986; Robb et al., 1991), thus fortifying the wall structure of affected cells. All the previous studies suggested that anionic peroxidases are involved in the polymerization of suberin monomers in pathogen attack or wounding. However, studies with the antisense *tap1* tomato plants revealed no difference in the cell wall polymers estimated by gas-liquid chromatography/mass spectrometry. This result was attributed to the multiplicity of plant peroxidases so that another peroxidase has substituted the function of *tap1*. The t*ap1* expression is correlated with suberization of the tissues with pathogen attack and wounding conferring resistance. Thus we attempted for tissue specific overexpression of *tap1* driven by an ethylene inducible fruit specific promoter E8 in red ripened fruits of tomato, which are attractive for many fungal pathogens causing severe damage to the fruits after harvesting.

2.16 E8 promoter

In order to give rise the maximum expression and effect of the transformed genes, the timing and localization of the gene expression should be considered. Promoter as one of the major transcript regulators plays a crucial role in gene expression. The most employed promoter cauliflower mosaic virus 35S (CaMV 35S) promoter can efficiently drive foreign gene expression in plant cells (Jani et al., 2002). However, this promoter does not confer any specificity-neither tissue specificity nor plant developmental stage specificity on exogenous gene expression leading to lower expression levels (Smigocki and Owens 1988). On

the other hand, the transgenic overexpression using fruit specific promoters is increasing. Several fruit-specific promoters such as E4, E8, PG and 2A11 were identified in tomato (Coupe and Deikman 1997; Deikman et al., 1998). These promoters have been mostly used to investigate the role of ethylene in fruit ripening. The E8 promoter is one of the most extensively characterized ripening-specific tomato promoters. The deletion studies of flanking DNA sequences on E8 gene expression in transgenic tomato fruit showed that the E8 promoter has at least two main regions contributing to its transcriptional regulation: the region of –2181 to –1088 containing DNA sequence that confer ethylene responsiveness in unripe fruit but are sufficient for E8 gene expression during ripening, and the 'downstream' region of –1088 to the transcriptional start site is sufficient for ripening-specific transcription in the absence of ethylene synthesis (Deikman et al., 1992). Some researches used the E8 promoter to drive the expression of exogenous genes in transgenic tomato fruits (Sandhu et al., 2000; Krasnyanski et al., 2001; Mehta et al., 2002; Yakoby et al., 2006). As ripened tomato fruit is more susceptible to various fungal pathogens, we aimed at fruit specific overexpression of tomato anionic peroxidase.

2.17 The objectives framed for the study of chapter 7

1. A*grobacterium* mediated genetic transformation of tomato with pBI101-E8-*tap*1 to facilitate ethylene inducible fruit specific overexpression of tomato anionic peroxidase.
2. Confirmation of transgenic plants by PCR, Southern and northern analyses.
3. Evaluation of transgenic tomato plants for fungal resistance.

Chapter 3

Materials and methods

3.1 Cell culture maintenance and transfection studies

3.1.1 Reagents

RPMI-1640, fetal bovine serum (FBS), penicillin, streptomycin and geneticin (G418) were purchased from Gibco BRL (California, USA). Trypsin-EDTA, protease inhibitor cocktail, caspase 3 and 8 substrates (AC-DEVD-AFC and AC - IETD - AFC) were purchased from Sigma (St. Louis, USA). p50, p65, pERK, pJNK, p38, iNOS, Cox-2, cyclin D1, c-Myc and β-actin antibodies were purchased from Cell Signaling Technology, USA. Enzyme immunoassay kit for measurement of NF-κB and Lipofectamine^{TM}2000 were obtained from Invitrogen Inc., (CA, USA). All secondary antibodies were purchased from Genei Pvt. Ltd. (Bangalore, India). pcDNA 3.0 vector was purchased from Upstate Biotechnology (Lake Placid, NY).

3.1.2 Cell culture and stable transfection

Human U373 glioblastoma cell line was obtained from National Centre for Cell Science, Pune, India. Cells were grown in RPMI 1640 media supplemented with 10% FBS, 100 IU ml^{-1} penicillin and 100µg ml^{-1} streptomycin. Cells were maintained in humidified atmosphere with 5% CO_2 at 37°C. For transfection studies, 1×10^5 cells were seeded in 35 mm culture dishes. After 24 h, 5 µg of linearized pcDNA-NPR1 and empty pcDNA plasmids were transfected into U373 cells using lipofectamine^{TM}2000 as per manufacturer's instructions. After 48 h, stable transfected cells were selected by growing them in the culture media containing G418 (500 µM), and the selected cells were used in further analysis.

3.1.3 Preparation of cytosolic and nuclear extracts

Control (U373) and transfected (U373-NPR1) cells were grown in 100 mm culture plates. Briefly, cells were harvested and washed in ice-cold PBS, lysed in 400 µl of cold buffer A [HEPES 10 mmol l^{-1}, pH 7.9, KCl 10 mmol l^{-1}, 1 mM EDTA, phenylmethanesulphonylfluoride (PMSF) 1 mmol l^{-1}, 1 mM EGTA, dithiothreitol (DTT) 1 mmol l^{-1}, aprotinin 1 mg l^{-1}, leupeptin 1 mg l^{-1} and

pepstatinA 1 mg l^{-1}]. After 15 min incubation on ice, 0.1% NP-40 was added to the lysates and the tubes were vigorously rocked for 1 min. Then, the lysates were centrifuged (20,800g, 5 min) at 4°C. The supernatant was collected as cytosolic fraction. The nuclear pellets were washed once with cold buffer A, resuspended in 50 µl of cold buffer B (HEPES 20 mmol l^{-1}, pH 7.9, NaCl 420 mmol l^{-1}, edetic acid 0.1 mmol l^{-1}, egtazic acid 0.1 mmol l^{-1}, PMSF 1 mmol l^{-1}, DTT 1 mmol l^{-1}, aprotinin 1 mg l^{-1}, leupeptin 1 mg l^{-1} and pepstatinA 1 mg l^{-1}) and vigorously rocked at maximum speed for 30 min at 40°C. The solution was centrifuged at 20,800g for 5 min, and the supernatant was collected as nuclear fraction. The protein concentration was determined according to the Bradford method.

3.1.4 RT-PCR and Northern blotting

Total cellular RNA was extracted from U373 and U373-NPR1 cells cultured in a 100 mm culture dishes using TRI-Reagent according to manufacturer's instructions (Sigma-Aldrich, USA). Purified total RNA was dissolved in DEPC treated water and stored at -80°C before use. Total RNA (5 µg) was treated with RNase free-DNase I and reverse transcribed to cDNA using oligo $(dT)_{18}$ primer and M-mLV reverse transcriptase enzyme (Invitrogen, Sandiego, CA, USA). PCR analyses were performed to detect the transcript level of *BjNPR1* and *GAPDH* (as an internal standard). The amplification conditions were initial denaturation at 94°C for 3 min followed by 30 cycles of 94°C for 1 min; 58°C for 45 s; 72°C for 45 s.

For northern blotting, total cellular RNA (20µg) was electrophoresed on 1% formaldehyde denaturing agarose gel and transferred onto Hybond N^+ membrane. The cDNA of *NPR1* for use as hybridization probe was prepared using Prime-a-Gene radiolabelling kit (Promega Corporation, USA) according to manufacturer's instructions and used in hybridization of the membrane at 65°C for 24 h followed by washing and exposure in auto radiography.

3.1.5 Western blotting

Equal amounts of cytosolic and nuclear proteins from control and transfected cells were mixed with SDS sample buffer, boiled for 5 min and subjected to electrophoresis on 10% SDS-polyacrylamide gels. The separated proteins were transferred to nitrocellulose membranes. The membranes were blocked with 5% non-fat dry milk in Tris Buffered Saline (TBS) (10mM Tris (pH 7.5), 150 mM NaCl) for 1 h at room temperature and incubated with primary antibodies at 4°C for 12-16 h. Then membranes were incubated with respective secondary antibodies for 1-2 h at room temperature. Before and after incubation of blots with secondary antibodies, blots were washed with TBS and TBST (TBS containing 0.1% Tween-20). Immunoreactivity was detected using western blot detection reagents.

3.1.6 Determination of NF-κB levels

NF-κB levels were measured in cytosolic and nuclear extracts by using Enzyme immunoassay kit (EIA) kit according to the manufacturer's instructions.

3.1.7 Caspase-3 and Caspase-8 activity assay

Caspase-3, and Caspase-8 activities were measured in U373 and U373-NPR1 cell lysates using synthetic fluorogenic substrates (Ac-DEVD-AFC; substrate for Caspase-3, Ac-IETD-AMC, substrate for Caspase-8) as described by the manufacturer's (BD Biosciences) protocol. Briefly, the cells were washed with PBS and lysed in the lysis buffer (100mM NaCl, 5μg ml^{-1} aprotinin, 10 μg ml^{-1} leupeptin, 0.1 mM EDTA, 50 mM HEPES, 0.1% CAHPS, 0.1 mM DTT, 100 μM PMSF). Subsequently 50 μg of the protein and 8 μM of the substrate were added to 1 ml of the assay buffer (20 mM Pipes, 100 mM NaCl, 10 mM dithiothreitol, 1 mM EDTA, 0.1% (w/v) CHAPS and 10% sucrose) and incubated for 1 h at 37°C. Measurements were made on a spectrofluorometer with a λ_{ex} of 380 nm and a λ_{em} of 460 nm for Caspase-8 and with a λ_{ex} of 400 nm and λ_{em} of 480–520 nm for Caspase-3.

3.1.8 Co-immunoprecipitation

Cytosolic extracts from U373-NPR1 cells were immunoprecipitated with NPR1 antibody and immune complex was probed with p50 and p65 antibodies. Briefly, cytosolic proteins were pre-cleared using protein A-agarose and incubated with 1 µg of NPR1 primary antibody overnight at 4°C. After the addition of 20 µl of protein A-agarose, samples were further incubated for 2-3 h at room temperature. Then the immune complex was washed thrice with a wash buffer containing 20 mM Hepes (pH 7.4), 500 mM NaCl, and 10 mM $MgCl_2$ and suspended in 40 µl of rinse buffer containing 20 mM Hepes (pH 7.4), 150 mM NaCl, and 10 mM $MgCl_2$ and the beads were resuspended in SDS sample buffer and subjected to western blot analysis.

3.1.9 Immunofluorescence

U373-NPR1 cells were seeded onto glass coverslips in 12-well plates. After reaching confluency, cells were washed with PBS and fixed with 4% paraformaldehyde. Fixed cells were permeabilized with 0.2% Triton X-100 and washed with PBS twice. Cells were then incubated with a cocktail of two primary antibodies (mouse polyclonal p65 and rabbit polyclonal NPR1) for 1-2 h at room temperature. After three washes with PBS, cells were incubated with a mixture consisting FITC-conjugated anti-mouse and TRITC-conjugated anti-rabbit secondary antibodies for 1 h at room temperature. Cells were washed three times with PBS and coverslips were mounted with 90% glycerol and examined under a Leica confocal microscope.

3.1.10 Cell proliferation assay

The proliferation potential of U373 and U373-NPR1 cells was determined by measuring the reduction of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) to formazan. Cleavage of MTT (a tetrazolium salt) by mitochondrial-associated dehydrogenase enzymes in metabolically active cells yields a colored formazan product that can be measured spectrophotometrically. Briefly, U373 and U373-NPR1 cells (1×10^4 cells/well)

were seeded in 96 well plates. After 48 h, cells were incubated with MTT (25 μg/well) for 4 h. Following this, 0.04 N acidic isopropanol was added to each well to dissolve the precipitates, and absorbance was then measured at 570 nm on a multi-well plate reader. Appropriate controls lacking cells were included to determine the background absorbance.

3.2 Chemicals

All the chemicals used in the present study were procured from Sigma, St. Louis, MO, USA; Amersham Biosciences, UK; Promega Life Science, Madison, WI, USA; Fermentas, Germany; Himedia chemicals, Mumbai, India and Qualigens fine chemicals, Mumbai, India.

3.3 Plasmid DNA vectors

The vectors and bacterial strains used in the cloning and transformation studies were listed below.

3.3.1 pTZ57R (MBI Fermentas, Germany)

This vector is used in the cloning of PCR amplified fragments of various genes used in our study. It also has a bacterial selectable marker gene, β-lactamase and therefore can be selected on ampicillin.

3.3.2 pcDNA3.0 (Upstate Biotechnology, NY)

This is a mammalian expression vector that is ideal for transient or stable transfection in mammalian systems. This vector possesses human cytomegalovirus immediate-early (CMV) promoter for high level expression in mammalian cells and neomycin resistance gene for selection of stable cell lines. *E.coli* transformants can be selected on Ampicillin plates.

3.3.3 pEGFP-N1 (Clontech, USA)

This is a mammalian transfection vector, which facilitates transient or stable transfection in mammalian cells. Stable transformants can be selected using G418. It contains Human cytomegalovirus immediate-early (CMV) promoter and EGFP coding sequence, which can be used as a transfection marker. It confers resistance to Kanamycin, used as a bacterial selection reagent.

3.3.4 pERFP-N1 (Clontech, USA)

This is a mammalian transfection vector used for transient or stable transfection in mammalian cells. Stable transformants can be selected using G418. It contains Human cytomegalovirus immediate-early (CMV) promoter and ERFP coding sequence, which can be used as a transfection marker. It confers resistance to Kanamycin, used as bacterial selection reagent. These vectors were employed in standarsing the transfection in cell lines.

3.3.5 pRT100 (Töpfer et al., 1987)

This is a plant expression vector used for cloning of cDNA at a multiple cloning site that was flanked by CaMV35S promoter and the polyadenyation signal. pRT100 has a gene encoding for β-lactamase for the selection in bacteria on ampicillin.

3.3.6 pCAMBIA2300 (CAMBIA, Australia)

pCAMBIA 2300 is a binary vector used to clone *AnnBj1* and *BjNPR1* for transformation into *Agrobacterium tumefaciens*, used for transforming cotton plants. This binary vector has *nptII* gene encoding for kanamycin resistance for selection on bacteria and as a plant selectable marker respectively.

3.3.7 pBI101.1 (Clontech, USA)

This is a binary vector used to clone E8-TAP1 for transforming tomato plants. This binary vector possess *nptII* gene providing kanamycin selection to facilitate selection in both bacteria and plants.

3.4 Bacterial strains used in the study

The bacterial strain *Escherichia coli* DH5α was used in the maintenance of the above plasmid clones. The *Agrobacterium tumefaciens* strains GV2260 and LBA4404 were used for transformation procedures of cotton and tomato plants.

3.5 Growth of microorganisms

The different strains of *E. coli* were incubated and cultured either in LB (Luria Broth) medium (Himedia, Mumbai, India) with continuous shaking at 200 rpm or on the solid LA (Luria Agar) medium at 37°C while the *Agrobacterium*

strains were incubated in liquid LB medium with continuous shaking at 200 rpm or on the solid LA medium at 28°C. These bacterial strains were incubated and selected with appropriate selection markers.

3.6 Restriction enzymes, modifying enzymes and Markers

The restriction enzymes, modifying enzymes such as Calf intestinal Alkaline phosphatase (CIAP), Klenow polymerase and T4 DNA polymerase and their corresponding buffers were from Sigma, St. Louis, MO, USA; Promega Life Science, Madison, WI, USA; Fermentas, Germany. The DNA markers (Lambda-*Hind*III + *Eco*RI or Lambda-*Eco*RI digest) and pre-stained protein standard marker (100 kDa) were from Fermentas, Germany.

3.7 Software programs

Adobe photoshop 6.0
ClustalX program
ClustalV program of Laser gene from DNASTAR
Genedoc Software
Sigma Plot
Sigma Stat
Image J 1.42

3.8 Plant growth conditions and stress treatments

3.8.1 Explant preparation and transformation

Cotton seeds of the cultivar Durga (JK Agrigenetics, Hyderabad, India) and Bharani (Manish Agribiotech, Hyderabad) were used in the transformation experiments with hypocotyl explants. These were cultured on the shoot induction medium (SIM) i.e., MS medium (Murashige and Skoog, 1962) with NN vitamins (Nitsch and Nitsch, 1969) augmented with TDZ (0.1mg l^{-1}), NAA (0.05mg l^{-1}) and activator charcoal (1g l^{-1}) followed by co-cultivation after treating with an overnight grown *Agrobacterium* culture (GV2260) harboring the binary vector

pCAMBIA2300-*AnnBj1* (Jami *et al.* 2008 Fig 1a) at an optical density (OD) of 0.4 using vacuum infiltration for 10 min.. Illumination was provided by cool white fluorescent lamps at a light intensity of 70μE cm^{-2} s^{-1}. The putative transgenic shoots grown on selection medium containing 75 mgl^{-1} Kanamycin (Sigma-Aldrich, Missouri, USA) were cut at the internodal position and cultured on root induction medium (RIM) supplemented with indole -3- butyric acid (IBA, 1.0 mg l^{-1}).

For tomato transformation Arka Meghali cultivar (ANGRAU, Hyderabad) was used. Cotyledons from 8-10 d old seedlings were cultured on shoot induction medium followed by rooting medium with selection marker kanamycin (50mgl^{-1}). *Agrobacterium* strain LBA4404 harboring pBI101.1 was used in the transformation experiments.

The putative transgenic plantlets with well developed roots were acclimatized and grown in green house.

3.8.2 Extraction of plant genomic DNA

Total genomic DNA was isolated from the fresh and young leaves of 4-week old cotton plants grown in green house, according to the method of CTAB procedure (Doyle and Doyle, 1990). The leaf material (0.5 gm) was ground to a fine powder in liquid nitrogen using pre-chilled mortar and pestle. The powder was added to 2 ml of pre-warmed DNA extraction buffer and incubated at 65°C for 1 h in a water bath with occasional mixing by gentle swirling. The mixture was suspended and mixed by several inversions to emulsify with one volume of chloroform: isoamyl alcohol (24:1 ratio) and centrifuged at room temperature for 15 min at 12,000 rpm. The aqueous phase was then mixed with 0.1 volume of 3 M sodium acetate (pH 5.2) and 2/3 volume of isopropanol and, mixed by inversion and incubated at room temperature for 1 h. The mixture was centrifuged as above and the supernatant was carefully discarded. The pellet was rinsed in 70% (v/v) ethanol for 10 min, air-dried and resuspended in 0.5 ml of TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA).

3.8.3 Purification of DNA

To the DNA sample, 5 µl of RNaseA (10 mg/ ml) was added and incubated for 2 h at 37°C. To this, 0.5 ml of Phenol: Chloroform: Isoamyl alcohol (25:24:1) was added and mixed by inversion for 10 min and the suspension was centrifuged at 12,000 rpm for 15 min at room temperature. The supernatant was mixed with 0.1 volume of 3 M sodium acetate (pH 5.2), 2.5 volumes of chilled absolute alcohol and incubated at -20°C for 2 h or overnight. DNA was pelleted by centrifugation at 12,000 rpm for 15 min at room temperature and the pellet was washed in 70% (v/v) ethanol for 10 min, air-dried and resuspended in 50-100 µl of TE buffer and stored at -20°C. DNA extraction buffer: 2% (w/v) CTAB, 100 mM Tris-HCl, pH 8.0, 1.4 M NaCl, 20 mM EDTA, 0.2 % (v/v) β-mercaptoethanol, which should be added *in-situ* (before DNA extraction).

3.8.4 Extraction of total RNA

The plant material of 0.2 g from the stress-treated and unstressed leaves/fibers and the tissues such as stem, roots and flowers were frozen in liquid nitrogen and ground to a fine powder. The total RNA was extracted using TRI reagent according to the manufacturer's instructions (Sigma, USA). Finally the pellet was resuspended in 40 µl of RNase-free water and stored at -70°C.

3.8.5 Spectrophotometric estimation of nucleic acids

The quantity and purity of extracted nucleic acids in solution (DNA and RNA) were spectrophotometrically determined by measuring the absorbance at 260 nm and 280 nm. A value of OD_{260} =1 corresponds to 50 µg/ml for DNA, while OD_{260} =1 correspond to 40 $\mu g ml^{-1}$ for RNA. For pure DNA extraction, the value of OD_{260}/ OD_{280} must be between 1.8 and 2.0. A value below 1.8 means the contamination of DNA with proteins and phenolic compounds. For the purity of RNA the value of OD_{260}/ OD_{280} must be higher than 2.0. A value below 2.0 means the contamination of RNA with proteins or phenolic compounds.

3.9 Cloning of DNA fragments

3.9.1 Primer designing for cloning

The melting temperature (T_M) of the primers should be according to Faust rules, T_M (°C) = 4 (G+C) +2 (A+T) where G, C, A and T represent the number of corresponding nucleotides in the primer. The annealing temperature (T_A) depends on the T_M value and is calculated as, $T_A = T_M$-5°C. The primers were designed to avoid self-complementation forming thereby a secondary structure. The forward and reverse primers of each reaction were designed to have approximately the same T_M.

3.9.2 Polymerase chain reaction (PCR)

For the amplification of DNA fragments, a total volume of 50 µl solution was prepared in a sterile 0.2 ml thin-wall PCR tube with 15 pmol/µl each of both forward and reverse primers, 100-150 ng of genomic DNA or 5 ng/µl plasmid DNA or PCR product or 2-5 µl of first strand cDNA as a template, 200 µM of each dNTP, 1.5 mM $MgCl_2$ and 2.5 U of Hot start *Taq* polymerase (Qiagen, Germany) or recombinant *Taq* DNA polymerase (Invitrogen, USA). Each PCR aliquot was mixed and the PCR reactions were performed in Eppendorf personal cycler or Eppendorf Mastercycler Gradient (Germany) and / or MJ Research Inc., (USA). The standard reaction conditions carried were initial denaturation at 94°C for 4 min, followed by 30 cycles of 94°C for 1 min, 55°C for 45 seconds, 72°C for 1 min and a final extension of 20 min at 72°C. An aliquot from the amplified mix was run on 1% agarose gel to check for amplification.

3.9.2 Gel extraction of DNA fragments

After the restriction digestion, or PCR amplification of plasmid DNA constructs, DNA bands or plasmid inserts were identified using standard molecular weight marker (λ-*Hind*III + *Eco*RI or λ- *Eco*RI digest). After the extraction, purification of DNA from the agarose gel pieces was performed with

Eppendorf perfectprep Gel cleanup kit (Eppendorf, Germany) according to the manufacturers instructions.

3.9.3 Restriction endonuclease treatments

DNA digestion was carried out in a reaction volume of 50 µl in 1 /10 end volume of appropriate reaction buffer (10×) and 5 U of restriction enzyme was used per 1µg of DNA to be digested, while for double digestions, the restriction digestions were carried out sequentially.

3.9.4 Dephosphorylation

The single digested DNA fragments were dephosphorylated at their 5'-ends with Calf intestine alkaline phosphatase (Fermentas, Germany) in order to avoid self-ligation of cohesive / blunt-end termini of plasmid DNA during DNA recombination. The reaction was made in a total volume of 50 µl comprising 5 µl dephosphorylation buffer (10×), 1 µl (1 unit) Calf intestine alkaline phosphatase and plasmid DNA. The mixture was made up to 50 µl with sterile distilled water. The reaction mixture was incubated at 37°C for 30 min and followed by heat inactivation at 85°C for 15 min and then purified by perfectprep Gel cleanup kit (Eppendorf, Germany).

3.9.5 Ligation

For plasmid DNA constructs, different DNA inserts were ligated in various independent experiments using T4 DNA ligase (Fermentas, Germany). The ligation reaction mixture was made in a total volume of 20 µl comprising 2 µl ligation buffer (10 x), appropriate volumes (in µl) each of linear digested plasmid DNA and insert DNA, 2 µl of 50% PEG 4000 solution and finally T4 DNA ligase (1-2 units for cohesive ends and 5 units for blunt ends). The reaction mixture was incubated for 16 h at 16°C for cohesive ends and at 22°C for blunt ends respectively.

3.9.6 Preparation of *E.coli* competent cells and transformation

A single colony of *E.coli* DH5α cells were inoculated into 5 ml of LB medium and incubated overnight with constant shaking at 37°C. One ml of the overnight culture was grown in 50 ml of LB medium with vigorous shaking until the OD_{600} reaches 0.5. The cells were cooled on ice for 10 min and pelleted by centrifugation at 4000 rpm for 5 min at 4°C. The pellet was suspended in 40 ml of ice cold 100 mM $CaCl_2$ and incubated on ice for 20 min and further centrifuged as above. The pellet was finally resuspended in 3 ml ice-cold 100 mM $CaCl_2$, 15% (v/v) sterile glycerol, mixed and stored at -70°C in aliquots of 0.2 ml of competent cells. One µl of the plasmid DNA (10-50 ng /µl) or the ligated plasmid DNA construct was added to competent cells, carefully mixed and incubated on ice for 30 min. The cells were subjected to heat shock at 42°C for 90 s, and then on ice for 1 min. LB medium (0.8 ml) was added to the treated cells and further incubated by shaking at 200 rpm at 37°C. Aliquots (100-200 µl) of the transformed cells were spread on selective plates and incubated at 37°C overnight.

3.9.7 Isolation of plasmid DNA (mini-prep) by alkaline lysis method (Birnboim and Doly, 1979)

For plasmid mini-prep, transformed *E.coli* colonies were inoculated in 5 ml of LB medium containing appropriate antibiotics and allowed to grow overnight with shaking at 200 rpm at 37°C. The bacteria were harvested by centrifugation at 12,000 rpm at room temperature for 1 min. The pellet was resuspended in 100 µl ice-cold solution-I (50 mM Glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA, 100 µg RNase A) and vortexed thoroughly. The cells were lysed by adding 200 µl freshly prepared solution-II (200 mM NaOH, 1% (w/v) SDS) and mixed by gentle inversion. To this lysate, 150 µl of pre-chilled solution-III (3.0 M potassium acetate, pH 4.8) was added, mixed well and incubated on ice for 5 min. The supernatant was collected by centrifugation for 15 min at 12,000 rpm at 4°C. To the supernatant an equal volume of phenol: chloroform: isoamylalcohol (25:24:1) was added, mixed gently and centrifuged as above. The DNA was precipitated by the addition of 0.1 volume of 3 M sodium acetate (pH

5.2) and 0.7 volumes of isopropanol to the supernatant after centrifucation. Finally the DNA was pelleted by centrifugation at 12,000 rpm for 15 min at room temperature and the pellet was washed in 70% (v/v) ethanol, vacuum-dried and resuspended in 50-100 µl of TE buffer and stored at -20°C.

3.9.8 Preparation of *Agrobacterium* competent cells and transformation

The recombinant vectors pCAMBIA2300-*AnnBj1*, pCAMBIA2300-*BjNPR1* and pBI101-*E8-tap1* carrying the expression cassettes and the marker gene for kanamycin resistance were transformed into *Agrobacterium tumefaciens* strain GV2260 by freeze-thaw method. A single colony of *Agrobacterium* was inoculated into 5 ml of LB medium and grown overnight with constant shaking (200 rpm) at 28°C. An aliquot of 2 ml of the overnight culture was grown in 50 ml LB broth with vigorous shaking at the same conditions until the OD_{600} reaches 0.5 to 1.0. The culture was cooled on ice for 10 min and centrifuged at 3000 rpm for 5 min at 4°C. The cell pellet was resuspended in 1 ml of ice-cold 20 mM $CaCl_2$, 15% (v/v) sterile glycerol, mixed and stored at - 70°C in aliquots of 0.2 ml in pre-chilled eppendorf tubes.

Transformation of *Agrobacterium* with the recombinant vectors was carried out by mixing 1 µg of DNA with the competent cells followed by immediate freezing in liquid nitrogen. The cells were thawed by incubating the Eppendorf tube in a 37°C water bath for 5 min and 1 ml of LB medium was added to the tube and incubated at 28°C for 2-4 h with gentle shaking. This period allows the bacteria to express the antibiotic resistance genes. Briefly, the cells were centrifuged for 30 s and the supernatant was discarded. The final pellet was resuspended in 0.1 ml LB medium and the cells were spread on LA plates supplemented with 100 mg l^{-1} carbenecillin, 100 mg l^{-1} rifampicin, 25mg l^{-1} streptomycin and 50 mg l^{-1} kanamycin and incubated at 28°C. Transformed colonies that appeared after 2-3 days were analyzed by PCR and also confirmed by restriction digestion of the purified recombinant plasmid.

3.10 Growth of transgenic cotton plants and molecular analyses

All transgenic and WT cotton plants were grown in a growth room under a photoperiod of 16 day/ 8 night regime at a temperature of 25 ± 2°C. The T_1 seeds, obtained from T_0 of the transgenic cotton plants by selfing, were germinated on MS medium containing kanamycin at a concentration of 75 mg l^{-1}. T_2 generation plants from kanamycin selected T_1 plants were raised in the greenhouse for all analyses. All physiological experiments were repeated at least thrice independently with the uniformly grown wild and transgenic plants of the same age.

3.11 The custom primer pairs used in our study

AN F: 5′-ATGGCGACTCTTAAGGTT-3′
AN R: 5′- TTAAGCATCATC TTCCCAGAG-3′ (~1.0 kb),
18S F: 5′-CCAGGTCCAGACATAGTAAG-3′
18SR: 5′-GTACAAAGGGCAGGGACGTA-3′ (0.3 kb)
SPS F: 5′-CGGGAAACGACTGGATAAACA-3′
SPS R: 5′-GCACAATCAACAAACTCAGG-3′ (~0.9 kb)
SUS F: 5′-CTCACTCGCGTCCACAGTCT-3′
SUS R: 5′-GCAAGGAGGCGACGATATT-3′ (~1.2 kb)
CelA1 F: 5′-GGAGTGATCGATATGCCAAT-3′
CelA1 R: 5′-CCTTGGAAGACGGCAAA-3′ (~1.0 kb).
NPTII F: 5’-GAGGCTATTCGGCTATGACTG-3’
NPTII R: 5’-ATCGGGAGCGGCGATACGTA-3’ (~0.7 kb)
NPR1 F: 5’-CATCCATGGAGACCATTGCTAGA-3’
NPR1 R: 5’-TCACCGACGCCGGTGAGAGG-3’ (~1.7 kb)
GAPDH F: 5’-CTTCACCACCATGGAGGAGG-3’
GAPDH R: 5’-TGAAGTCAGAGGAGAGGAGACCACC-3’ (~0.5 kb)
CHITINASE F: 5’-CAGGCCTTGTCAATTTTCCT-3’
CHITINASE R: 5’-TACATTGAGTCCACCGAGACT-3’ (~0.9 kb)
GLUCANASE F: 5’- AGATCACAGCACTTCCACAA-3’
GLUCANASE R: 5’-CGGATAATAGCCGAAACCAT-3’ (~1.2 kb)

PR F: 5'-TGGGTGTTGTCACTTATAACT-3'

PR R: 5'-TCGGGGTTAGCCAAGAGGTAA-3' (~0.5 kb)

TAP1 F: 5'-ATGGGTTTTCGTTTGAGTCAT-3'

TAP1 R: 5'-TCACATAGAAGCCACTGAAGT-3' (~1.0 kb)

3.12 Sequence and Phylogenetic analysis

The sequences were analyzed using the BLAST search (Altshul et al., 1990). Multiple sequence alignments were carried out with protein sequences retrieved from EMBL/GenBank databank at http:// www.ebi.ac.uk/ClustalW. The multiple sequence alignment of amino acid sequences was performed with either clustalW or clustalV program of Lasergene from DNASTAR. SMART analysis was used to find out the important functional domains in the sequences.

3.13 Southern Hybridization

All the basic molecular techniques were performed following the procedures described in Sambrook et al. (1989).

Restriction digestion of DNA

Restriction endonucleases recognize specific palindromic sequences and cleave a phosphodiester bond on each strand at that sequence. Total genomic DNA isolated from leaf samples of WT and transgenic plants (10-15µg) was digested with 30 units of required restriction enzyme and the reactions were allowed to proceed overnight at 37°C to facilitate complete digestion of DNA. Samples were electrophoresed on 0.8% gel for 12 h at 25V.

Southern blotting (Sambrook et al., 1989)

The gel was treated with depurination (10 min), denaturation (30 min) followed by neutralization solutions. The DNA was transferred on to Hybond N^+ nylon membrane (Amersham Biosciences, UK) by capillary blotting using 20×SSC (1.5 M NaCl, 0.15 M Sodium citrate, pH 7.0) buffer.

Hybrization

The DNA transferred on the membrane was UV-cross linked , prehybridezed at 65°C for 4-6 h in phosphate buffer, pH 7.2, 7% (w/v) SDS, 10mM EDTA and 1% BSA) and hybridized for 16 h with (α-^{32}P) ATP radio labeled DNA using Prime-a gene labeling system of Promega corporation, Madison, USA. After hybridization for 16-24 h, membranes were washed with 2×SSC, 0.1% SDS at 65°C for 10 min followed by 1×SSC, 0.1% SDS followed by 0.1×SSC and 0.1×SSC respectively. Later, membranes were exposed to X-ray films at -70°C and autoradiographed.

Depurination solution: 0.2 N HCl

Denaturation solution: 1.5 M NaCl, 0.5 M NaOH

Neutralization solution: 1 M Tris-HCL, pH-7.4, 1.5 M NaCl

20×SSC: 1.5 M NaCl, 0.15 M Sodium citrate, pH-7.0

3.14 Northern blot analysis

Total RNA was extracted from the frozen samples prepared from different abiotic stress treated and from unstressed samples as well as from the tissues such as stems, leaves, roots and flowers using TRI reagent (Sigma, USA) according to the manufacturer's instructions. Total RNA (20 µg) was separated by electrophoresis at 90 V for 2-3 h through 1.2% (w/v) formaldehyde agarose gel in formaldehyde gel buffer. The gel was washed in DEPC water for 30 min. Transfer of DNA onto Hybond-N$^+$ nylon membrane, hybridization and washings were carried out at 65°C as described in the protocol of southern blot analysis.

All glassware, plasticware, buffer solutions used for RNA work should be treated with 0.1% (v/v) DEPC.

1.2% formaldehyde gel (100 ml): To 1.2 g of agarose, 10 ml 10x formaldehyde gel buffer, RNase-free water to 100 ml was added and agarose was melted in the microwave oven. This was then cooled to 65°C and 1.8 ml of 37% (12.3 M) formaldehyde and 1µl of ethidium bromide (10 mg ml^{-1}) was added, mixed thoroughly and poured onto gel support.

10×formaldehyde gel buffer:

200 mM MOPS (free acid)

50 mM Sodium acetate

10 mM EDTA

pH adjusted to 7.0 with NaOH

1× Formaldehyde running buffer:

100 ml 10× Formaldehyde gel buffer

20 ml 37% formaldehyde (12.3 M)

880 ml RNase-free water

3.14.1 RNA sample preparation

The RNA samples were prepared by adding 1 volume of 5×RNA loading buffer per 4 volumes of RNA sample (for example: 10 µl of 5×RNA loading buffer and 40 µl of RNA). This was incubated for 3-5 min at 65°C, chilled on ice and loaded onto gel.

5x RNA loading buffer:

Saturated bromophenol blue -16 µl

0.5 M EDTA, pH 8.0 - 80 µl

37% formaldehyde (12.3 M) -720 µl

100% glycerol - 2ml

Formamide – 3084 µl

10× Formaldehyde gel buffer - 4ml

RNase-free water to 10 ml

3.14.2 Synthesis of P^{32} labeled DNA hybridization probes by random primer labelling

An appropriate size of a cDNA sequence was PCR amplified using specific forward and reverse primers. The PCR products were gel-purified using Perfectprep Gel cleanup kit (Eppendorf, Germany) and used as a probes for both DNA and RNA gel blot analysis. The protocol outlines the method of Prime-a-gene labeling system (Promega, USA). In an eppendorf tube, 25 ng of the denatured template DNA, 10 µl labeling buffer (5x), 2 µl of a mixture of unlabeled 5mM dNTPs (dGTP, dCTP and dTTP), 2 µl of nuclease-free BSA, 5 µl

of 5mM 3Ci [Q-^{32}P] dATP and 5 units of DNA polymerase I (Klenow) fragment were added and finally the volume of the reaction mixture was made to 50 µl. This mixture was then incubated at room temperature for 1 h. The reaction was terminated by heating at 95-100°C for 5 min, chilled on ice and finally 20 mM EDTA was added and used directly in a hybridization solution.

3.15 Western blotting

Western blotting was performed as described above in cell culture with the equal loading of 50µg protein from control and transgenic plants.

3.16 Immunoflourescence

For immunoflourescence studies, fresh young leaves and fruits at the stage of 10 DPA were collected from WT as well as transgenic plants. Small pieces were fixed with 4% (w/v) paraformaldehyde and 0.1% (v/v) Triton X-100 in PBS (135mM NaCl, 3mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4). After dehydration in graded ethanol series, material was embedded in paraffin and cross sections at 6 µm thickness were cut. They were immunolabelled with rabbit with anti-NPR1 primary antibody (diluted 1:10 in PBS) and secondary anti-rabbit IgG antibody conjugated with TRITC (diluted 1:500). Immunoflourescence detection was carried out using a Leica confocal microscope.

3.17 Scanning Electron Microscope studies

The proximal parts of the hypocotyls were fixed in 2.5% Glutaraldehyde at 4ºC for 4 h. These tissues were removed, washed with double distilled water and again fixed in 1% osmium tetra oxide (OsO_4) for 2 h at 4°C. After a few washes in double distilled water, the specimens were dehydrated in graded Ethanol solutions. Tissues were mounted on specimen stub. Critical point drying was done in Palaron Jumbo Critical Dryer. Gold sputter coating was applied on the samples under reduced pressure in an inert argon gas atmosphere (Agar sputter coater powder). After coating, the tissues were examined under Phillips (15 1B) Scanning Electron Microscope.

3.18 Analysis for abiotic stress tolerance with cotton seedlings

Seeds were collected, totally de-linted and surface-sterilized with 0.1% (v/v) mercuric chloride followed by 4-5 washes with sterile distilled water. They were incubated in sterile tissue culture bottles with wet tissue paper for *in vitro* germination. After 7 d, uniformly germinated seedlings were chosen and transferred in to separate bottles containing tissue paper wet with different treatment solutions and incubated further for a period of 7 d. A total of 20 seedlings from each of the transgenic plants (4, 5, and 11) along with WT were used in different stress treatments. Shoot length, root length and number of lateral roots were measured for each seedling at the end of the stress treatments. The experiments were randomized with different concentrations of NaCl, mannitol and hydrogen peroxide (H_2O_2), and repeated three times independently.

3.18.1 Estimation of relative water content (RWC)

Relative water content of the WT and transgenic seedlings after different stress treatments was determined as described below:

RWC = (Fresh weight [FW] – Dry weight [DW])/ (Turgid weight [TW] – Dry weight [DW]) ×100

Fresh weight [FW] of each seedling was determined just before the initiation of every treatment. The dry [DW] weight was obtained after drying the seedling tissues for 48 h at 72°C immediately after finishing the each treatment. Turgid weight [TW] was determined from the seedlings after incubation with double distilled water for 72 h.

3.18.2 Leaf disc senescence assay

Uniform leaf discs of approximately 1.0 cm diameter were excised from healthy, green and fully expanded fourth nodal leaves of 3 wk old WT and transgenic plants (4-3, 5-7, and 11-1) using a cork borer before the salt stress on whole plants has been initiated. They were floated on 10 ml of each of the respective treatment solutions to mimic salt, osmotic and oxidative stresses.

Simultaneously, leaf discs of both WT and transgenic plants also were mock-treated with distilled H_2O as control. The treatments were carried out in continuous white light of 30 µmol m^{-2} s^{-1} at 27 ± 1°C for 72 h. The different treatments included NaCl (100 mM-400 mM), mannitol (100 mM-400 mM), H_2O_2 (10 mM-40 mM), and PEG (10%-40%)

3.18.3 Chlorophyll estimation

Total chlorophyll was extracted with 80% ethanol from the leaf discs of WT and transgenic plants, after different treatments for 72 h and content was measured spectrophotometrically as described by Arnon (1949).

3.18.4 Lipid peroxidation

Lipid peroxidation was assessed by estimating thiobarbituric acid reactive substances (TBARS) using the method of Heath and Packer (1968). Leaf discs after different stress treatments were collected and frozen in liquid nitrogen for further use. The leaf extract was used to read absorbance at 532 nm and 600 nm. The melandialdehybde (MDA) levels were estimated using the extinction coefficient 155 $mM^{-1}cm^{-1}$.

3.18.5 Stress treatment to WT and transgenic cotton plants

Transgenic T_1 (progeny of 4, 5 and 11) generation and untransformed plants were grown in pots (15 cm/20 cm/15 cm) containing a mixture of soil and vermiculite (1:1) with a 16/8 h (day/night) photoperiod (about 120 mmol $m^{-2}s^{-1}$) at 26/24°C (day/night). Two-month-old plants were exposed to salt stress (20 replicates). Salt stress was conducted by irrigating the plants with 100 mM NaCl for 4 wk. All the transgenic plants were restored to normal growth conditions after their respective treatments. The cotton plants that survived the stress treatments were counted and divided by the total plant number to define the survival rate. Phenotypic differences were observed regularly.

3.18.6 Proline estimation

The proline content in the NaCl (100 mM-400 mM) treated leaf discs of WT and transgenic plants was estimated using the standard procedure described by Bates et al. (1973). The absorbance of the leaf extract was read at 520 nm, using toluene as blank. Proline concentration was measured as μmole g^{-1} FW, using L-proline for standard curve.

3.18.7 Determination of carbohydrate content

Carbohydrate content was estimated from finely ground leaf discs (100mg) after treatment with 300 mM NaCl for 72 h. The samples were extracted with 80% ethanol at 80°C for 30 min followed by re-extraction with 50% ethanol for 15 min. The supernatant collected after centrifugation at 10,000 rpm for 10 min and mixed with an equal volume of chloroform. The aqueous phase was separated, vacuum dried and dissolved in distilled water. This solution was subsequently used for measuring the sucrose, glucose and fructose contents enzymatically by determining the reduction of NADP at 340 nm after the addition of glucose-6-P-dehydrogenase, hexokinase, phosphoglucose-isomerase and invertase (King et al., 1997, Singh et al., 2005). The pellet was used to measure the starch in the form of converted glucose content after incubation at 60°C with α-amylase and amyloglucosidase for 3 h.

3.18.8 Fiber cellulose estimation

WT and transgenic plants of the same age (3 wk) were irrigated daily with 100 mM aqueous NaCl solution after the initiation of flowering for 8 wk. After the treatment, 10 bolls (30 DPA) from each treated plant were hand harvested and 100 mg fiber samples from each boll were used for determining total cellulose content (three replicates in two different experiments). Fibers were treated with 10 ml boiling acetic/nitric reagent to extract all lignin and hemicellulosic material except crystalline cellulose (Updegraff, 1969), which was further washed in ethanol, freeze dried and weighed (Haigler et al., 2007). The cellulose content was expressed as percent of initial weight of the fibers.

3.18.9 Estimation of total peroxidase Peroxidase assay

The peroxidase activity was estimated using Amplex Red reagent (Molecular probes, Leiden, the Netherlands) with a spectroflourometer. Leaf tissue (1gm) was ground in liquid nitrogen, and homogenized in 10 volumes of an extraction buffer (10 mM sodium phosphate and 1% sodium metabisulphite, pH 6.0). The supernatant was collected after centrifugation at 12,000 × g for 30 min at 4°C, and stored at -70°C until used. The assay buffer consisted of 50 mM potassium phosphate buffer (pH-7.4), 2 mM H_2O_2 and 100 µM Amplex red reagent per sample from each plant (Gorecka et al., 2005). Fluorescence emission of the oxidative product of the Amplex red reagent, Resorufin was read at λ_{em}-590nm and λ_{ex}-560nm. The enzyme activity was expressed in terms of µmoles of Resorufin formed $min^{-1}g^{-1}FW$.

3.18.10 Quantification of H_2O_2 levels

Cotyledonary samples (1 g fresh weight) from WT and T_2 transgenic seedlings with and without NaCl treatment were homogenized in 5 ml cold acetone. The homogenate was centrifuged at 1250 g^{-1} and the chlorophyll contents in the supernatant were adsorbed by activated carbon. Then 200µl of supernatant was added to a 1 ml of reaction buffer [0.25 mM $FeSO_4$, 0.25 mM $(NH_4)_2SO_4$, 25 mM H_2SO_4, 1.25 mM xylenol orange, and 1 mM sorbitol] at room temperature for 1 h H_2O_2 levels were quantified at 560 nm absorbance and were calculated as described by Xue et al. (2009) with three individual repetitions of the experiment.

3.18.11 Detection of ROS using confocal microscopy

Ten day old WT and T_2 seedlings germinated on MS medium were incubated in 100mM NaCl for 12 h and ROS accumulation was monitored by treating with 10µM 2', 7'- dichlorodihydroflourescien diacetate (H_2DCFDA, Molecular Probes) for 10 min. A control experiment was performed without NaCl followed by staining with H_2DCFDA to detect the basal levels of ROS in the roots. The fluorescence (λ_{ex} – 488nm and λ_{em} – 530nm) was picturized by Leica

Laser Scanning Confocal Microscope (Leica, TCS SP2 with AOBS, Heidelberg, GmbH, Germany) and the experiment was repeated thrice with n=50 for each plant.

3.18.12 Light microscopic studies of fiber cross sections

Cotton fruit age was determined by tagging the flowering plants when the flower was fully open (anthesis) from NaCl treated WT and transgenic plants. Cross sectional images of 30 DPA fibers from bolls of WT and transgenic plants were observed under light microscope operated in transmission mode. Sections were stained with crystal violet before imaging. More than 300 digitized images from each plant were analyzed to determine the perimeter, secondary wall area, immature fiber content (IFC %) using ImageJ 1.42 software (NIH, USA). Correspondingly Theta [$(4\times\Pi\times \text{area})/\text{perimeter}^2$] and Maturity ratio (Theta/0.577) were calculated to assess the quality of the fibers.

3.19 Statistical analysis

The data were analyzed by Chi-square and analysis of variance (one-way ANOVA). The significance in the mean values were compared by Duncan's multiple range test or Student Newman-Keuls statistical method. In all cases significance was indicated at $P \leq 0.05$.

Chapter 4

Heterologous expression of *Brassica juncea NPR1*, a mammalian IκBα homologue in human glioblastoma cell lines (U373)

4.1 Results

4.1.1 *NPR1* shows structural homology to IκB proteins in mammals

Previous findings showed that all the IκB proteins possess 5-7 ankyrin repeats for binding and inhibiting NF-κB. In this regard, recent finding suggested that NPR1 a plant transcription regulatory protein involved in SAR signal transduction pathway that is homologous to NF-κB in higher organisms and evolutionally conserved disease resistant pathway. *Brassica juncea* NPR1 (BjNPR1) shows striking similarity with the mammalian IκB proteins. Sequence comparison with all the other subunits of IκB revealed a maximum similarity with IκBα subunit which plays a crucial role in the regulation of NF-κB activation. The aminoacid sequence alignment between the NPR1 protein (AAV52267) and mouse (NP_035037), pig (NP_001005150), rat (NP_001099190) and human (NP_065390) IκBα showed significant sequence similarity. In essence, NPR1 possesses important structural domains of IκBα throughout the length of the protein. As displayed in the Fig 4.1, amino acid identities are highlighted in black whereas conserved and semi conserved substitutions are represented by dark and light gray colors. Altogether NPR1 shares 78.3% homology with human IκBα, 78.1% homology with mouse IκBα, 77.7% homology with pig IκBα and 78.1% homology with rat IκBα respectively. The phosphorylated serine residues important in IκBα function are conserved in NPRl within a large contiguous block of conserved sequence from amino acids 44 to 89 along with two N-terminal serines (amino acids 45 and 53 of NPRl), a pair of lysines (amino acids 215 and 216 in NPRl), and an acidic C terminus. In addition, a high extent of homology exists among NPR1 and IκBα sequences in the serine threonine-rich C-terminal region, which is important in basal turnover rate.

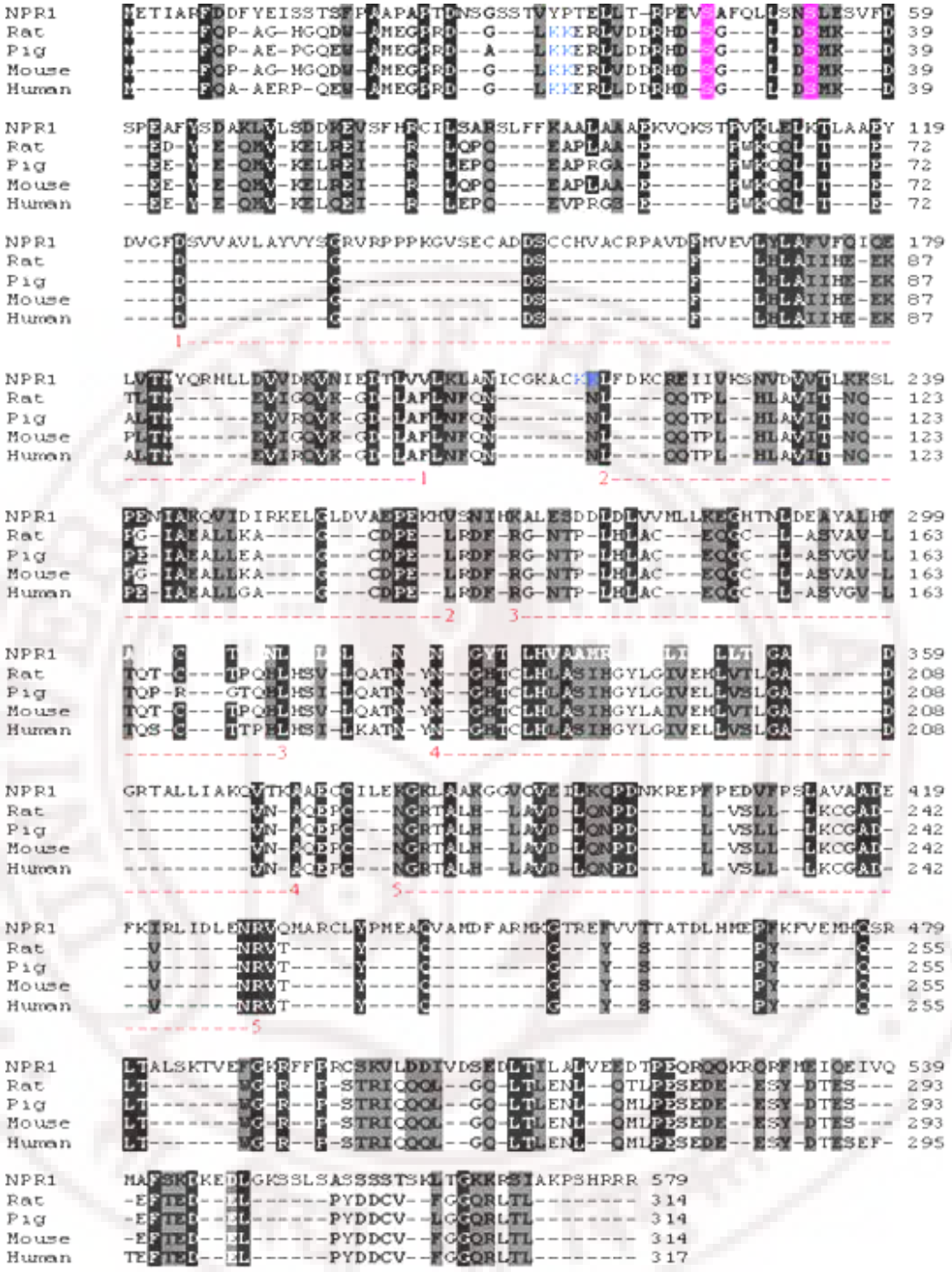


Figure 4.1 Amino acid sequence alignment of NPR1 with mammalian IκBα proteins using ClustalV of lasergene software from DNASTAR. Identical aminoacid sequences were highlighted in black color and conserved and semi-conserved substitutions were indicated with dark gray and light gray colors respectively. Ankyrin repeat positions were identified in IκB sequences by SMART analysis. The five ankyrin repeats in IκBs are indicated by the dashed lines under the sequence in red color. The lysines required for ubiquitination of IκB are represented in blue color where as phosphorylation sites were kept in pink color. Amino acids are numbered relative to the NPRl protein.

4.1.2 Cloning of *Bjnpr1* in pcDNA3.0 vector for stable transfection

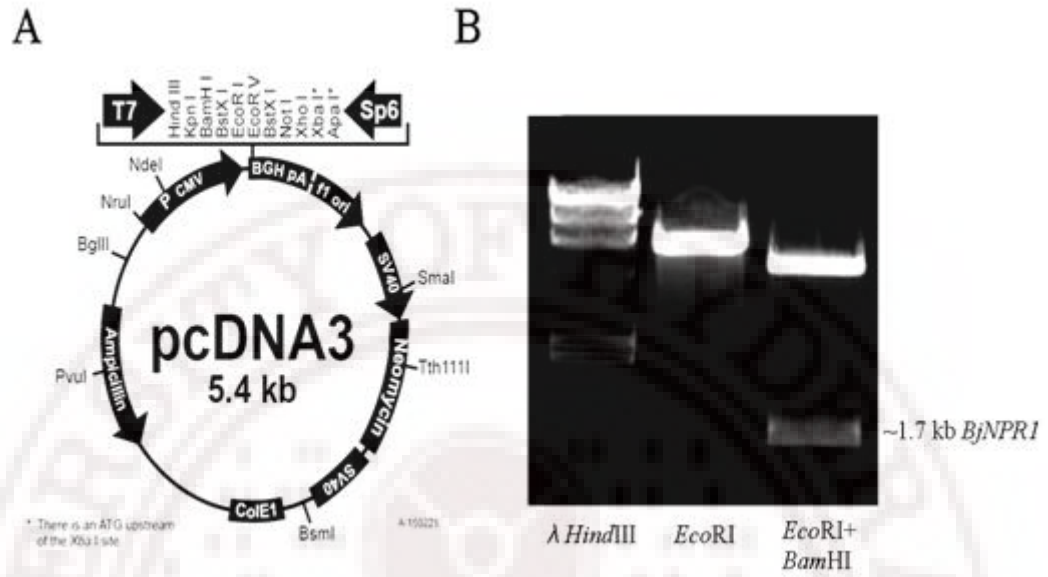


Figure 4.2 A pcDNA vector map. B Cloning of *BjNPR1* in pcDNA. Digestion with *Eco*R1 linearizes the plasmid containing *BjNPR1* and double digestion with *EcoRI* and *Bam*HI releases the 1.7 kb fragment of *BjNPR1*.

Recently *Brassica juncea NPR1* (*BjNPR1*) has been cloned, which is showing striking amino acid sequence similarity with the animal IκBα proteins, regulatory proteins in NF-κB activation. The *Brassica juncea NPR1* was cloned in the MCS of mammalian transfection vector pcDNA3.0 (5.4 kb) (Fig. 4.2A). The entire coding sequence (1740bp) has been amplified using specific primers from *Brassica juncea* using RT-PCR. At 5' end of the sequence, Kozak sequence has been inserted to facilitate the expression in mammalian systems. The recombinant plasmids were screened with PCR showing amplification of NPR1 as well as double digestion with *Eco*R1 and *Bam*HI releases 1.74 kb fragment of *NPR1* (Fig. 4.2B).

4.1.3 Confirmation of NPR1 overexpression in U373 cells.

The sequence analysis showed that NPR1 possessed structural domains important for IκB function. This important finding tempted us to investigate whether this protein acts in a way similar to IκB and overexpression of this

protein may inhibit the tumor cell proliferation, as it is reported that NF-κB is a major signal transducer in tumor cell proliferation. Therefore we have stably transfected the full length cDNA of *NPR1* in human U373 glioblastoma cell lines. Several G418 resistant clones were isolated and developed which showed the stable integration and expression of *NPR1* as confirmed northern blotting (Fig. 4.3A).

Each clone is showing varying levels of NPR1 expression as revealed by northern blotting. Among these, the high expressing clone U4 was chosen for further studies. The expression of NPR1 protein (~65.5 kDa) was confirmed by western blotting (Fig. 4.3B) and immunofluorescence studies of the transfected cell lines using npr1 antibodies (Fig. 4.3C). Importantly, when compared to U373 cells, U373-NPR1 cells showed significant reduced proliferation rates of more than 20% in culture.

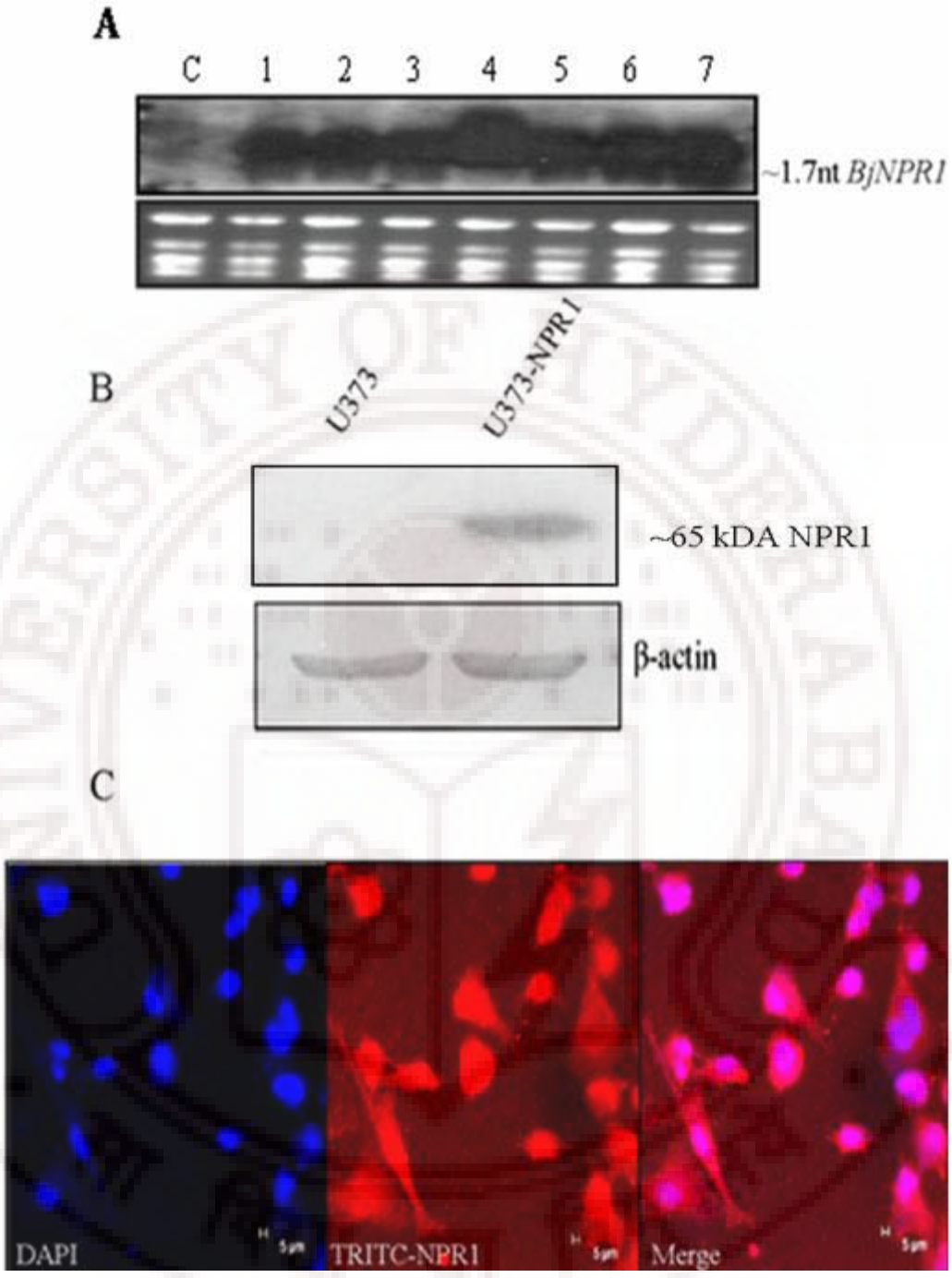


Figure 4.3 Stable expression of BjNPR1 in rat C6 glioma cell lines. C6 cells were stably transfected with pCDNA 3.0-NPR1 plasmid, and transfected cells were selected with 500uM G418. Expression of NPR1 was confirmed by northern blotting (A), western blotting (B) and immunofluroscence (C).

4.1.4 NPR1 binds to NF-κB and inhibits its nuclear translocation.

We found that NPR1 formed a complex with p50 and p65 in the cytosol. Further, we performed western blot analysis for p50 and p65 in cytosol and nuclear fractions of control and transfected cells (Fig. 4.4A). In normal cells, the p50 and p65 protein levels were present in cytosol and in nucleus, but in transfected cells most of the p50 and p65 proteins were confined to cytoplasm and lesser was found in nucleus (Fig. 4.4B,C).

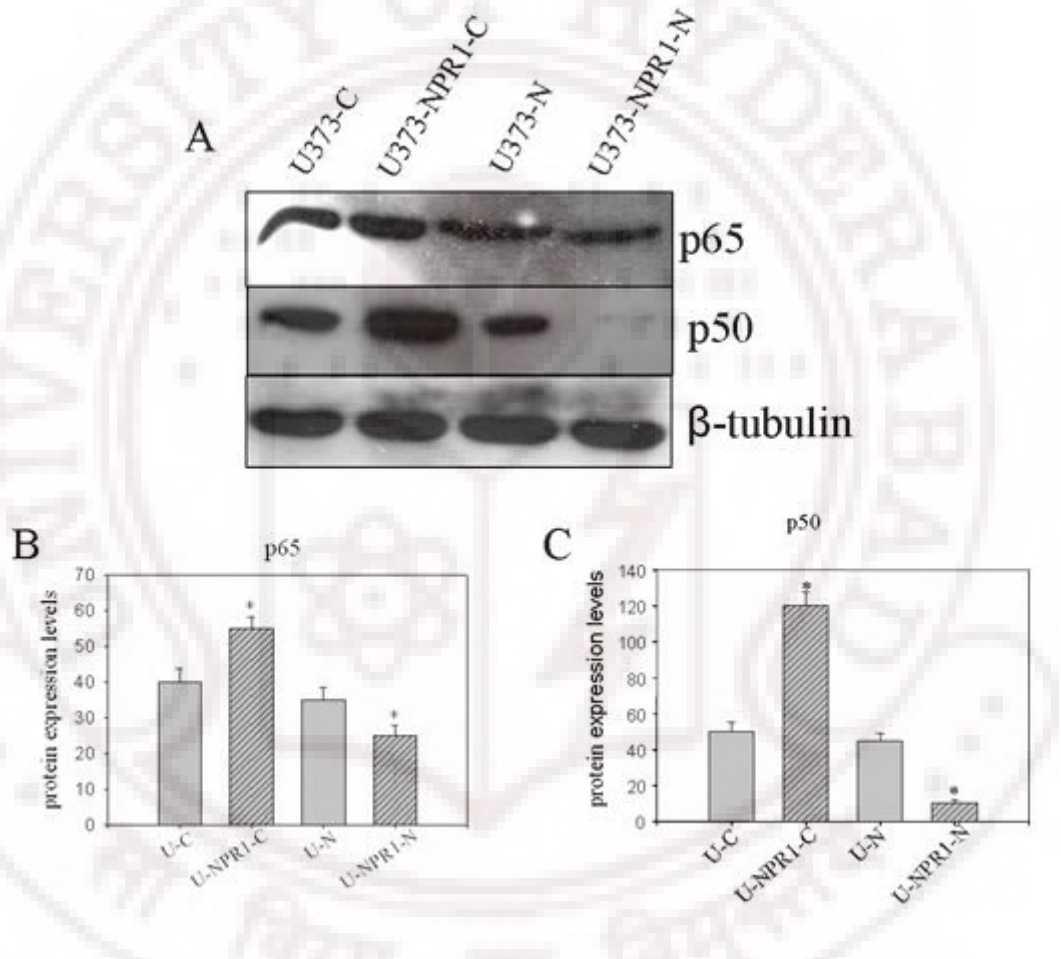


Figure 4.4 NPR1 inhibits NF-kB activation and its target gene expression. A. Effect of NPR1 overexpression on activation and nuclear translocation of NF-kB. NPR1 inhibited the nuclear translocation of p50 and p65. Cytosolic and nuclear fractions were prepared from control (U373) and transfected (U373-NPR1) cells and the p50 and p65 levels were analyzed by western blotting. The blots were representative of three independent experiments. B,C. The band intensities of p50 and p65 were represented as histograms with mean ± SD. The asterisk (*) represents a statistically significant difference (P<0.05) from the control (U373) cells. Amount of labeled protein immunoprecipitated was quantitated (in pixels) by densitometry using NIH Image software and was expressed as percent above background.

Moreover NF-κB (p65) activity showed that NPR1 inhibited the nuclear translocation of p65 in transfected cells compared to normal tumor cells (Fig. 4.5). These findings suggested that overexpressed NPR1 was able to bind to NF-κB and inhibited its nuclear translocation.

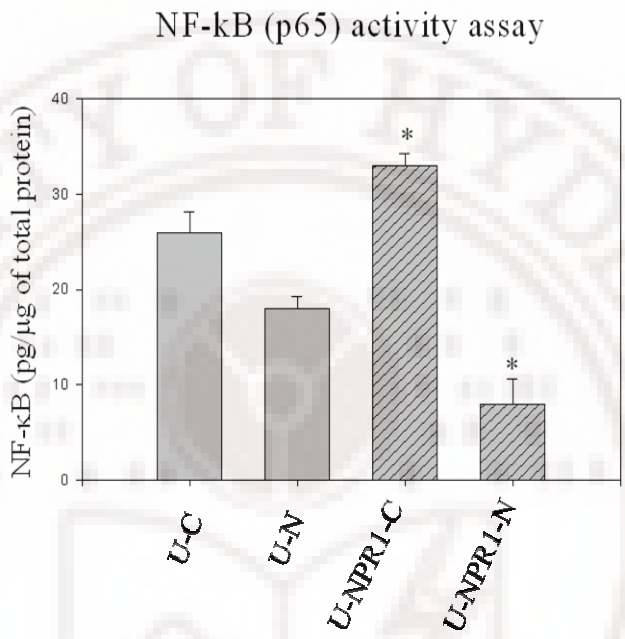


Figure 4.5 NPR1 inhibited the NF-kB activity. Cytosolic and nuclear NF-kB (p65) levels in control (U373) and tranfected (U373-NPR1) were estimated by EIA method as described in materials and methods. Data represents mean ± SD of three independent experiments.

In U373-NPR1 cells NPR1 was colocalized with NF-κB and most of the cells showed colocalization pattern in cytosol. Further we isolated cytosol and nuclear extracts from transfected cells and performed co-immunoprecipitation experiments with cytosolic fraction. Co-immunoprecipitation was observed when immunoprecipitated with NPR1 antibodies and this immunocomplex was probed with anti-p65 and anti-p50 antibodies where IgG was loaded as a negative control (Fig. 4.6A and B). U373 cells transfected with NPR1 were seeded on glass coverslips and fixed in paraformaldehyde: methanol fixative and blocked by incubating in a mouse IgG blocking solution for 1 h. A monoclonal antibody that recognizes active Nf-kB sub unit p65 was applied at a dilution of 1:100 and the slides were incubated at 4°C overnight. Nf-kB p65 immunoreactivity was

detected using a fluorescent secondary antibody and observed under confocal microscope. NPR1 was detected by anti rabbit-NPR1 antibody (red) and p65 was detected by anti mouse p65 antibody (green). Colocalization of NPR1 with p65 was observed in the cytoplasm and nucleus of transfected cells (Fig. 4.6C).

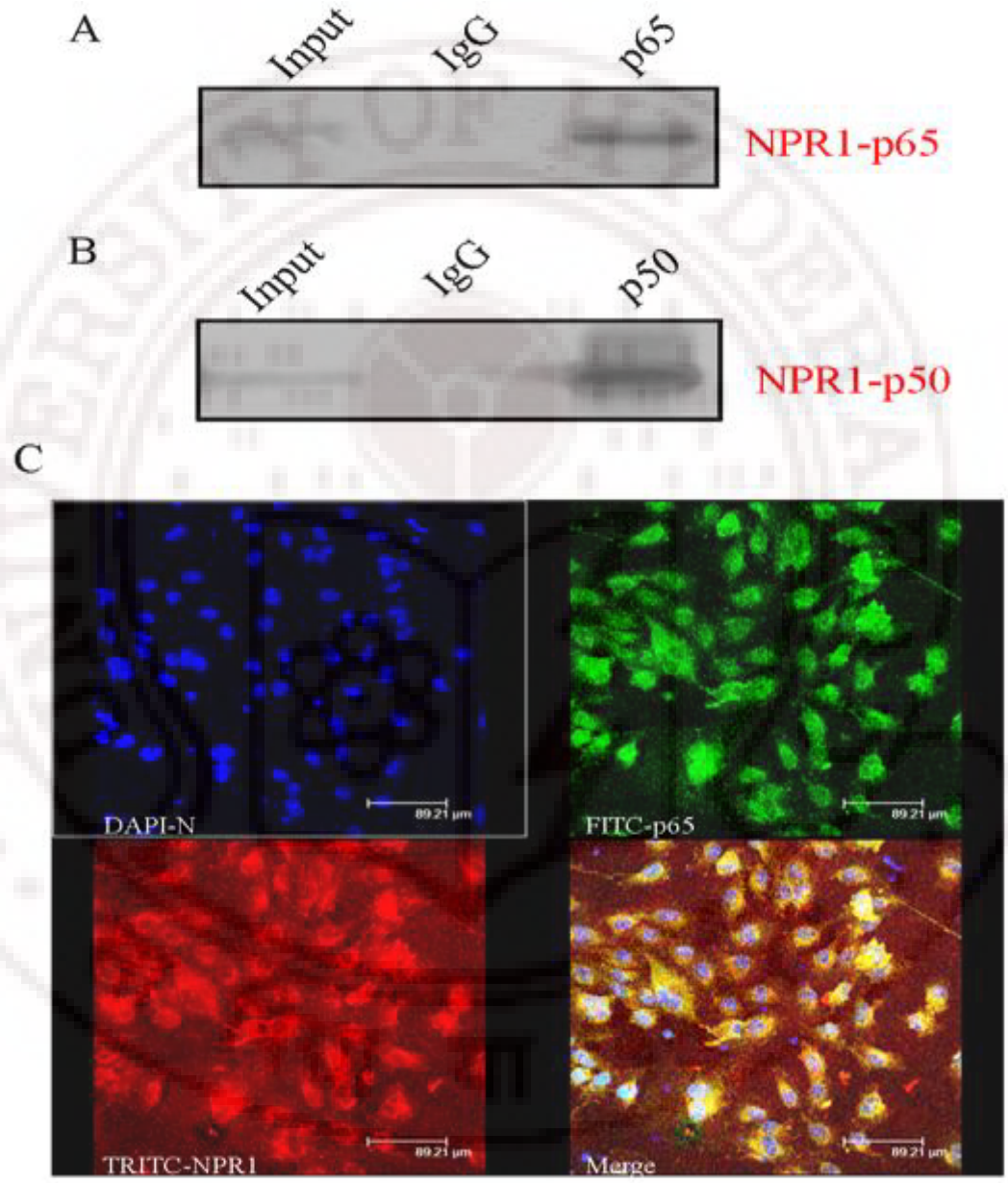


Fig. 4.6 NPR1 interacts with NF-κB. A,B. Cell lysates from U373-NPR1 cells were immunoprecipitated with NPR1 antibodies and the immune complex was probed with anti-p50 and anti-p65 antibodies. IgG serve as negative control.C. U373-NPR1 cells were fixed with paraformaldehyde and incubated with p65 and NPR1 primary antibodies overnight at 4°C and anti-FITC and anti-TRITC secondary antibodies for 1-2 h at room temperature. Fluorescence was captured under Leica confocal microscope.

4.1.5 Expression levels of NF-κB target genes iNOS, Cox-2, c-Myc, cyclin D1 and PKC

iNOS, Cox-2, PKC, c-Myc and cyclin D1 play critical roles in inflammation, cell cycle progression and cell proliferation and their expression is controlled by NF-κB. In the present study, we found that NPR1 overexpression significantly reduces the protein levels of iNOS, Cox-2, c-Myc, cyclin D1 (Fig. 4.7) and PKC in transfected cells than in control cells. This clearly suggests that NPR1 inhibited NF-κB based transcription and finally reduced the proteins involved in tumor cell proliferation.

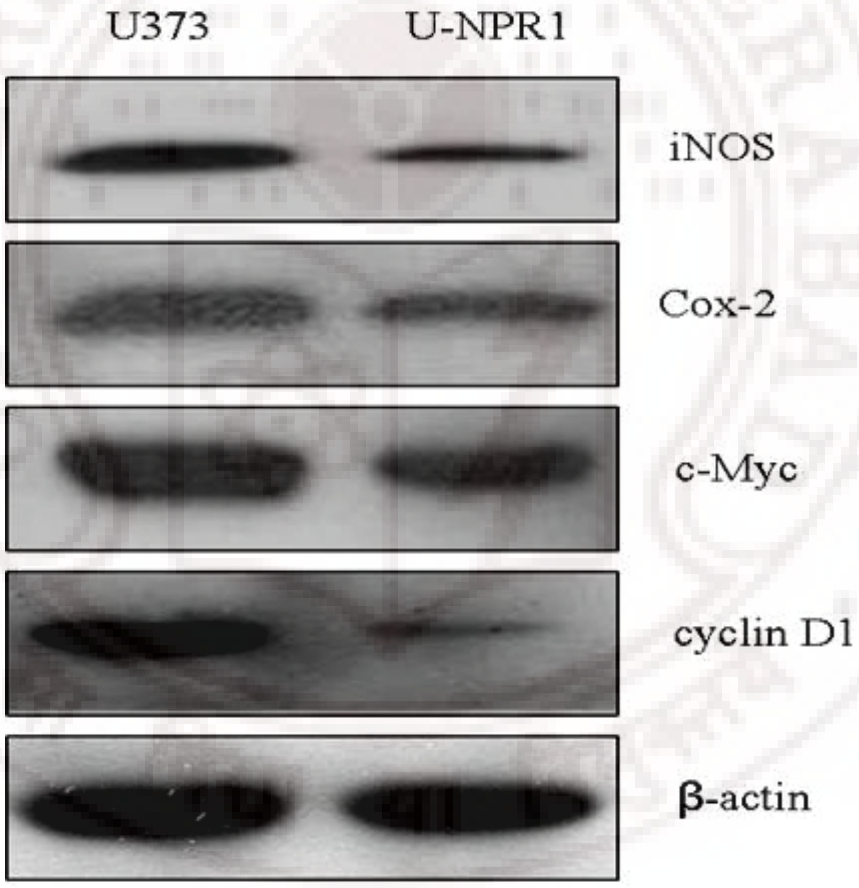


Figure 4.7 Effect of NPR1 overexpression on NF-kB target genes. Whole cell lysates were prepared from control (U373) and transfected cells (U373-NPR1) and analyze the protein levels of iNOS, Cox-2, c-Myc and cyclin D1.

4.1.6 NPR1 overexpression attenuated the MAP kinases and increased the Caspase 3 and Caspase 8 activities

Mitogen activated proteins play critical roles in cell proliferation, differentiation and they control cellular responses to various growth factors and stress. In addition, they play a critical role in the modulation of NF-κB activity. The MTT assay revealed the reduced proliferation rate in the transfectants, which is in correlation with the inhibition of NF-κB levels (Fig. 4.8A).

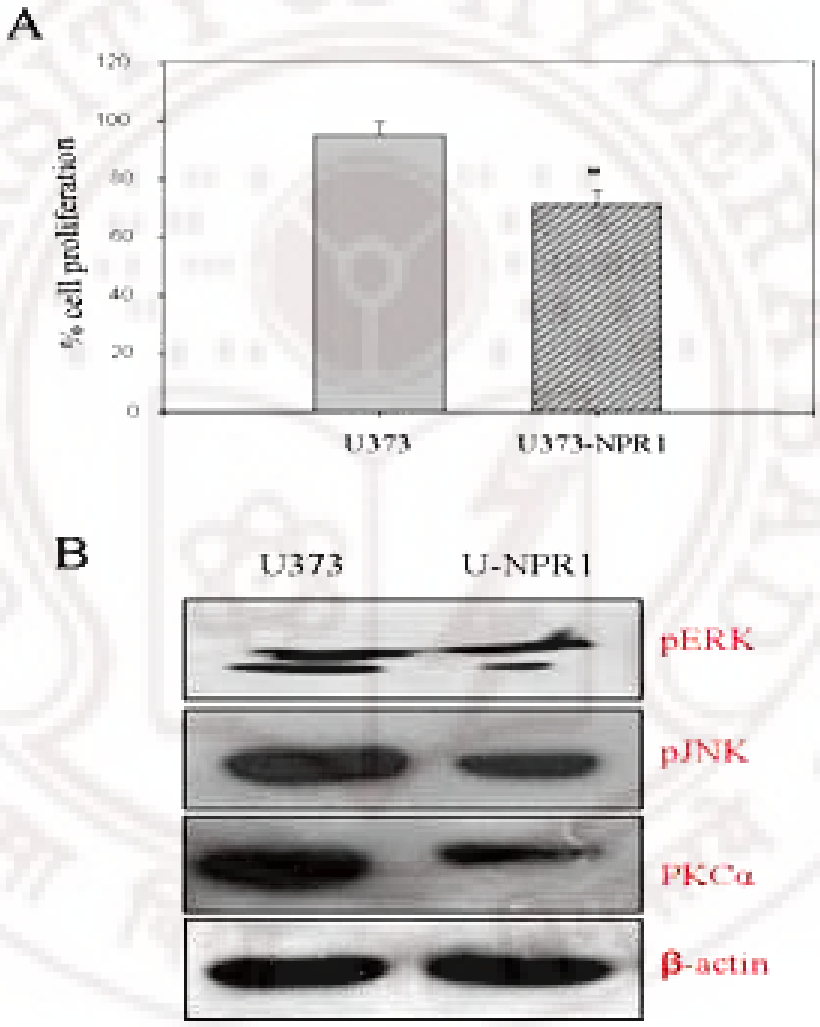


Fig. 4.8 NPR1 overexpression inhibits the proliferation of U373 cells. A. Effect of NPR1 overexpression on proliferation of U373 cells. Both U373 and U373-NPR1 cells were seeded in equal number in 96 well plates and after 48 hours subjected to MTT assay. Data is representative of three independent experiments. Asterisk (*) indicates significant difference compared to control (U373) cells (P<0.05). B. Effect of NPR1 overexpression on MAP kinases and PKC. Whole cell lysates were prepared from U373 and U373-NPR1 cells and subjected to western blot analysis of PKCα, pERK, pJNK. The blots were representatives of at least three independent experiments.

To investigate the molecular mechanism of NF-κB inhibition by NPR1 in U373 cells, we studied the inhibition of the phosphorylation of pERK, pJNK and p38. Western blot analysis demonstrated the levels of phosphorylated ERK, JNK were significantly reduced in transfected cells compared to control cells. While phospho p38 levels were unaffected in transfected cells (Fig. 4.8 B).

Caspases are the critical mediators of apoptosis in all organisms. To study the effect of *NPR1* overexpression on apoptosis we studied the caspase 3 and 8 activities in control and transfected cells. Compared to control cells, transfected cells bear more Caspase-3 and Caspase-8 activity (Fig. 4.9). Moreover, we also checked the levels of various proteins involved in cell death like bcl-2, bax, bid, bim. But we found that NPR1 does not have any effect on expression of these proteins.

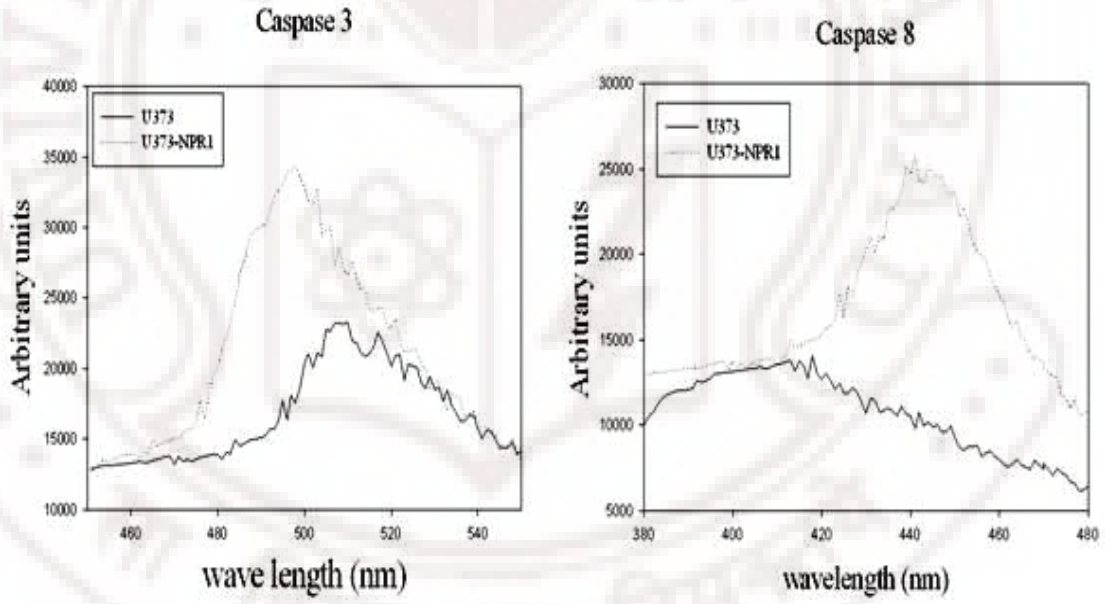


Figure 4.9 Effect of NPR1 overexpression on Caspase-3 and Caspase-8 activities. Caspase-3 and Caspase-8 activities were estimated from control and transfected cells lysates.

4.2 Discussion

Activation NF-κB and its translocation to nucleus plays an important role in the activation of various NF-κB-dependent genes participating in cell proliferation, tumorigenesis, apoptosis etc (Karin et al., 2002). With the exception of mature B cells, virtually all normal cells have an inactive form of NF-κB in the cytoplasm sequestered by IκBα (Zhou et al., 2003). The IκB protein functions in signal transduction by binding to the transcription factor NF-κB and prevents it from entering the nucleus (Baeuerle and Baltimore, 1996; Baldwin, 1996). When the signal transduction pathway is activated, IκBα is phosphorylated at two serine residues (amino acids 32 and 36 of mouse IκBα). Approaches to suppress NF-κB activation in malignant cells have been considered a potential treatment for cancer. NF-κB targets many genes that facilitate tumor progression, inflammation, cellular immortality, cell survival, angiogenesis, proliferation, tumour promotion, and metastasis. The expression of target genes which help in the tumour promotion, Cox2, iNOS, c-myc, cyclinD1 were studied in detail in the control and transfected cells.

Structurally, IκBα is composed of an N-terminus, a central domain containing five ankyrin repeats, and a highly acidic C-terminus. The ankyrin repeats are responsible for the binding of IκBα to the p65 and p50 subunits of NF-κB protein, whereas the C-terminus physically interacts with p53 (Chang, 2002). In response to apoptotic stimuli such as Dox, IKK kinase is activated, which in turn phosphorylates IκBα at N-terminal serines 32 and 36. The phosphorylated serine residues important in IκBα function are conserved in NPRl within a large contiguous block of conserved sequence from amino acids 35 to 84. The phosphorylation programs ubiquitination at a double lysine (amino acids 21 and 22 of human IκBα). In contrast to IκBα in which the double lysine is located -15 amino acids toward the N terminus of the protein, the lysine pair is located toward the C-terminal end in NPR1 (amino acids 214 and 215 of NPR1). Ubiquitin modifications of proteins can occur in multiple forms (Haglund and Dikic 2005). In the simplest way, a single ubiquitin molecule is attached to a single lysine residue in a substrate, which is defined as mono-ubiquitination. NF-κB has been

considered a target for cancer treatment (Garg and Aggarwal, 2002). Phosphorylated IκBα dissociates from the NF-κB/IκBα complex, and is degraded by the proteasome/ubiquitin pathway, permitting activated NF-κB to translocate to the nucleus. After ubiquitination, the NF-κB/IκBα complex is routed through the proteosome, where IκBα is degraded and NF-κB is released to the nucleus. In addition, a high degree of homology exists between NPR1 and IκBα in the serine threonine-rich C-terminal region, which has been shown to be important in basal turnover rate (Sun et al., 1996). Based on the analysis of structural homology and the presence of elements known to be important for IκBα function, NPRl may function like the IκBα sub class of proteins.

NF-κB is activated by a variety of signals through mechanisms that result in phosphorylation and degradation of the inhibitory IκBα protein. Methods to stabilize IκBα, such as the use of protease inhibitors or transfection of the dominant-negative mutant IκBα, have been employed to inhibit NF-κB activation in order to sensitize cancer cells to therapy-induced apoptosis. A previous study with the overexpression of IκBm (dominant-negative mutant), which is not susceptible to phosphorylation has been proved to downregulate the NF-κB levels in cancer cells (Zhou et al., 2003). The similarity of the plant transcription regulator with mammalian IκB recommends the future exploitation of this protein in inhibiting the NF-κB activation, which is an important target in cancer therapy. Downregulation of NF-κB by transfection of IκBα homologue NPR1 may directly affect the expression levels of NF-κB regulated genes such as CoX2, iNOS, CyclinD1 etc.

4.3 Summary

We have successfully developed the clones of transfected human glioblastoma cell lines which stably expressed the *Brassica juncea NPR1*, a mammalian IκBα homologue. In summary, our studies demonstrated that the plant NPR1 protein was homologous to mammalian IκBα and functions like IκB by inhibiting NF-κB translocation directly and reduced the levels of various proteins that control tumor cell proliferation. Heterologous expression of NPR1 significantly reduced constitutive NF-κB activity in human gliobalstoma cell lines U373, resulting in decreased cell growth.

The physical interaction of NPR1 with NF-κB was assessed by *in vitro* coimmunoprecipitation assay. These results suggested the NPR1 expressed in the cell lines could coimmunoprecipitate with antisera of both the p65 and p50 and form an immunocomplex . When western blotting was done with the NPR1 pulled down sample and developed with p65 and p50, there was a corresponding band formation at 65 KDa and 50 KDa respectively. The reverse experiment with NF-κB pull down and detecting the immunocomplex with NPR1 showed the 65.5 KDa band formation. Taken together the above findings showed the interaction of NPR1 with subunits of NF-κB, which further provides a strong evidence of the sequence as well as functional similarity of NPR1 with IκB proteins. Our studies may promise that inhibition of NF-κB by NPR1 may have a therapeutic significance in various inflammatory diseases and malignancies.

Chapter 5

Optimization of regeneration and development of transgenic cotton plants expressing *Brassica juncea NPR1*

5 Results

Germination frequencies varied among the three varieties with 94%, 88%, and 72% in Bharani, Durga, and JKCH-99 respectively. The germination percentage was higher, if seeds were incubated in culture bottles containing filter papers moistened with liquid half-strength MS solution when compared to germination on MS agar medium that resulted in lower frequency.

5.1 Standardization of regeneration in cotton

5.1.1 Effect of age and preparation of explant

Among different explants tried for *de novo* regeneration, hypocotyls gave a better response. Age of the seedlings from which explant is prepared was found to influence the regeneration frequency considerably. In the beginning, 2 - 20 d old seedlings were tested and among them explants from 8 - 10 d old seedlings were chosen for further analysis because of their best morphogenic ability. When hypocotyl sections between 0.5 - 1 cm with apical bud strictly removed and placed horizontally on the medium, both ends of the hypocotyls bulged and proximal ends differentiated shoot buds by the end of second week of culture. These microscopic buds differentiated into individual shoots at the proximal region where as it is a simple cell division and minor callus formation at the distal end of the explant.

5.1.2 Effect of culture medium

Among all the vitamin combinations in MS basal medium along with 2.0 mg l^{-1} TDZ and 0.05 mgl^{-1} NAA tested, MS salts with NN vitamins was most successful in producing highest regeneration response at the end of 5 wk of culture (76%), which prompted us to select this medium for further studies (Fig. 1). The order of vitamin combinations influencing the regeneration was NN, Mc Cown, MS, B_5, LS and the least response was observed in LS vitamin supplementation in the medium.

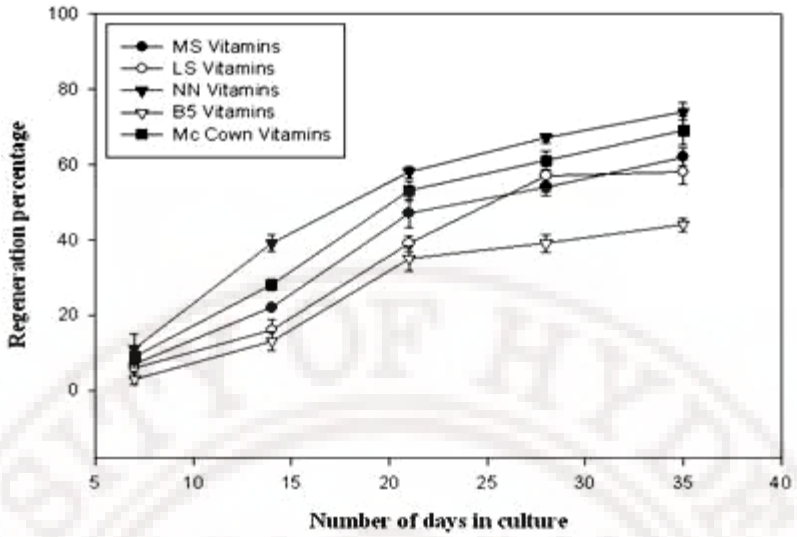


Figure 5.1 Regeneration of hypocotyl explants using different vitamin (according to media MS, LS, NN, B5 and Mc Cown) added to MS basal medium comprising 2 mg l^{-1} TDZ, 0.05 mg l^{-1} NAA, 5 mg l^{-1} $AgNO_3$ and 1 g l^{-1} Activated charcoal as constant components. Regeneration percentage has been calculated as the percentage of the responding explants out of the total explants cultured. Vertical bars represent SE of the means (n = 20).

5.1.3 Effect of various growth regulators

Randomized experiments with TDZ (0.5-3.0 mg l^{-1}), BAP and Kinetin (1-5 mg l^{-1} each) and an auxin source of NAA (0.01-0.1 mg l^{-1}) resulted in maximum regeneration response. Besides attaining maximum number of responding explants, the highest number of shoot primordia (19.2 per responding explant) (Table. 1) and a maximum number of shoots yielded per explant (10.6) with high regeneration percentage up to 76% has been recorded on 2.0 mg l^{-1} TDZ, 0.05 mg l^{-1} NAA combination (Table. 2, Fig. 2). This was followed by the combination of BAP and KIN (12.3 shoot buds/responding explant). Lower levels of TDZ induced the formation of more number of visible shoot primordia (Fig. 3a, b) and a maximum of them were converted into individual shoots whereas numerous shoot primordia appeared at higher levels of TDZ. However, shoot primordia on higher TDZ concentrations developed into hyperhydrated shoots associated with fasciated water soaked callus subsequently. Hence, explants with shoot primordia from TDZ medium were transferred to 2.0 mg l^{-1} BAP, 0.1 mg l^{-1} NAA after two subcultures to obtain optimal growth of shoot buds into individual shoots. Shoots

cultured on TDZ medium also have been observed to show delayed rooting response by more than eight weeks with 1-2 roots per shoot only.

Table 5.1 Effect of growth regulators on regeneration in hypocotyl explants of cotton. Data represents means three replicates ± SE from 4 wk old cultures. Means denoted by different letters differ significantly at $P \leq 0.05$.

GR [mg l^{-1}]				Bharani		Durga		JKCH-99	
TDZ	BAP	KIN	NAA	No. of explants forming shoot primordial	No. of shoot primordial [explant^{-1}]	No. of explants forming shoot primordial	No. of shoot primordial [explant^{-1}]	No. of explants forming shoot primordia	No. of shoot primordia [explant^{-1}]
0.5	-	-	0.01	3.7 ± 0.0kl	5.7 ± 0.8	3.3 ± 1.2	3.9 ± 0.1	3.8 ± 0.2	3.0 ± 1.1
1.0	-	-	0.01	5.6 ±.0.6jk	9.8 ± 1.9	4.6 ± 2.0	6.9 ± 2.1	4.0 ± 0.4	4.8 ± 2.3
1.5	-	-	0.01	11.8 ± 0.6e	12.5 ± 0.5	11.1 ± 2.2	8.8 ± 1.7	9.2 ± 0.1	8.1 ± 2.1
2.0	-	-	0.01	14.2 ± 0.8ab	16.9 ± 0.1	11.6 ± 2.0	12.1 ± 0.0	10.9 ± 0.0	9.7 ± 0.6
2.5	-	-	0.01	10.8 ± 1.7de	10.0 ± 0.8	9.3 ± 1.6	9.6 ± 0.5	9.1 ± 0.3	5.8 ± 0.2
3.0	-	-	0.01	5.7 ± 0.1ij	7.8 ± 1.2	6.2 ± 0.2	4.2 ± 0.8	5.0 ± 1.6	3.8 ± 0.0
0.5	-	-	0.05	4.6 ± 1.0k	7.1 ± 1.4	4.5 ± 0.0	5.6 ± 1.8	4.0 ± 2.0	3.2 ± 2.1
1.0	-	-	0.05	8.4 ± 1.2g	12.5 ± 0.1	8.3 ± 0.2	8.8 ± 0.1	5.3 ± 1.1	5.6 ± 1.2
1.5	-	-	0.05	11.6 ± 0.3cd	14.8 ± 0.4	11.3 ± 0.9	10.4 ± 0.8	9.1 ± 1.8	8.8 ± 0.6
2.0	-	-	0.05	15.5 ± 0.4a	19.2 ± 0.7	12.2 ± 1.3	14.7 ± 0.4	12.0 ± 1.0	9.8 ± 1.9
2.5	-	-	0.05	13.7 ± 0.5bc	13.0 ± 0.2	11.9 ± 1.1	10.2 ± 0.6	9.2 ± 0.2	6.7 ± 2.0
3.0	-	-	0.05	8.0 ± 0.1gh	9.6 ± 0.5	7.8 ± 1.9	6.8 ± 1.3	5.4 ± 0.7	4.9 ± 1.8
-	1.0	-	0.1	2.8 ± 0.8lm	3.2 ± 0.2	2.8 ± 1.0	2.9 ± 1.9	2.0 ± 0.1	2.4 ± 0.4
-	2.0	-	0.1	5.2 ± 1.0k	6.0 ± 1.1	4.5 ± 1.4	5.1 ± 1.2	3.4 ± 1.2	4.6 ± 0.5
-	3.0	-	0.1	8.7 ± 0.7fg	9.5 ± 0.4	8.1 ± 0.2	8.2 ± 0.4	6.9 ± 1.6	7.7 ± 0.8
-	4.0	-	0.1	7.4 ± 1.2ghi	8.0 ± 0.2	6.8 ± 0.8	6.4 ± 0.9	6.3 ± 0.1	5.4 ± 0.1
-	5.0	-	0.1	4.6 ± 2.2k	5.2 ± 0.3	3.8 ± 1.7	4.7 ± 0.1	3.5 ± 2.0	3.2 ± 0.0
-	-	1.0	0.1	0.0 n	-	0.0	-	0.0	-
-	-	2.0	0.1	1.9 ± 0.7m	3.8 ± 0.6	1.8 ± 0.2	2.6 ± 0.8	0.0	-
-	-	3.0	0.1	2.6 ± 1.9m	6.4 ± 1.3	2.6 ± 0.8	5.7 ± 1.0	2.2 ± 0.8	5.3 ± 0.4
-	-	4.0	0.1	1.7 ± 0.4m	4.3 ± 1.4	1.7 ± 1.4	3.4 ± 0.2	1.6 ± 1.8	3.0 ± 0.4
-	-	5.0	0.1	1.2 ± 0.4m	1.3 ± 0.9	1.2 ± 1.8	2.2 ± 0.8	1.3 ± 0.4	1.8 ± 1.0
-	0.5	0.5	-	4.0 ± 1.8kl	3.2 ± 0.2	3.5 ± 0.8	2.4 ± 0.5	2.8 ± 0.7	2.0 ± 2.2
-	1.0	1.0	-	4.9 ± 0.1k	5.7 ± 0.3	3.8 ± 1.5	5.1 ± 0.9	3.7 ± 0.4	4.4 ± 0.9
-	1.5	1.5	-	7.9 ± 0.5fg	9.8 ± 0.6	7.5 ± 1.4	9.6 ± 1.0	7.6 ± 0.1	8.8 ± 1.3
-	2.0	2.0	-	12.3 ± 0.8bc	14.5 ± 1.2	11.8 ± 0.0	12.6 ± 0.9	9.4 ± 1.6	10.8 ± 0.8
-	2.5	2.5	-	9.4 ± 0.8ef	11.4 ± 1.3	8.7 ± 1.8	9.9 ± 2.0	8.6 ± 0.4	7.1 ± 1.1

-	3.0	3.0	-	5.8 ± 1.4k	7.3 ± 1.0	3.9 ± 0.3	7.2 ± 0.4	3.6 ± 1.5	6.5 ± 1.2
0.5	0.5	0.5	-	3.0 ± 0.2lm	4.2 ± 0.7	2.3 ± 0.8	2.9 ± 0.1	2.2 ± 1.2	2.0 ± 1.2
1.0	1.0	1.0	-	4.8 ± 1.6k	7.7 ± 1.3	4.7 ± 0.8	6.5 ± 0.7	4.0 ± 1.6	6.1 ± 0.3
1.5	1.5	1.5	-	9.4 ± 0.9fg	11.9 ± 1.4	8.4 ± 1.3	10.3 ± 0.5	5.5 ±0.2	8.6 ± 0.2
2.0	2.0	2.0	-	7.1 ± 1.1hij	9.6 ± 0.6	6.4 ± 1.7	9.2 ± 1.1	4.2 ± 1.5	7.0 ± 1.8
2.5	2.5	2.5	-	6.6 ± 0.9k	5.4 ± 0.9	3.8 ± 0.1	3.6 ± 0.8	3.1 ± 1.0	3.6 ± 2.0
3.0	3.0	3.0	-	2.4 ± 0.4m	3.9 ± 0.8	2.1 ± 1.2	3.2 ± 1.8	1.8 ± 1.4	2.4 ± 0.7

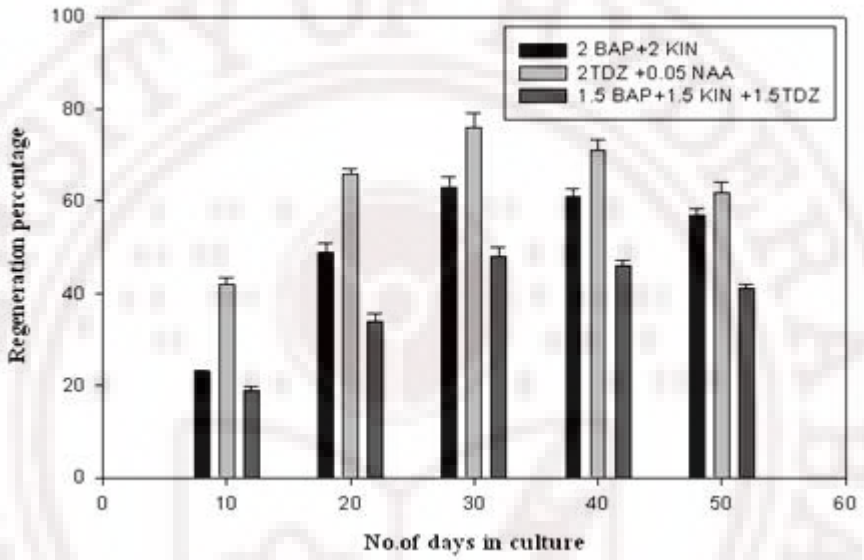


Fig. 5.2 Regeneration of hypocotyl sections in MS basal medium augmented with NN vitamins, 5 mg l^{-1} $AgNO_3$ and 1 g l^{-1} AC with different growth regulator combinations. *Vertical bars* represent SE of the means; $n = 20$.

The synergistic effect of BAP and KIN was found to be superior in enhancing the number of responding explants in comparison to their individual effect. The optimum level of both cytokinins for the synergistic effect was 2.0 mg l^{-1} each. Increased concentration of BAP and KIN also resulted in hyper-elongated shoots with very long internodes, small chlorotic leaves. The combined effect of TDZ, BAP and KIN also initially yielded considerable number of shoot buds but later associated with high amounts of callus suppressing their conversion into individual shoots (Table 2). Shoot elongation occurred on EM viz., 1.0 mg l^{-1} BAP and 2.0 mg l^{-1} GA_3 (Fig. 3c).

Table 5.2 Number of regenerated shoots from responding explant and the shoot regeneration response (%). Means were obtained from three replicates ± SE. Data were scored after 4 weeks of culture.

TDZ [mg l^{-1}]	BAP [mg l^{-1}]	KIN [mg l^{-1}]	NAA [mg l^{-1}]	No. of shoots [responding explant^{-1}]	Regeneration frequency [%]
0.5			0.05	4.5 ± 0.5	59.0
1.0			0.05	7.2 ± 1.4	65.2
1.5			0.05	10.1 ± 0.2	73.3
2.0			0.05	10.6 ± 0.3	76.7
2.5			0.05	9.2 ± 1.0	69.5
3.0			0.05	6.0 ± 1.2	66.0
	1.0		0.1	2.2 ± 1.1	28.0
	2.0		0.1	4.1 ± 0.3	40.0
	3.0		0.1	5.6 ± 0.5	55.1
	4.0		0.1	5.0 ± 1.0	53.0
	5.0		0.1	3.0 ± 0.2	42.4
		1.0	0.1	0.0 ± 0.0	0.0
		2.0	0.1	1.8 ± 0.2	21.6
		3.0	0.1	3.4 ± 1.6	32.8
		4.0	0.1	2.8 ± 0.8	26.5
		5.0	0.1	2.2 ± 0.8	25.4
	0.5	0.5		3.0 ± 1.6	44.0
	1.0	1.0		6.8 ± 2.0	50.5
	1.5	1.5		8.2 ± 0.8	62.4
	2.0	2.0		8.8 ± 0.6	70.5
	2.5	2.5		7.5 ± 1.0	68.0
	3.0	3.0		5.2 ± 1.1	55.5
0.5	0.5	0.5		3.0 ± 0.5	28.5
1.0	1.0	1.0		3.2 ± 0.5	41.0
1.5	1.5	1.5		6.2 ± 0.1	58.0
2.0	2.0	2.0		5.8 ± 1.0	48.8
2.5	2.5	2.5		3.6 ± 0.8	39.0
3.0	3.0	3.0		1.2 ± 1.4	16.5

Figure 5.3 Plant regeneration by organogenesis from hypocotyl segments in cotton (*Gossypium hirsutum* L.). A - Hypocotyl explants from 8 to 10-d-old seedlings on SIM; B - Shoot initiation from proximal ends of hypocotyls at the end of 3 weeks on SIM; C - Multiple shoot production and elongation on EM; D - Isolated and elongated shoot with developing roots when cultured on RIM; E - Well-rooted plant transferred to greenhouse; F - Regenerated plants showing flowers and bolls.

On the different media tried for rooting, highest rooting percentage (86%) was obtained on medium with 1.0 mgl^{-1} IBA (Table. 3, Fig. 3d). Though higher levels up to 10.0 mg l^{-1} IBA induced rooting on shoots, it was associated with callus at the cut end of shoots. The rooting percentage of shoots on RIM with

NAA was (75%) but with less number of roots per shoot than IBA. IAA was a very poor root inducer in cotton when compared to the other two auxins. The survival rate of IAA rooted plantlets also appeared to be less because of the callus formation.

Table 5.3 Root induction in *in vitro* regenerated shoots of cotton by different auxin concentrations. Data represents means from three replicates ± SE. Means denoted by different letters differ significantly at $P = 0.05$.

		Bharani			Durga		
GRs [mg l^{-1}]		No. of shoots responding	No. of roots [responding $shoot^{-1}$]	Rooting [%]	No. of shoots Responding	No. of roots [responding $shoot^{-1}$]	Rooting [%]
IBA	0.01	20.2 ± 2.2cd	3.1 ± 0.9	70	18.9 ± 1.8	2.2 ± 1.2	60
	0.1	22.9 ± 3.0ab	5.0 ± 0.5	83	22.5 ± 2.4	4.4 ± 0.8	73
	1.0	26.0 ± 3.2a	6.4 ± 0.6	86	24.2 ± 3.0	5.5 ± 0.2	80
NAA	0.01	16.9 ± 3.1e	2.8 ± 1.2	53	13.7 ±2.6	1.9 ± 1.0	43
	0.1	19.3 ± 1.8d	4.4 ± 1.4	63	17.0 ± 2.4	3.4 ± 0.4	56
	1.0	23.7 ± 1.8bc	5.3 ± 0.8	75	20.1 ± 1.6	4.3 ± 0.7	66
IAA	0.01	9.5 ± 1.6g	1.2 ± 0.2	30	6.1 ± 2.0	2.4 ± 1.5	20
	0.1	11.2 ± 2.4f	3.8 ± 0.6	36	10.2 ± 1.2	3.1 ± 0.9	33
	1.0	15.8 ± 2.2e	4.2 ± 0.8	50	14.3 ± 2.2	3.8 ± 1.2	46

5.1.4 Role of activated charcoal

Activated charcoal was also helpful in the maximum recovery of shoot primordia into elongated shoots with good internodal length. Leaves were large in size and dark green in color. Activated charcoal in combination with silver nitrate greatly enhanced regeneration capability and shoot yield with almost similar response in all the three varieties. It was observed to have a promotive effect on the root initiation and elongation may be due to the immediate adsorption of phenolics at the cut end. Complete plantlets with root system having well developed lateral roots survived upon transfer to pots in the green house and

appeared morphologically normal (Fig. 5.3e, f).

5.1.5 Role of Silver nitrate

Silver nitrate was shown to have a marked influence on the growth and differentiation of hypocotyl tissues *in vitro.* An optimum level of response in increasing multiple shoots (Fig. 5.4) was noticed at 3-5 mg l^{-1}. We observed that the phenomenon of hyperhydricity has been decreased by silver nitrate and the quality of shoot primordia as well as elongated shoots has improved when compared to shoot regeneration on media without silver nitrate The effect of silver nitrate was observed to be genotype dependent and it varied in three varieties considerably (data not shown). Increase in concentration of silver nitrate beyond 5.0 mg l^{-1} disturbed the organization of shoot primordia leading to the formation of friable callus. Visual observations indicated that the shoots appeared healthy and well elongated with inclusion of silver nitrate in the culture medium. Though the number of shoot primordia has not increased significantly by silver nitrate, the conversion rate into individual shoots has been improved significantly.

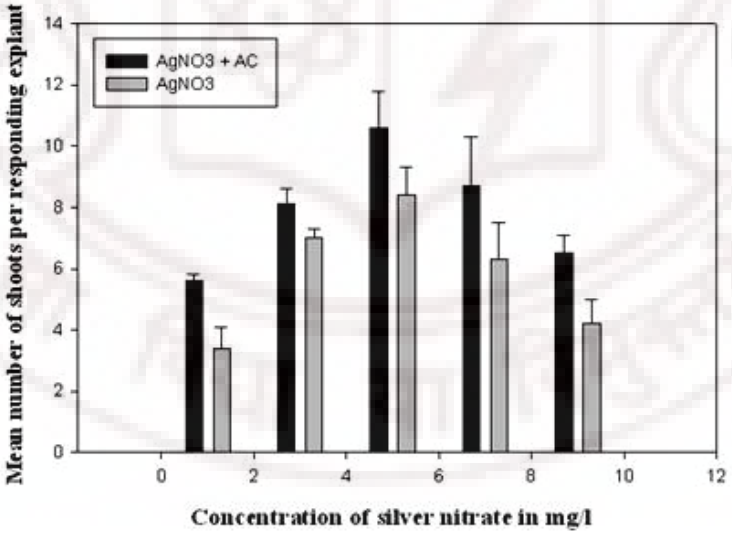


Figure 5.4 Effect of different concentrations of $AgNO_3$ with or without AC on multiple shoots induction on TDZ medium. The vertical bars represent SE of the means.

Scanning electron micrographs of control explants grown on MS medium without growth regulators did not show any developing shoot buds (Fig. 5a). Where as, the proximal parts of *in vitro* cultured hypocotyls showed clusters of shoot primordia obtained *de novo* on shoot induction medium (Fig. 5.5b).

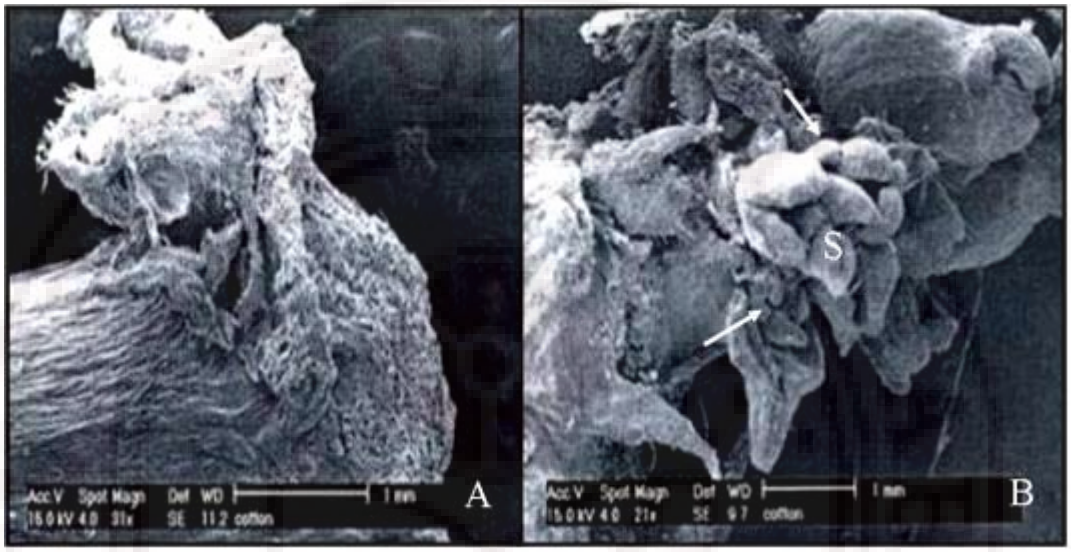


Figure 5.5 Scanning electron microscopic studies of the *in vitro* cultured explants. A - Control explant grown on MS medium without growth regulators, $AgN0_3$ and AC showing callus formation; B - Acropetal end of hypocotyl with clusters of shoot primordia when cultured on MS medium. S - shoot primordia.

5.2 Genetic transformation of cotton (*Gossypium hirsutum* L.) with *Brassica juncea NPR1*

5.2.1 Agrobacterium mediated transformation of cotton using hypocotyls sections

The cultivar Bharani (Manish Agritech, Hyderabad) was used in transformation experiments. Transformation was carried out as described above in materials and methods section. Kanamycin resistant putative transgenic shoots were obtained from hypocotyls sections after *Agrobacterium* mediated transformation, which were rooted on 75mg l^{-1} kanamycin supplemented rooting medium. Thirty two putative transgenic plants were obtained which were transferred to greenhouse and grown for further molecular analysis.

5.3 Molecular confirmation transgenic plants

5.3.1 PCR analysis and Southern hybridization

The presence of the *BjNPR1* gene was confirmed in all the plants by using the specific primers for ~1.7kb *BjNPR1* for amplification in PCR (Fig. 5.6). Southern blot analysis was performed to check the stable integration in T_0 transformants. Genomic DNA of transgenic plants was digested with the enzyme *Eco*R1 and hybridization with the *NPR1* probe revealed the integration of the transgene in transgenic plants.

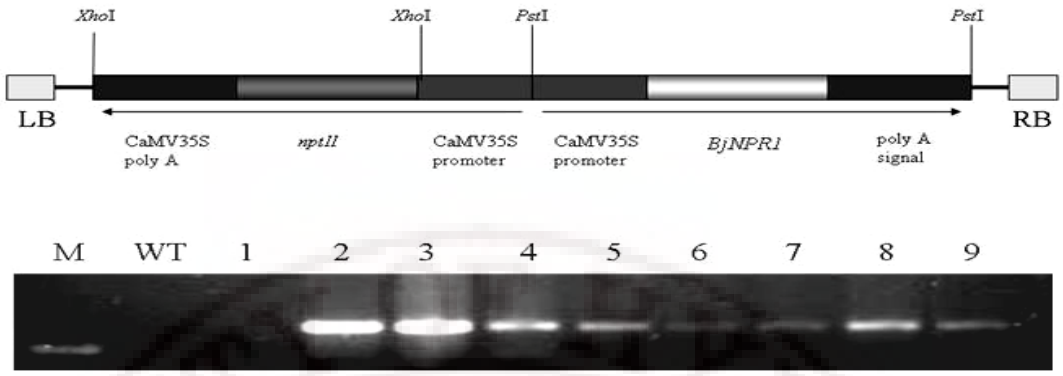


Figure 5.6 Partial map of pCAMBIA 2300 showing T-DNA cloned with *BjNPR1*. PCR analysis in T_0 plants showing the amplification ~1.7kb *NPR1* by using *BjNPR1* primers.

No hybridization signals were detected in DNA of control plants. Multiple copies of integrated transgene were identified in plant no. s 2, 3, 4, 5 with 3 copies. Plants 7, 8 and 9 are showing single copy insertion with 7 and 9 showing similar pattern and 8 is showing individual integration from that of others (Fig. 5.7).

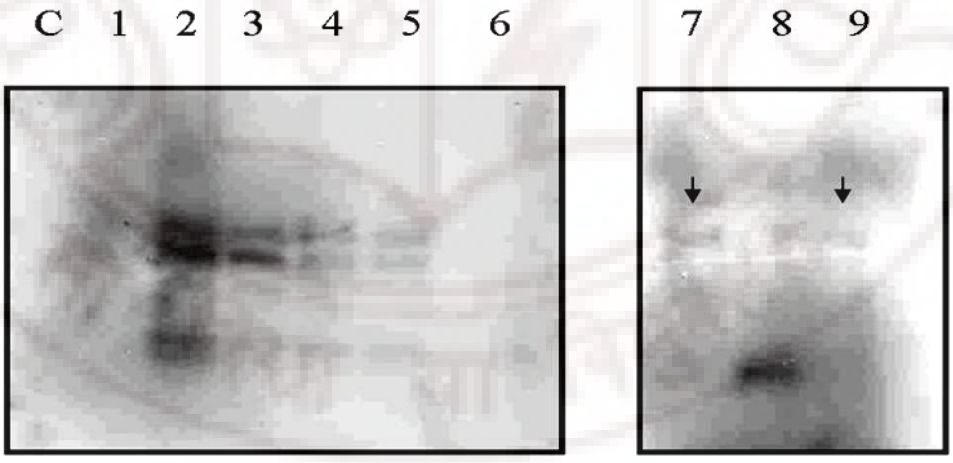


Figure 5.7 Southern blot analysis in T_0 transgenic cotton plants transformed with pCAMBIA-*BjNPR1*. Plants 2, 3, 4 and 5 were showing three copy integration of the transgene. Plants 7, 8 and 9 are the single copy plants with Plant 8 showing an independent integration from the other two.

5.3.2 Northern blot analysis

The expression of transgene *BjNPR1* in the T_0 transgenic plants was observed by northern blotting. All the 8 T_0 plants tested were showing varied expression levels of the transgene (Fig 5.8).

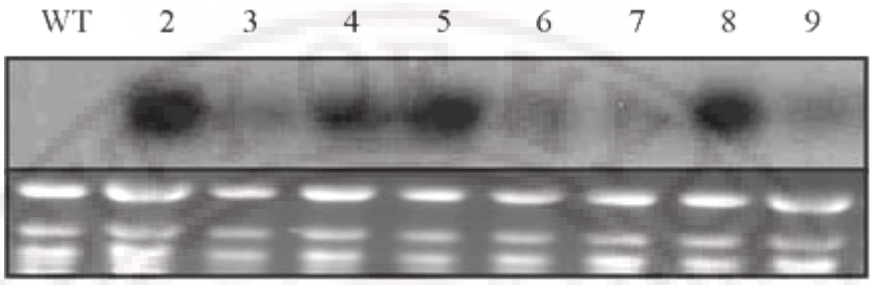


Figure 5.8 Northern blot analysis using PCR amplified *NPR1* probe which confirmed the expression of *BjNPR1* in transgenic plants. The plants 2, 5 and 8 are the high expression plants, with plant-4 is showing the moderate expression. Plants 3, 6, 7 and 9 are showing low expression.

Among these plants, 2, 5 and 8 were identified to be high expression transgenic plants and plant 4 is showing moderate expression. The plants 3, 6, 7, and 9 were identified as the low expression lines as evidenced by northern analysis. The progeny of single copy plants 7, 8 and 9 were analyzed further.

5.3.3 Western blotting

Preliminary studies with kanamycin germination assays of the seeds of T_0 generation displayed their Mendelian inheritance pattern of transgene (3:1) by showing kanamycin sensitivity. Transgenic plants at mature stage were further analyzed in T_1 generation for the expression of BjNPR1 using western blot analyses. When 50 μg protein was loaded and developed with anti rabbit anti-NPR1 antibodies all the transgenic plants were showing a NPR1 band corresponding to ~65.5 KDa (Fig 5.9). The progeny of plant no. 8 i.e. 8a and 8b were identified as the high expression lines followed by 9a, 7a and 7b.

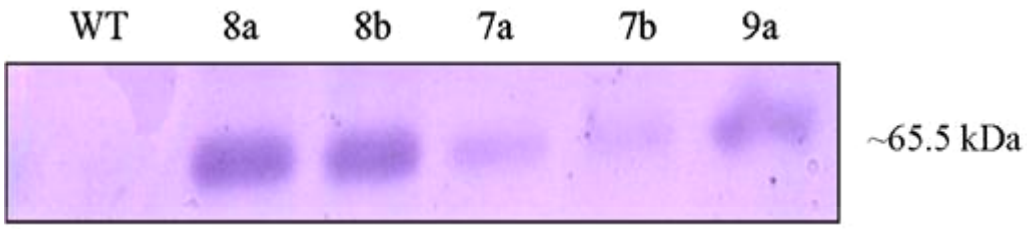


Figure 5.9 Western blotting in T_1 generation of transgenic plants with anti-rabbit-NPR1 primary antibody (1:50 dilution) and anti-rabbit-IgG secondary antibody (1:1000 dilution). Total protein extracts (50 µg per lane were loaded). WT plant does not show any signal whereas all the transgenic plants showed varied expression levels of the NPR1.

5.3.4 Immunoflourescence

For immunoflourescence microscopy, leaves and immature seeds of wild-type and transgenic seeds were tested with anti-NPR1 antibody. These results showed that no immunoflourescence signal was detected in non-transgenic fruit (Fig 5.10A) and leaf cross sections (Fig 5.10B), while strong fluorescence signals of BjNPR1 expression were detected in the cells of leaves as revealed by leaf cross section (Fig 5.10C) as well as distributed in the folded cotyledons in seeds, by checking the cross section of the immature fruit of transgenic plants (Fig 5.10D). The immunoflourescence signal of the NPR1 was also stronger in the outer portion of the fruit wall as visible in the cross section of the immature 10DPA boll (Fig 5.10E).

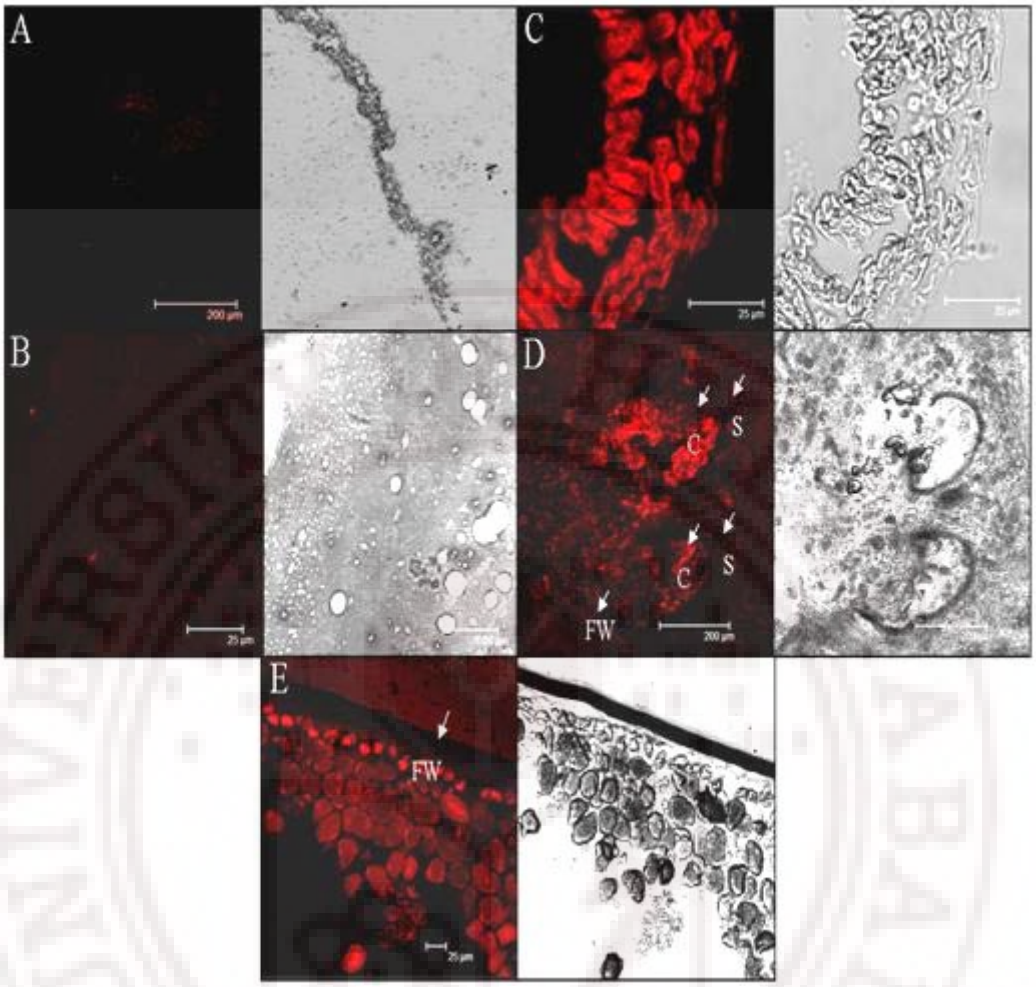


Figure 5.10 Confocal images showing the immunoflourescence signal in the tissues of WT (A and B) and high expressing transgenic line 8a (C, D and E). A. WT immature fruit (10 DPA). B. WT leaf. C. Leaf. D. Immature fruit (10DPA) with seeds. E. Fruit wall.
S- Seed, C- Cotyledon, FW-Fruit wall.
Figure shows the Immunoflourescence localization of NPR1 protein in leaf and immature fruit sections. Sections were incubated with anti-NPR1 antibody and the arrows indicate regions of high expressions of overexpressed NPR1 protein in transgenic cotton plants. Flourescence is almost absent in transgenic sections.

5.4 Enhanced expression of PR genes in transgenic plants

The T_1 generation transgenic plants were analyzed for the expression levels of *PR* genes. As NPR1 is the transcription regulatory protein, which localizes to the nucleus and activates the expression of *PR* genes, their expression levels were studied in WT and *BjNPR1* overexpressed transgenic plants. All the transgenic plants showed enhanced *1, 3- β-glucanase, Chitinase* and *PR-5*

expression levels (which can confer enhanced resistance towards pathogens) as revealed by northern blotting, when compared to WT plants (Fig 5.11).

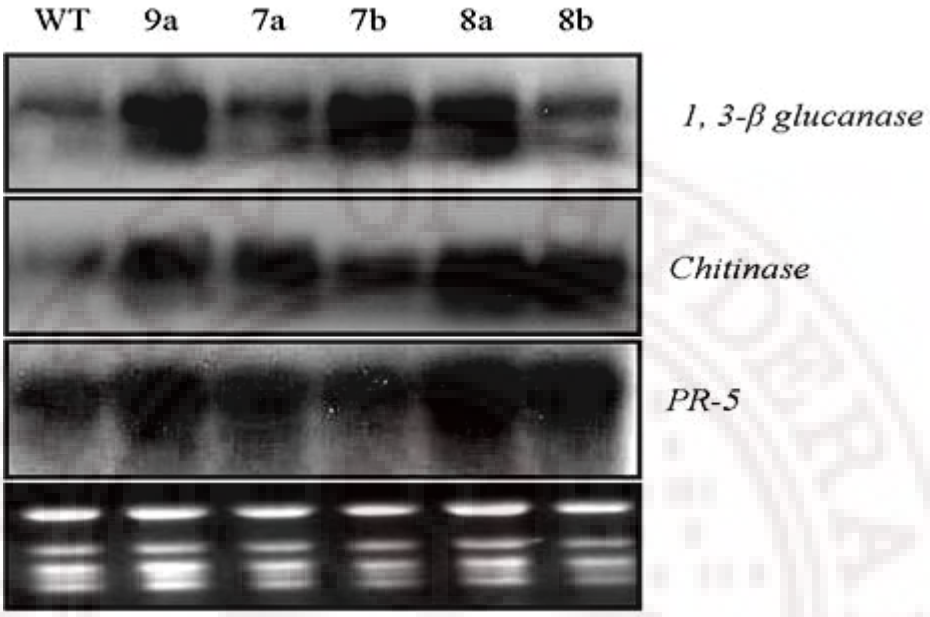


Figure 5.11 Expression of *PR* genes in T_1 transgenic plants. Blots contain total RNA (20µg) isolated from wild type (WT) and transgenic plants which were probed with cDNA probes of *1, 3-β-glucanase* (Z68154), *chitinase* (U60197) and *PR-5* (AY301283). The ethidium bromide-stained rRNA in gels used for northern blot analysis is shown to display equal loading of RNA.

5.5 Disease screens in transgenic lines

The transgenic plants were tested against the following diseases. Five transgenic plants at T_1 stage were checked for their resistance levels in comparison to wild type plants.

5.5.1 Seedling damping-off by *Rhizoctonia solani*

Seedlings of WT and transgenic plants were tested for the seedling damping-off studies in *R. solani* mixed soil. The survival rate transgenic plants were up to 76% when compared to only 8% survival rate in WT. (Fig 5.12)

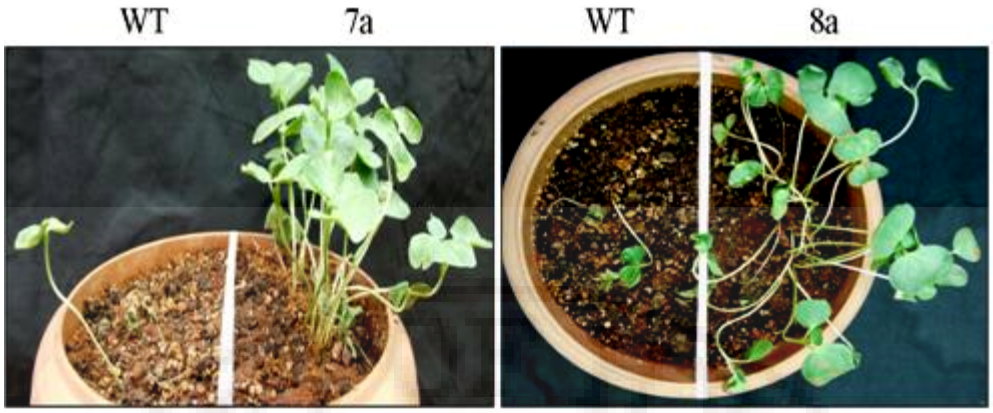


Figure 5.12. Seedling germination assay of WT (left half) and transgenics (right half) in soil mixed with *R..solani*.

The seedling damping-off was observed in WT seedlings in response to *R. solani* whereas transgenic seedlings were not affected by the fungal treatment (Fig 5.13). The economically important fungi, which are responsible for the severe loss to the cotton crop at seedling stage are *Rhizoctonia solani* (seedling damping-off) and *Fusarium oxysporum* (Fusarium wilt). Each pot was divided into equal half and WT and transgenic seeds (n=30 each) were kept for germination. Each pot was covered with transparent plastic cover to maintain high humidity. After 2 weeks, the germination frequency was observed. The transgenic seedlings were not affected by the fungal attack and showed almost normal germination rate as in case of control conditions (*Rhizoctonia*- 76%, *Fusarium*-81%).

Figure 5.13. Seedling growth studies in *R. solani* inoculations. A. Growth pattern of WT (Left) and transgenic seedlings (8a, 8e and 9a) in *R. solani* containing soil. B. Arrow shows seedling damping-off in WT seedling. C-E- Healthy root system in transgenic seedlings.

5.5.2 Evaluation of resistance to *Fusarium* wilt (FW)

For FW evaluation caused by *Fusarium oxysporum pv vasinfactum*, inoculum was prepared. The entire contents of 7 d old potato dextrose agar cultures were mixed with 100 ml of sterile distilled water per plate. The slurry contained about 10^5 conidia /ml. Cotton seedlings (8 d old) were uprooted and

immersed in the slurry for 5 min and transferred to individual sections in the plastic pots and the temperature was maintained 25°C ± 1 in the greenhouse. A total of 20 plants per line were maintained in randomized design. The score was rated 21 days post inoculation as 0-no symptoms, 5- stunted with vascular browning, 7.5- severely stunted with vascular browning, 10- completely wilted beyond recovery. The transgenic seedlings showed 2-7.5 scores while WT seedlings were severely affected showing 5-10 scores (Fig. 5.14).

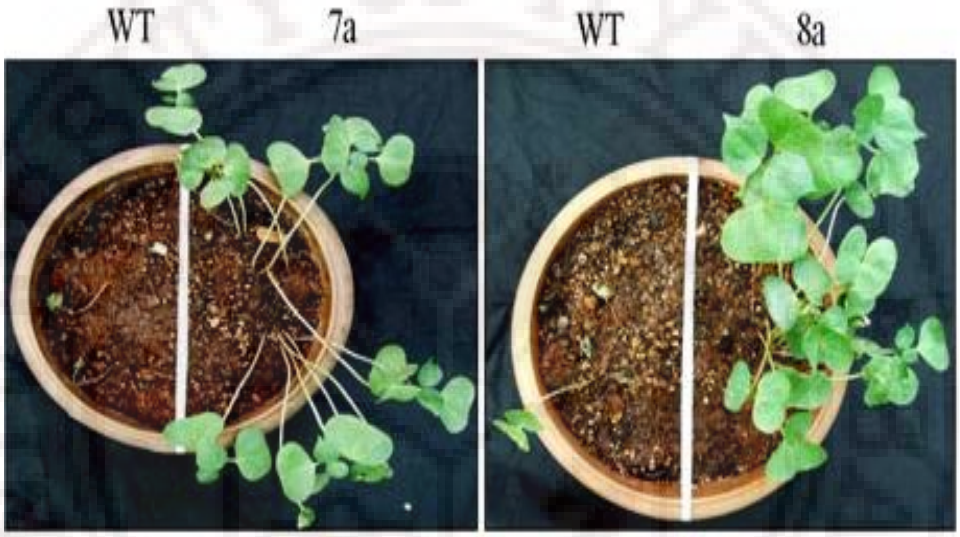


Figure 5-14.Fusarium wilt (FW) by *Fusarium oxysporum* f.sp. *vasinfectum.*WT (left half) and transgenic plants (right half) were analyzed for the resistance towards FW.

The T_1 transgenic plants showed varied levels of enhanced resistance to FW. The high expression lines 8a and 8b showed lower disease severity compared to the WT plants. The transgenic plants with moderate or low *BjNPR1* expression showed almost similar or slightly higher severity than WT plants. These observations suggest that threshold expression levels of *NPR1* might be required for disease resistance in transgenic plants. The survival rate of transgenic seedlings was up to 81%, which is significantly higher when compared to the survival rate of WT seedlings which is only 15%.

5.5.3 Evaluation of resistance to boll rot

For boll rot evaluation, sporangial suspension (5×10^4 sporangia/ml) of *Phytophthora infestans* isolate was prepared from 10 d old agar cultures and chilled at 12°C for 2-3 h for zoospore release. Plants with bolls (10-20 DPA) were sprayed with the chilled suspension until run-off. Controls were kept without spraying. Inoculated plants were kept at 20°C and for 10-15 d. The severity of the disease was scored from 0-5, where 0- means no symptoms and 5- means 95-100% fruit affected by the disease.

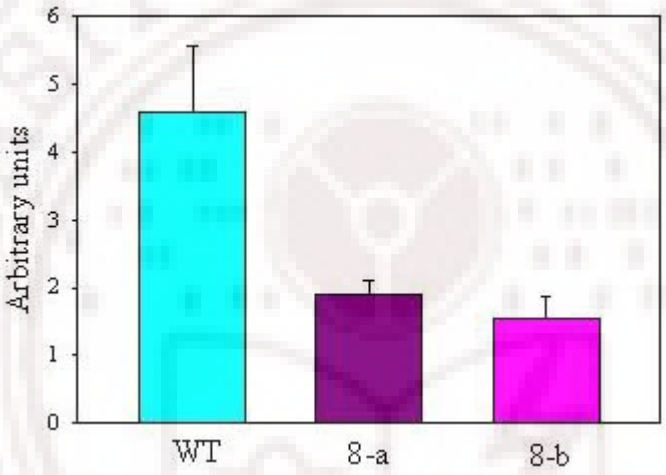


Fig. 5.15. Scoring of Boll rot caused by spraying *Phytophthora infestans.*

The transgenic score showing the disease severity was recorded as from 0-3 and the WT plants were with damaged bolls with 3-5 scores indicating fruit damage. The average values of 10 plants each of WT and transgenics were plotted (Fig 5.15)

5.6 Discussion

Genetic manipulation of disease resistance to biotic stress tolerance through transferal of plant defense related genes into commercial crops is effective in terms of cost, efficacy and reduction in pesticide usage. In the studies involving plant-pathogen interactions to improve disease resistance, systemic acquired resistance is an interesting phenomenon. SAR is one of the best understood mechanisms of induced resistance in plants upon pathogen attack. SA is an essential signal and NPR1 is a key regulator in the activation of *PR* genes in response to pathogen assault. Till now no single PR gene is shown to be important in SAR, the protection provided by a single specific PR gene is usually very limited in its spectrum, degree and duration compared to that of native SAR response. *NPR1* overexpression in plants can be closely related to the natural SAR as it is the important transcription regulator activating many *PR* genes. Transgenic cotton plants were developed by using the optimized regeneration protocol (Divya et a., 2008)

Overexpression of NPR1 activates PR genes

Overexpression of *AtNPR1* in *A. thaliana*, rice, tomato, and wheat has been demonstrated to induce enhanced fungal and bacterial resistance (Cao et al 1998; Chern et al. 2001; Fitzgerald et al. 2004; Friedrich et al. 2001; Lin et al. 2004; Makandar et al. 2006). In *A. thaliana,* overexpression of *AtNPR1* and enhanced resistance are correlated with both enhanced and quicker *PR* gene expression, suggesting that *NPR1* induces enhanced resistance through elevated expression of *PR* genes (Cao et al. 1998; Friedrich et al. 2001). On the other side expression of *AtNPR1* which regulates the activation of systemic acquired resistance, when expressed in the Fusarium head blight-susceptible wheat caused by *Fusarium graminearum* confers a heritable, type II resistance. It was shown to be associated with *PR1* expression and is induced rapidly to a high level in the fungus-challenged spikes of the *AtNPR1*-expressing wheat.

Overexpression of NPR1 in Arabidopsis has been shown to lead to a non-specific resistance to *Peronospora parasitica* and *Pseudomonas syringae pv.*

maculicola in a dosage-dependent fashion, without obvious detrimental effects on the plant morphology (Cao et al., 1998). Overexpression of NPR1 also results in enhanced efficacy of fungicides in Arabidopsis (Friedrich et al., 2001). Transgenic rice plants overexpressing the *AtNPR1* gene exhibit enhanced resistance to the rice bacterial blight pathogen *Xanthomonas oryzae pv. oryzae* (Chern et al., 2001). Overexpression of apple *MpNPR1* conferred resistance to a broad spectrum of diseases and showed enhanced activation *PR* genes (Malnoy et al., 2007). Overexpression of *AtNPR1* in transgenic tobacco plants conferred enhanced insect tolerance (Gargi et al., 2008). Our results showed the elevated expression levels of *PR* genes in transgenic plants when compared to WT and enhanced resistance to two potent fungi (*Rhizoctonia solani* and *Fusarium oxysporum pv vasinfectum*) causing seedling root rot in the most important commercial fiber yielding crop, cotton. Transgenic plants also showed enhanced resistance to boll rot caused by *P. infestans*.

5.7 Summary

We have successfully engineered the expression of *BjNPR1* under 35S promoter in cotton. The integration and expression of the transgene were confirmed by Southern, northern and western blot analyses. Immunoflourescence studies indicated high expression of overexpressed NPR1 proteins in leaf and fruit sections in transgenic plants. Northern blot revealed the stronger activation of *PR* genes in transgenic plants. Resistance to seedling disease complex has been manifested in the *NPR1* overexpressed transgenic cotton plants. Transgenic seedlings displayed resistance to *Rhizoctonia* seedling damping-off, *Fusarium* wilt and boll rot caused *Phytophthora*. The *BjNPR1* overexpression is a promising approach in the production of disease resistant cotton plants.

Chapter 6

Transgenic cotton plants expressing *Brassica juncea* annexin with improved abiotic stress tolerance and fiber quality under stress

6 Results

6.1 Development of transgenic cotton plants overexpressing mustard annexin

A total of 52 putative T_0 transgenic plantlets were obtained by screening on the selection medium. After transfer to the green house, 36 plants showed the 954 bp annexin amplification, by PCR, whereas no amplification was observed in WT plants.

Southern hybridization analyses were performed to check the stable integration and copy number of transgene in 7 putative transgenic plants. Restriction digestion of genomic DNAs with *Xho*I cuts the T-DNA region near left border that contains entire cauliflower mosaic polyA and coding region of *nptII*. Upon hybridization a probe of *nptII* ORF detected a 0.9kb fragment release, which indicates the presence of selectable marker gene in the transgenic plants. Complete digestion of the genomic DNAs with *Pst*I and probing with the probe of coding region of AnnBj1 released the expected cassette of ~ 1.7 Kb, which contains the entire cauliflower mosaic virus promoter and polyA along with the coding region of *AnnBj1* (Fig 6.1).

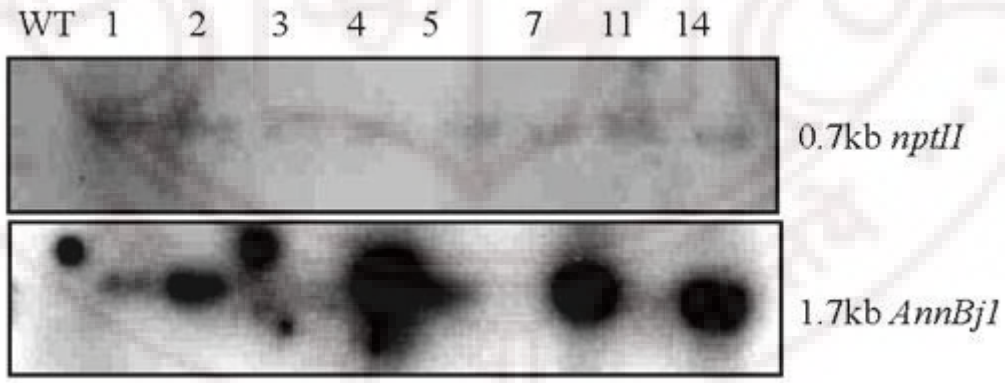


Figure 6.1 Southern blot analyses showing the fragment release of ~0.7kb *nptII* and ~1.7kb *AnnBj1* upon restriction digestion of genomic DNA with *Xho*I and *Pst*I respectively.

To assess the copy number of the transgenes, the genomic DNA was digested with *Eco*RI, which has a single restriction site in the T-DNA, and probed

with the *AnnBj1* cDNA. This revealed variation in the integration pattern of the transgenes in different plants showing random integration in the cotton genome. Plants 1, 4, 5, 9 and 11 showed single copy integration, where as the plant 14 showed two-copy integration (Fig 6.2A).

All southern positive plants showed variable annexin transcript levels. The amplification of the transcripts for 18S rRNA was used as an internal control (Fig 6.2B). Based on this, the single copy transgenics 4, 5 and 11 were chosen for further characterization at various levels. As revealed by northern blot analysis, 4 and 11 are the high expression lines and plant no. 5 is low expression line. T_1 seeds of these plants after self pollination (T_2 progeny) were collected and used for various functional analyses.

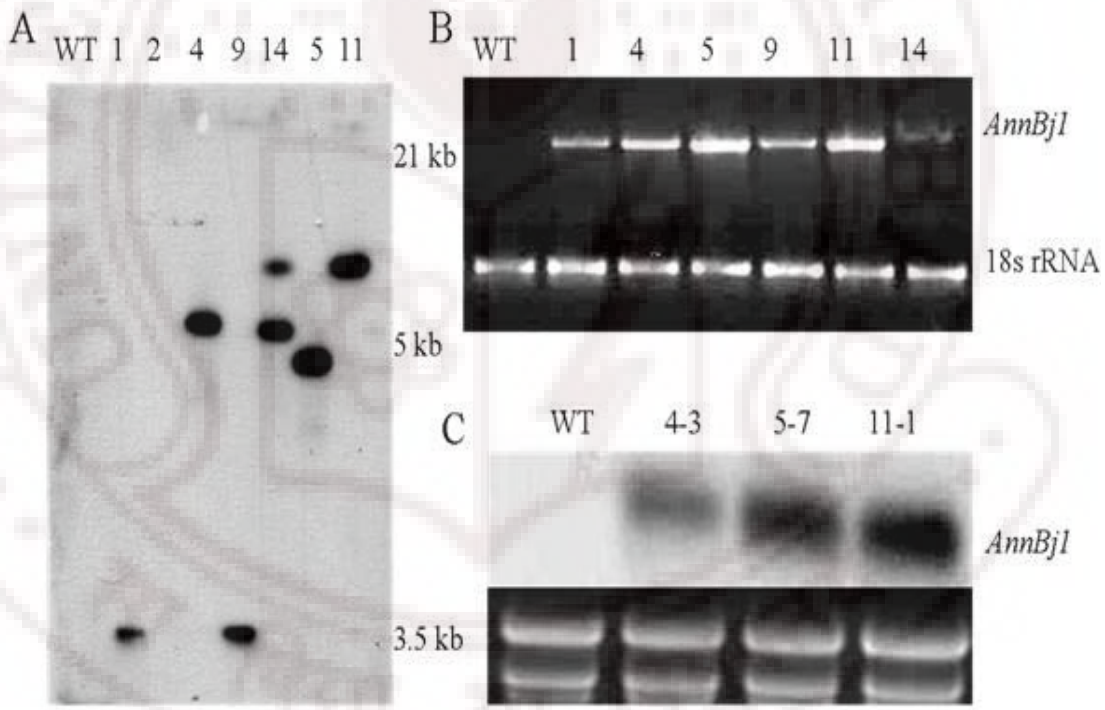


Fig. 6.2 (A) Southern analysis of T_0 transgenic cotton for transgene copy number using *Eco*RI, with a single restriction site on the T-DNA and probing with the PCR amplified *AnnBj1*. (B) RT-PCR analysis in T_1 plants showing expression levels of *AnnBj1*. 18S rRNA was used as a control. (C) Northern analysis of RNA (20 μg) extracted from the leaf samples of 8-week-old plants. Hybridization of the RNA blots was carried using *AnnBj1*.

Table 6.1 Segregation analysis in progeny of T_0 transgenic cotton lines as assessed by kanamycin sensitivity test.

T_0 Plant	Number of seeds tested	Number of seeds germinated[a]		χ^2-value[b]
		Kanamycin (+)	Kanamycin (-)	
4	56	43	13	0.071
5	53	40	13	0.004
11	45	36	9	0.450

6.2 Molecular characterization of the T_1 generation

Northern analysis illustrated variation in the expression levels of the annexin transcript among these transgenics; where 4-3 was observed to be a comparatively low expression plant, the expression levels in 5-7 and 11-1 were significantly high (Fig 6.2C). Homozygous T_2 progeny were obtained by selfing these plants (as confirmed by uniform 100% germination on kanamycin containing medium. These transgenic T_2 lines were used in abiotic stress tolerance studies.

6.3 Assessment of abiotic stress tolerance in transgenic plants

6.3.1 Mannitol stress tolerance

Surface sterilized *in vitro* germinated seedlings (7 d old) of WT and transgenic plants were treated with mannitol (300mM) on sterile tissue paper for a further period of 7 d. A control experiment with mock-treatment in water was performed in parallel to these seedlings experiment (Fig 6.3A). The WT seedlings showed retarded growth and transgenic seedlings showed longer roots with profuse lateral root system. The root length and number of lateral roots formed at the end of the treatment were significantly high in the transgenic seedlings (Table 6.2). At this stage the cotyledons were fully open and the cotyledonary lamina was larger and green in transgenics in comparison to WT seedlings. The WT

seedlings only showed a single tap root without any lateral root formation with stunted growth with mutilated cotyledons and shoot length was also reduced (Fig 6.3B).

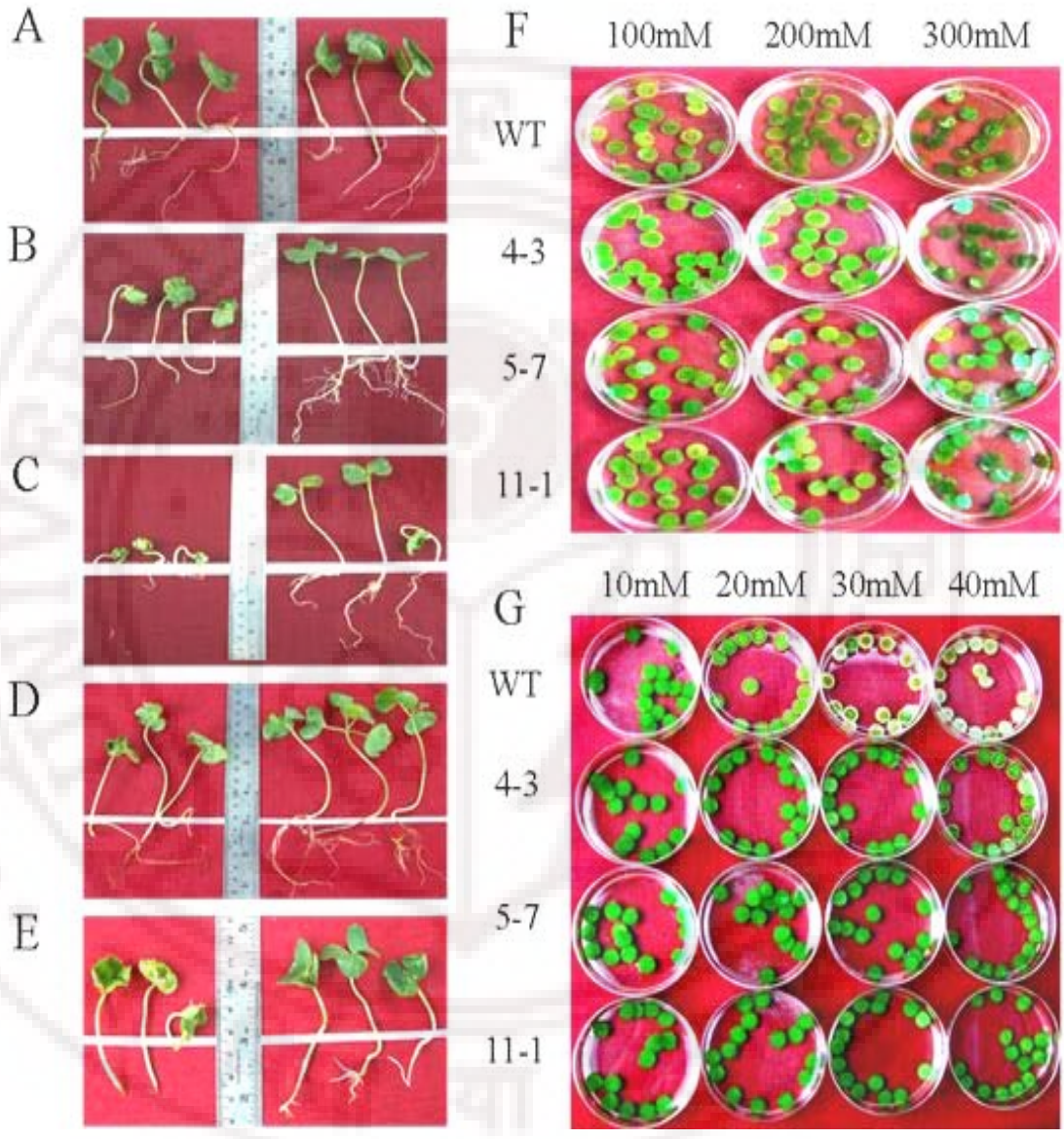


Figure 6.3. Assessment of stress tolerance at seedling stage by imposing various stress conditions with different treatments. (A) Growth of WT and transgenic seedlings on water for two weeks as control experiment.(B) Effect of 300 mM mannitol, (C) 300mM NaCl, (D) 10% PEG and (E) 10mM H_2O_2 respectively on the growth of 7 d old WT and T_2 seedlings (4-3, 5-7, 11-1) further incubated on sterile tissue paper moistened with respective treatment solutions and grown for another 7 d. (F) and (G) Leaf disc assay of WT and transgenic plants at mature stage on different concentrations of NaCl and H_2O_2. There is visible loss of chlorophyll pigment in WT leaf discs which is comparatively less in transgenics.

6.3.2 Salt stress tolerance

Similar to mannitol, treatment with NaCl (300mM) also showed that the transgenic seedlings possessed well developed root system with very long roots, significantly enhanced root length and number of lateral roots (Fig 6.2C, Table 6.2). The WT seedlings were severely damaged by the completion of the stress treatment with delayed growth rate after germination, substantial chlorosis, smaller cotyledon lamina and hindered root growth and development, when compared to transgenic seedlings.

6.3.3 Dehydration stress tolerance

When the seedlings of WT and transgenic lines 4-3, 5-7 and 11-1 were treated with 10% PEG inducing high osmotic stress the seedlings of WT were severely damaged by not producing lateral roots and showing wilted appearance. Where as the transgenic seedlings were healthy with significant secondary leaf and lateral root formation and better development in terms of shoot and root elongation indicating that the transgenics possessed enhanced dehydration tolerance (Fig 6.2D).

6.3.4 Oxidative stress tolerance

The treatment with 10mM H_2O_2 for the induction of oxidative stress conditions resulted in considerable loss of the chlorophyll in all the seedlings, but the WT seedlings bleached completely, whereas transgenic seedlings retained significantly higher chlorophyll content with partial bleaching. Upon transfer to the green house condition, the transgenic seedlings had a recovery and survival of 84 %, where as none of the WT seedlings recovered after treatment leading to their complete death (Fig 6.2E). Simultaneous experiments were conducted to check the tolerance levels of transgenic plants at mature stages by performing leaf disc assays on different stress treatment solutions (Fig 6.2F, G).

Table 6.2 Average root parameters in stress treatments of WT and T_2 transgenic cotton seedlings with and without stress. Values represented by mean ± SE followed by different superscripts differ significantly.

Stress imposed (**days)**	**Root length (cm)**		**No. of lateral roots**	
	Before	**After**	**Before**	**After**
H_2O (Control)				
WT	3.8±0.1	10.6±0.2	2.0±0.4	4.9±0.5
Transgenic	3.6±0.05	10.8±0.8	2.0±0.5	5.0±0.5
NaCl (300mM)				
WT	3.2±0.08^a	4.6±0.6^b	2.2±0.1^a	2.8±0.5^b
Transgenic	3.0±0.12^a	12.2±0.3^c	2.4±0.2^a	6.8±0.3^c
Mannitol (300m				
WT	4.4±0.8^a	6.6±0.2^b	1.8±0.1^a	2.6±0.3^b
Transgenic	4.1±0.6^a	18.6±0.8^c	1.6±0.1^a	16.2±0.8^c
PEG (10%)				
WT	4.8±0.04^a	7.6±0.3^b	2.4±0.2^a	4.0±0.1^b
Transgenic	5.0±0.10^a	10.2±0.4^c	2.4±0.1^a	12.0±0.4^c
***P* value**	≤0.01	≤0.01	≤0.01	≤0.01

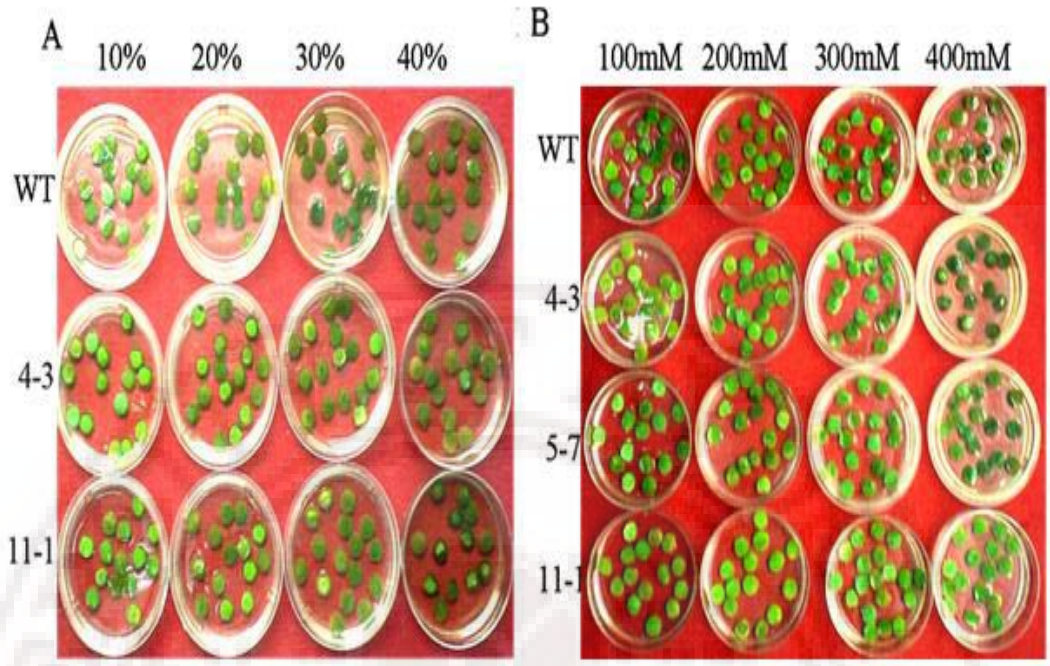


Figure 6.4. Leaf discs on (A) Mannitol (100mM- 400mM) and (B) Polyethylene glycol (10%-40%). Damage to leaf discs was more in WT plants when compared transgenic plants.

6.3.5 Estimation of total Peroxidase activity

The total peroxidase activity was assayed from the leaves of WT and transgenic plants (4-3, 5-7, 11-1) using the amplex red reagent. Transgenic plants displayed 2.1-2.8 fold increase in the peroxidase activity, as recorded by the formation of resorufin, in comparison to the WT plant at ($P \leq 0.02$, Fig 6.5).

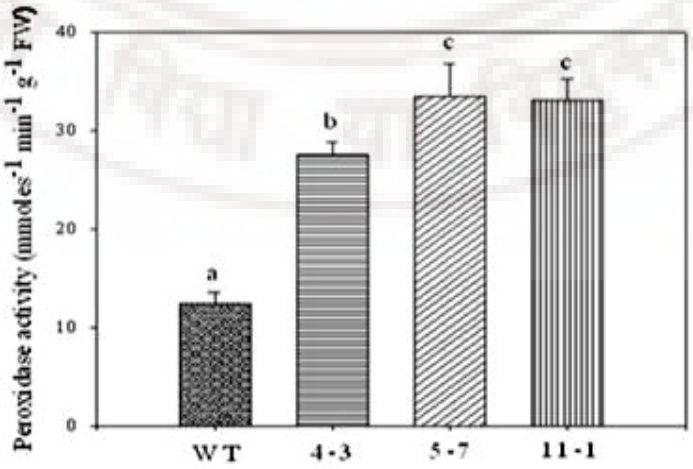


Figure 6.5 Total peroxidase activity in the leaf samples of WT and transgenic lines 4-3, 5-7 11-1. Data was represented as mean ± SE and the bars represented by different letters differ significantly at $P<0.02$.

6.3.6 Detection of ROS using confocal microscopy

Without NaCl incubation only very minimal level of fluorescence was observed which was similar in untransformed and transformed roots (Fig 6.6 A-D). With the NaCl treatment increased fluorescence was recorded in untransformed WT roots where as in transformed roots only a slight enhancement in the ROS is observed which is almost one-third of that in the WT roots (Fig 6.6 E-H). Flourescence was quantified using ImageJ based on internal density (Fig 6.7)

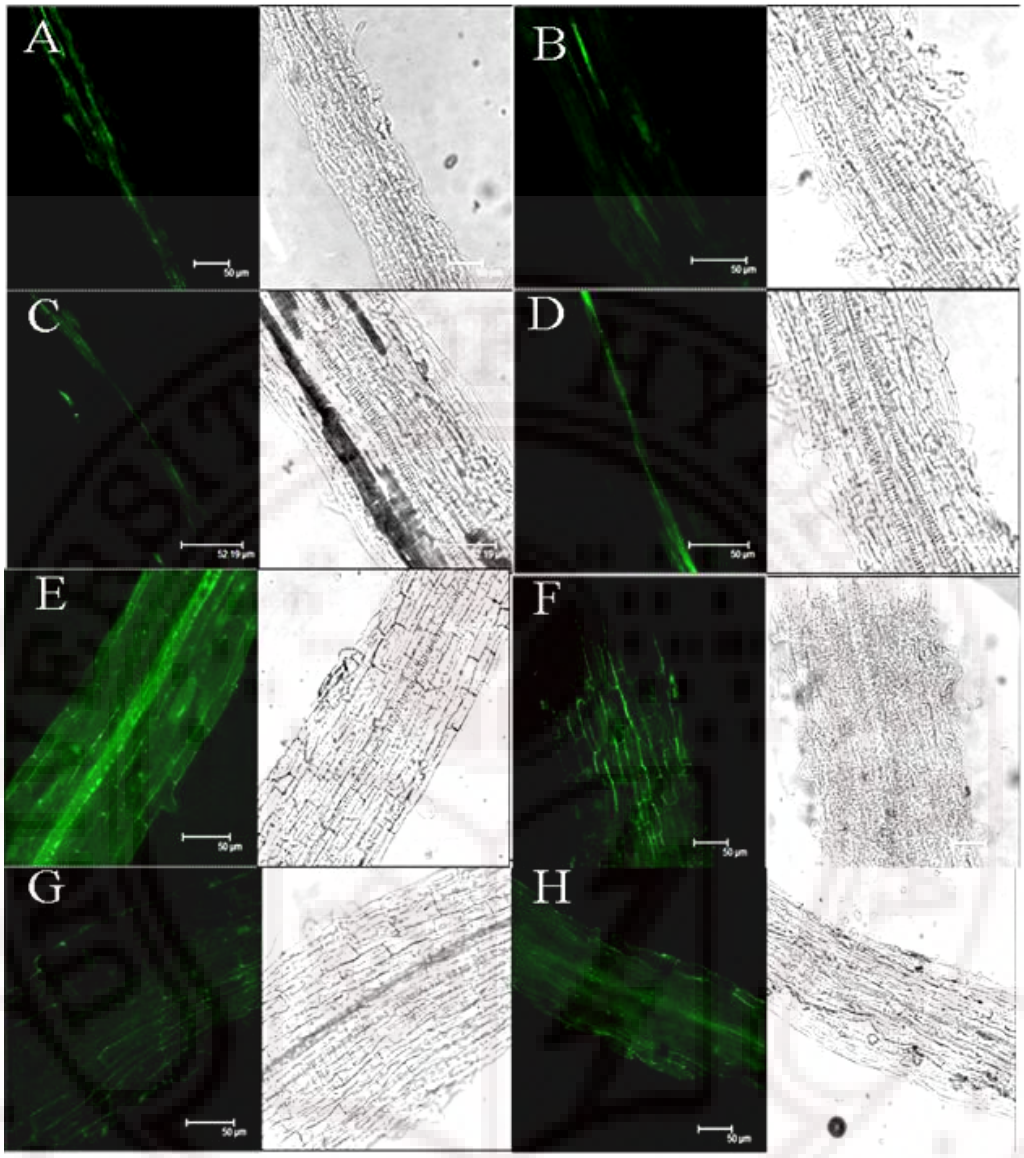


Figure 6.6 A-D Root confocal images of WT (A) and T_2 transgenic cotton seedlings 4-3 (B), 5-7 (C), 11-1 (D) stained with H_2DCFDA on control treatment without NaCl. Number of seeds tested for each sample, n=50. (E-H) ROS accumulation in roots of WT (E) and T_2 transgenic cotton seedlings 4-3 (F), 5-7 (G), 11-1 (H) with 100mM NaCl treatment.

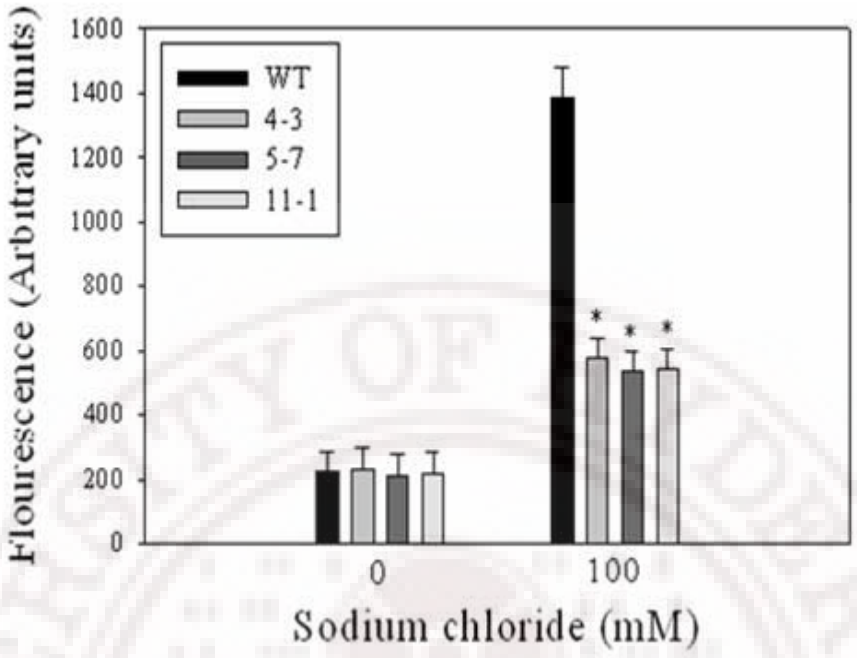


Figure 6.7 Quantification of ROS production using Image J 1.42, as relative fluorescence intensity when stained with H_2DCFDA with and without 100mM NaCl in roots of ten day old seedlings of WT, 4-3, 5-7, 11-1 (T_2 generation). Data was represented as mean ± SE. Bars represented by asterisks are significantly different at $P<0.02$.

6.3.7 Determination of H_2O_2 content

In an attempt to determine the level of ROS scavenging in WT and transgenic plants during salt stress, H_2O_2 levels in leaf samples were estimated before and after imposing salt stress. In control conditions without stress, the H_2O_2 levels in both WT and transgenic plants were more or less similar, but in NaCl treatment, there was a striking elevation in H_2O_2 level in WT plants. The transgenic plants showed a minimum elevation in the H_2O_2 content when compared to WT plants, which indicated that the enhanced stress tolerance might be due to the ROS scavenging activity of overexpressed *AnnBj1* in transgenic plants (Fig 6.8). The enhanced H_2O_2 levels in WT cotyledons were in correspondance to the increased ROS accumulation levels in the roots.

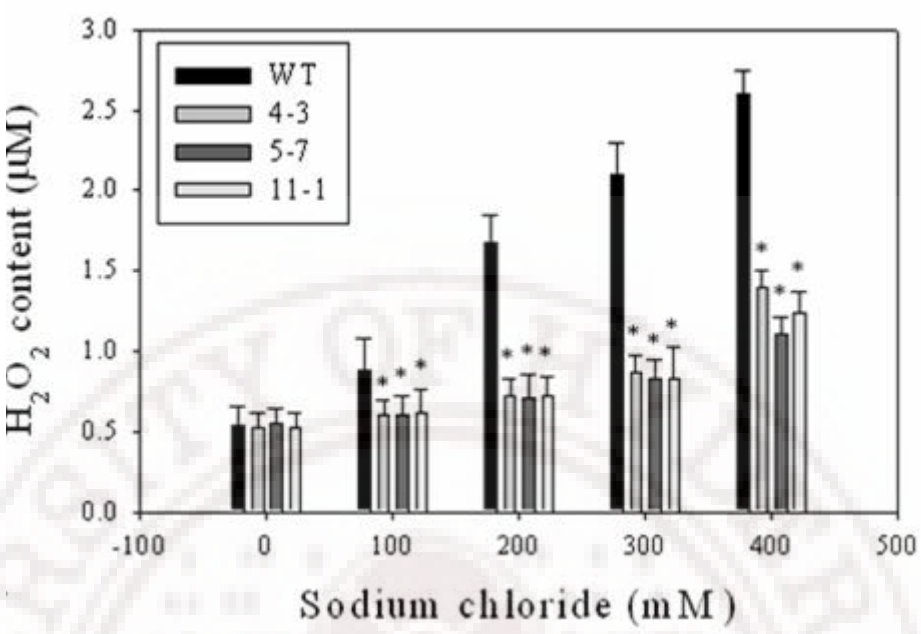


Figure 6.8 Estimation of H_2O_2 levels in leaf samples of WT and transgenic plants (4-3, 5-7 and 11-1) with and without NaCl stress (100mM-400mM). Data represents mean ± SE of three individual experiments. Asterisk represent the significant difference in the values from WT plants (P<0.01).

6.3.8 Relative water content of seedlings after salt treatment

When the relative water content (RWC) WT and transgenic seedlings were calculated after different stress treatments, the diminution in the RWC in the different transgenic plants was significantly less when compared to WT seedlings indicating less damage to the transgenic plants under salt stress. The fresh weight (FW) and dry weight (DW) of seedlings after different stress treatments were measured and there was an increase in the DW in all the transgenics (Table 6.3).

Table 6.3 Relative water content (RWC), fresh weight (FW) and dry weight (DW) in stressed seedlings. Values are represented as mean ± SE, different superscript denote the significant difference at following *P* values.

T_2 seedlings	Type of stress imposed	RWC (%)	FW (mg)	DW (mg)
	H_20 (Unstressed)			
WT		92.0 ± 1.2^{a}	678 ± 5.8^{a}	55 ± 1.1^{a}
4-3		91.5 ± 3.4^{a}	653 ± 8.3^{a}	54 ± 1.0^{a}
5-7		92.2 ± 1.0^{a}	672 ± 3.5^{a}	55 ± 2.4^{a}
11-1		90.0 ± 2.2^{a}	663 ± 5.4^{a}	56 ± 2.2^{a}
	300mM NaCl			
WT		61.0 ± 2.8^{c}	512 ± 7.2^{c}	58 ± 2.8^{a}
4-3		80.0 ± 1.0^{b}	558 ± 3.6^{b}	78 ± 3.2^{d}
5-7		83.5 ± 2.4^{b}	569 ± 4.5^{b}	69 ± 1.8^{c}
11-1		84.2 ± 3.3^{b}	561 ± 6.0^{b}	72 ± 3.3^{c}
	300mM Mannitol			
WT		59.0 ± 3.5^{c}	522 ± 4.8^{c}	63 ± 1.2^{b}
4-3		83.0 ± 2.2^{b}	536 ± 5.1^{b}	75 ± 1.4^{c}
5-7		88.0 ± 2.0^{b}	541 ± 2.2^{b}	83 ± 0.8^{d}
11-1		86.5 ± 2.0^{b}	559 ± 1.9^{b}	80 ± 2.2^{d}
	10%PEG			
WT		53.2 ± 3.2^{c}	468 ± 7.0^{c}	59 ± 1.3^{a}
4-3		79.1 ± 3.6^{b}	547 ± 3.4^{b}	71 ± 1.4^{c}
5-7		79.9 ± 4.0^{b}	558 ± 3.3^{b}	73 ± 1.1^{c}
11-1		78.5 ± 3.3^{b}	550 ± 6.5^{b}	68 ± 1.0^{b}
*P*value		≤0.012	≤0.015	≤0.005

6.3.9 Estimation of total chlorophyll content and TBARS

The stress tolerance in whole plants of the WT and transgenic lines was analyzed by performing the leaf disc assay in different concentrations of NaCl (100mM-400mM), mannitol (100mM-400mM), H_2O_2 (10mM-40mM) and PEG (10%- 40%). Tolerance levels were also checked in the form of estimation of total chlorophyll content and TBARS at end of 72hr. Though there was a dose dependent reduction in the total chlorophyll and an increase in the TBARS in all the treatments, the overall loss in chlorophyll content of WT plant was

significantly higher than transgenics in all the treatments ($P \leq 0.01$).(Fig 6.9A, B, C and D).

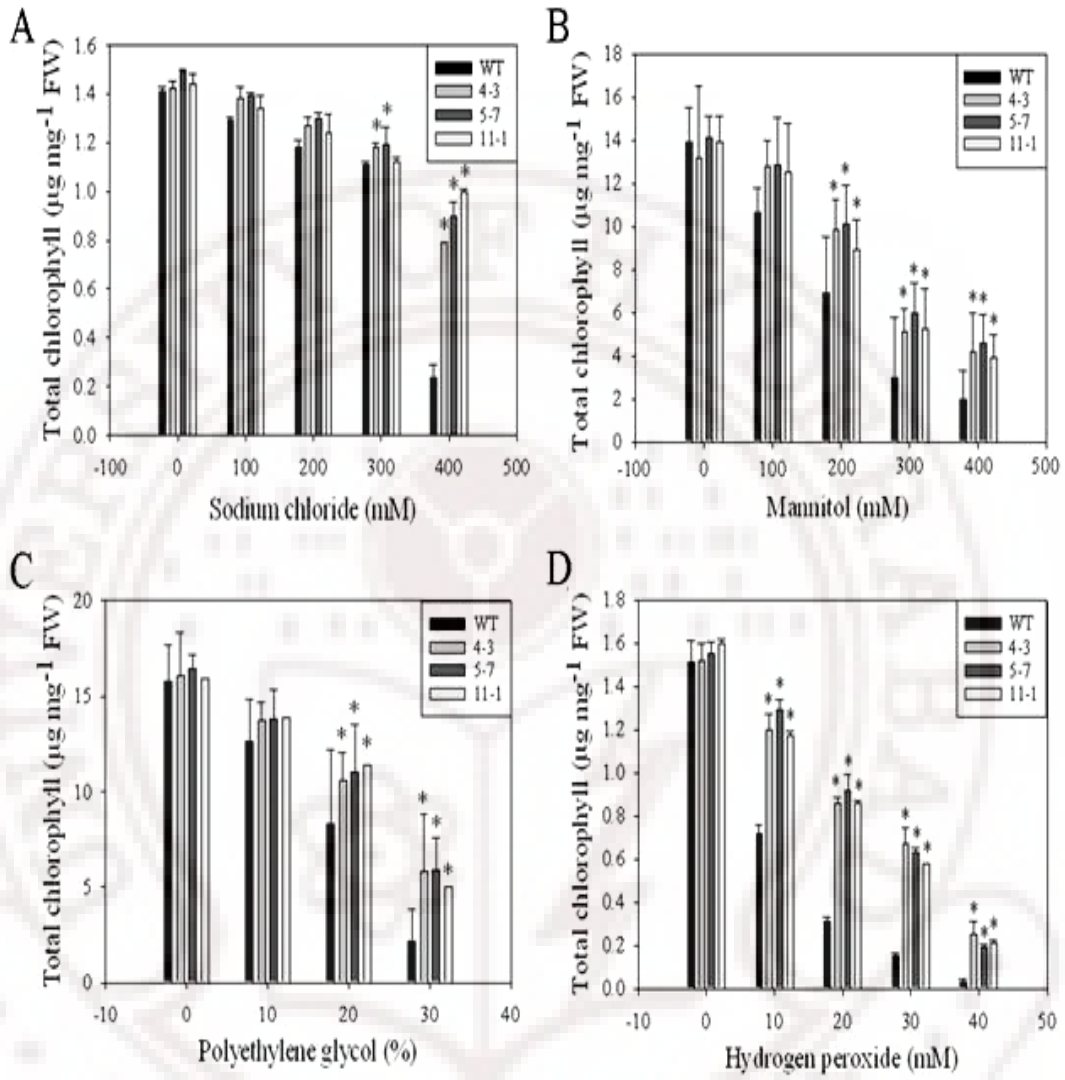


Figure 6.9 The chlorophyll content (µg mg^{-1} FW) in the leaf-discs after 72 h of different treatments: (A) NaCl (100mM-300mM); (B) Mannitol (100mM-300mM); (C) PEG (10%-40%) and (D) H_2O_2 (10mM-40mM). Values of transgenic plants are significantly different from WT ($P<0.01$).

The fold increase in TBARS content in all the treatments was significantly very high in the WT in comparison to transgenics in all leaf disc stress treatments showing that the transgenic plants were protected from cellular membrane damage in stress conditions ($P \leq 0.01$, Fig 6.10 A, B, C and D).

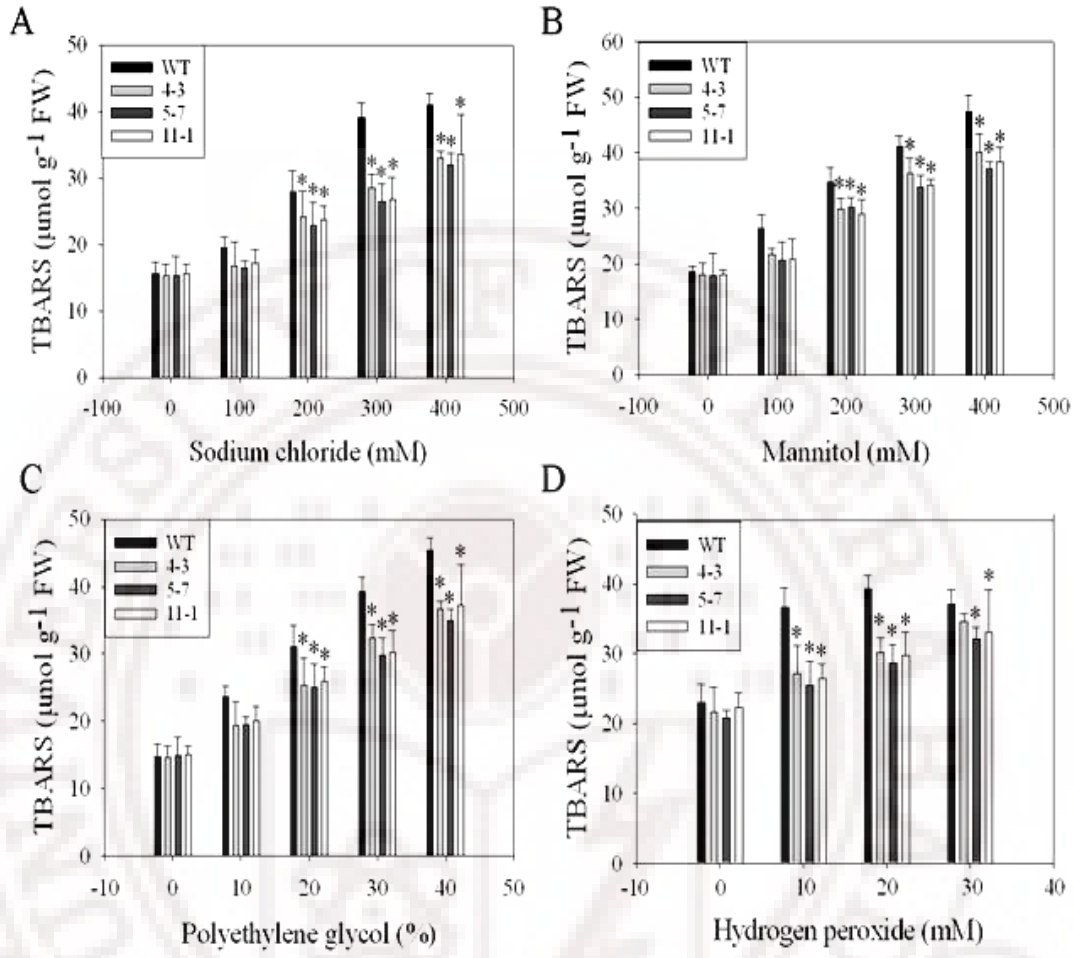


Figure 6.10 TBARS (μmol g^{-1}FW) in leaf-discs after 72 h of different treatment: (A) NaCl (B) Mannitol; (C) PEG and (D) H_2O_2. Values of WT are significantly different from transgenic plants (P<0.01).

6.3.10 Elevated levels of the osmoprotectants in the *AnnBj1* overexpressing transgenic plants

In plants, osmoprotectants such as proline and soluble sugars like sucrose play a major role in alleviating osmotic stresses. Proline is a well-known osmoprotectant with an important role in the maintenance of osmotic balance under the stress conditions. In order to find out the differences in the proline and sucrose contents of the WT and transgenic plants, we incubated their leaf discs in different concentrations NaCl.

Even though the difference in the proline content in the WT and transgenics was considerable in 100mM and 200mM concentration of NaCl, it was remarkably high in 300mM and 400mM NaCl treatments. The proline content at 400mM NaCl was increased up to 15-fold in transgenic lines, which was significantly high compared to the 8-fold increase in the proline content in the WT plants relative to basal level without treatment ($P \leq 0.02$, Fig 6.11A).

6.3.11 Leaf carbohydrate content

Carbohydrate content in the leaves of salt treated field grown WT, 4-3, 5-7 and 11-1 lines were also estimated. There was no significant difference recorded in the leaf carbohydrate content of WT and transgenic plants under control conditions (data not shown). A remarkable increase was recorded in the hexose (fructose and glucose) and sucrose levels in the transgenics after salt treatment, when compared to WT. The hexose levels in annexin transgenics were 36-61% with the greatest increase observed in transgenic line 5-7 followed by 11-1 and 4-3 (Fig 6.11B). A maximum of 1.4 times increase in the sucrose level was observed in 5-7 followed by 11-1 with 1.2 times and 4-3 with 1.17 times ($P \leq 0.02$), when compared to its salt treated WT counterpart (Fig 6.11C). The starch content decreased in all the transgenic plants under stress and the sucrose: starch ratio increased consistently (Fig 6.11D and E).

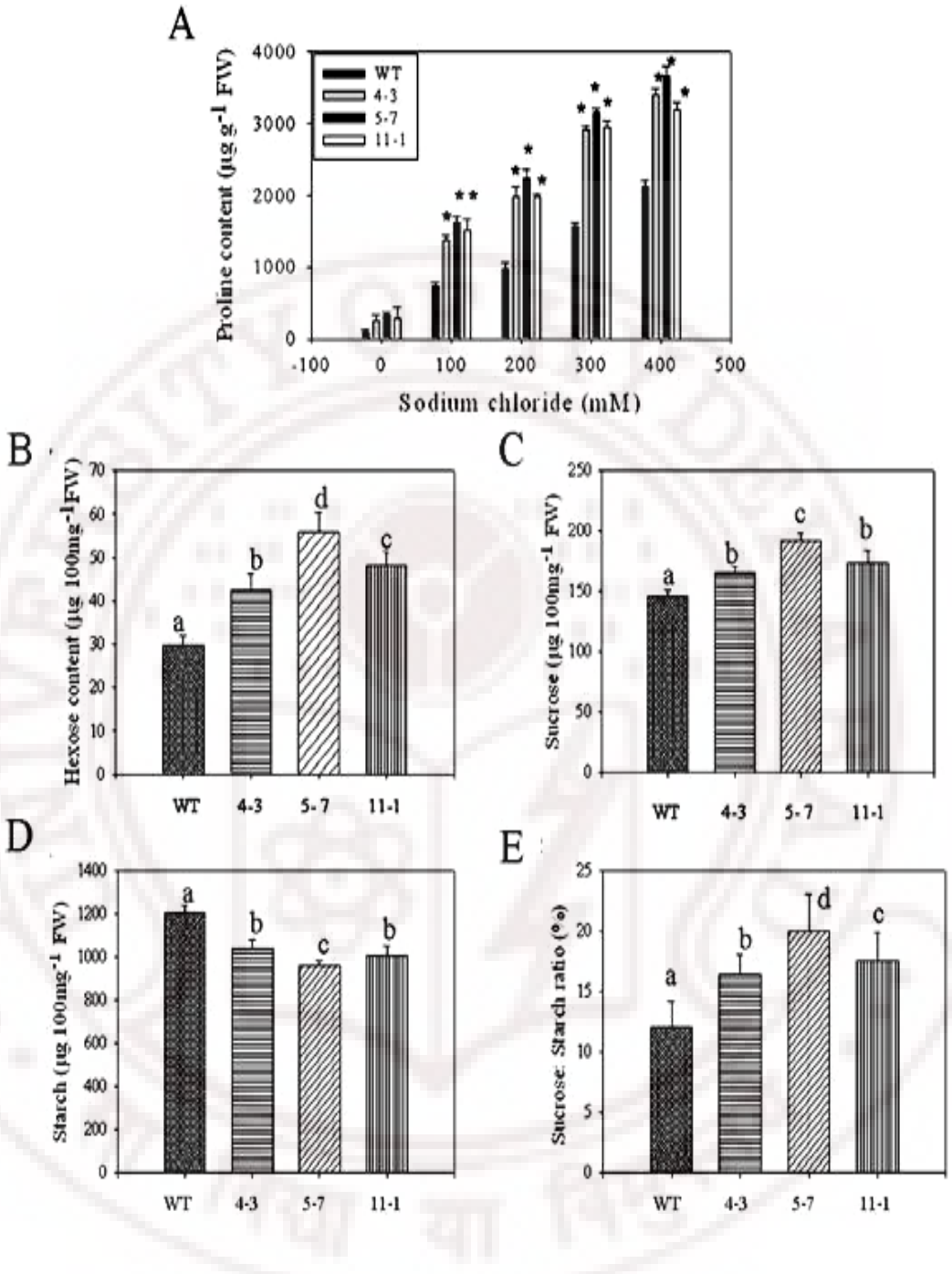
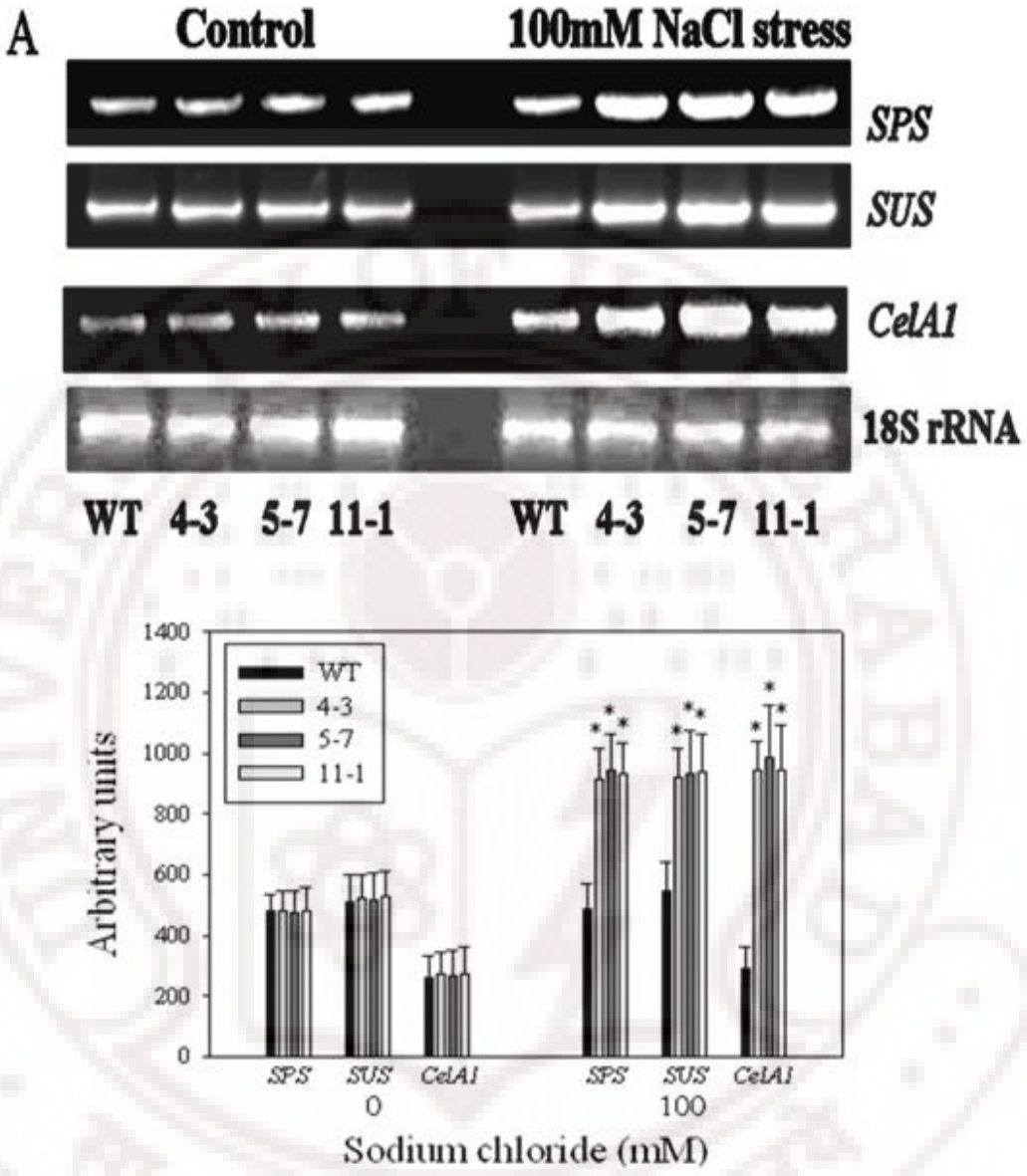


Figure 6.11 A. Proline content in NaCl treated WT and transgenic plants after 6-wk treatment. Differences between the WT and the transgenics are significant (P<0.02).
B, C, D, and E The pool sizes (µg 100mg^{-1}) (B) hexoses (glucose and fructose), (C) sucrose, (D) starch and (E) sucrose: starch ratio after irrigating plants continuously for 8-wk with 100mM NaCl. Differences are significant between WT and transgenics (P<0.02).

6.3.12 Upregulation of *SPS*, *SUS* and *CelA1* genes in the transgenics

Since the importance of sucrose levels in the development of cotton fiber was well established, and *AnnBj*1 is showing 71% identity to native annexin (*AnnGh*1) of cotton which is highly expressed in cotton fibers during initiation and elongation, we studied the expression of the three genes encoding sucrose phosphate synthase (*SPS*), sucrose synthase (*SUS*) and cellulose synthase (*CelA1*), the expression of which leads to the synthesis of sucrose and cellulose in transgenic lines expressing the mustard annexin. In a reversible way, the availability of sucrose controls the expression of SPS in the cell**.** The expression level of the *SPS*, *SUS*, and *CelA1* was analyzed using RT-PCR in the salt stressed WT and transgenic plants 4-3, 5-7 and 11-1. The expression levels of these three intermediate genes participating in the cellulose synthesis pathway converting sucrose to cellulose genes were similar in untreated WT and transgenic lines. However repetitive experiments revealed that there was a significant increase in the gene expression levels after treatment with 100mM NaCl in the transgenic plants, in correlation with the elevated levels of cellular sucrose content in leaf discs, where as there is no significant elevation was observed in WT plants (Fig 6.12A). Similar analysis with cotton fibers collected from these plants also indicated that the transcripts of the above genes were upregulated under stress in the transgenic plants (Fig 6.12B).


A
Control
100mM NaCl stress
SPS
SUS
CelA1
18S rRNA
WT 4-3 5-7 11-1
WT 4-3 5-7 11-1
Arbitrary units
1400
1200
1000
800
600
400
200
0
WT
4-3
5-7
11-1
SPS
SUS
CelA1
SPS
SUS
CelA1
0
100
Sodium chloride (mM)

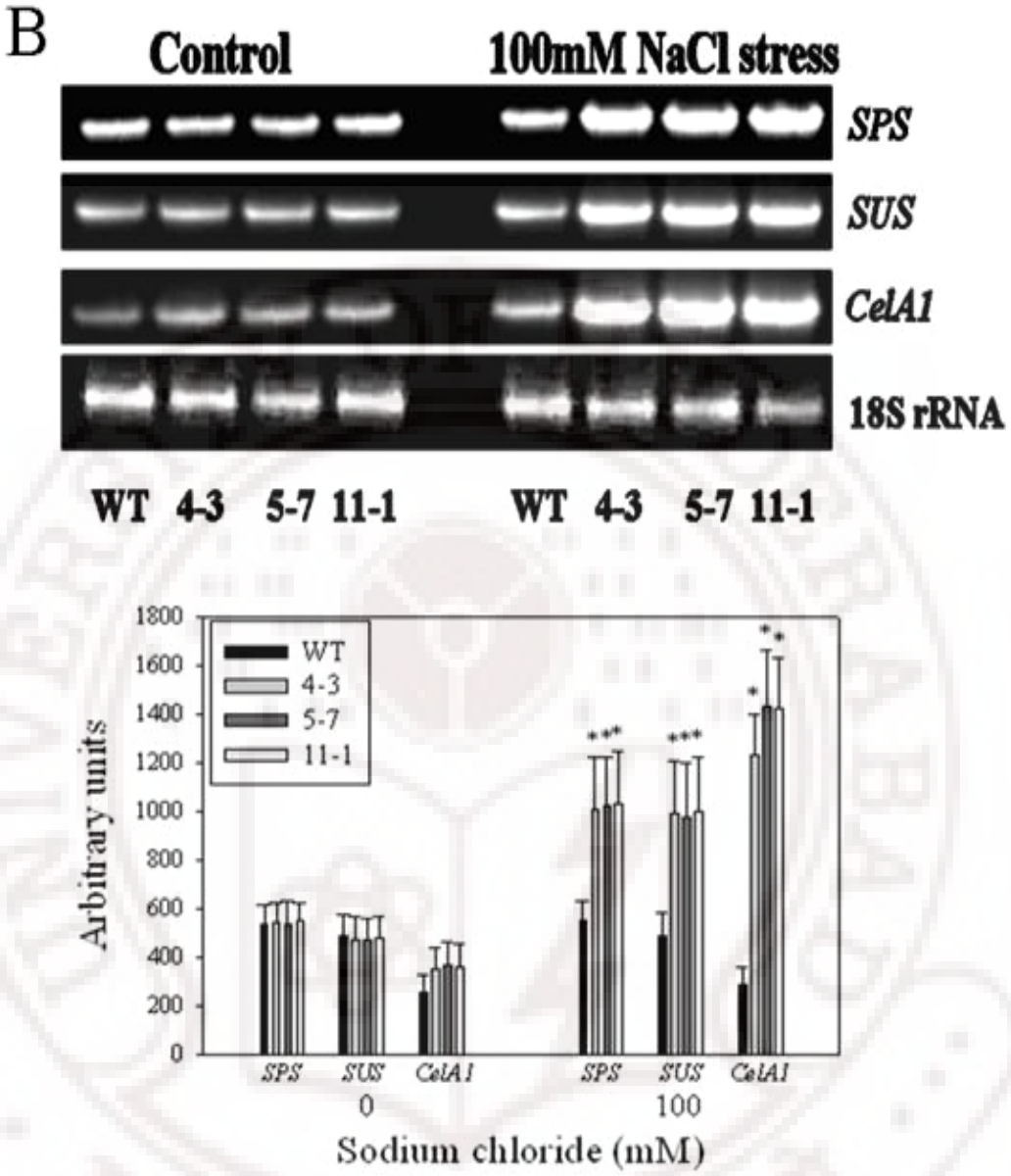


Figure 6.12 A. RT-PCR analysis showing the expression levels of Sucrose phosphate synthase (*SPS*), Sucrose synthase (*SUS*), Cellulose synthase A (*CelA1*) using RNA isolated from fresh leaves of untreated and 100mM NaCl treated WT and transgenic lines. 18S rRNA was used as internal control. B. Elevation of Sucrose phosphate synthase (*SPS*), Sucrose synthase (*SUS*) and *CelA1* genes in RT-PCR analysis performed from the fiber RNA from untreated and 100mM NaCl treated WT and transgenic plants. 18S rRNA was used as internal control.

6.3.13 Plant growth, cellulose content of the fiber and fiber quality assessment

When the WT and transgenic plants were grown under control conditions without any stress, there were no visible differences in the parameters such as phenotype, boll number and size, seed development, fiber yield and cellulose content etc. When the WT and transgenic plants (n=14) were treated continuously for 8 wk with 100mM NaCl and the phenotypic differences between them were notable; plant height was affected by salt treatment in WT, and boll size and number were decreased (Fig 6.13A and B). The seeds were shrunken with salt stress with diminished endosperm formation where as the seeds of transgenics line 5-7 were as normal as untreated control plant (Fig 6.13C).

In line with the sucrose levels and the upregulation of the genes involved in sucrose metabolism leading to the synthesis of cellulose, there was a significant increase (7-10%) in the cellulose content of the transgenic plants under stress. The cellulose content was almost unaffected by the salt stress treatment in transgenic plants, which was similar to untreated WT plants, but the WT plants treated with NaCl exhibited a drastic reduction in the cellulose content of the fibers (Fig 13D).

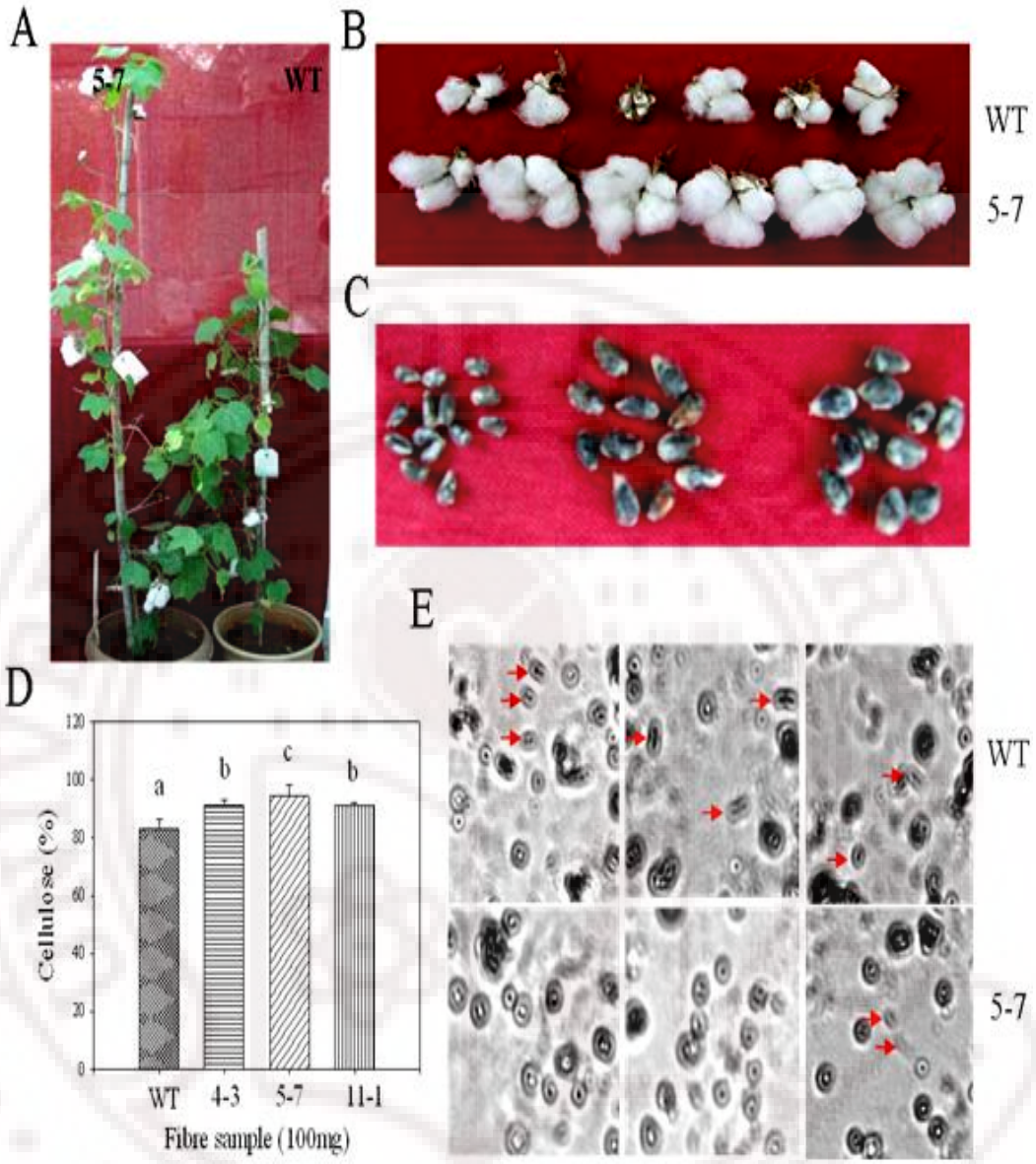


Fig. 6.13 A. Effect of NaCl stress on the phenotypic differences between WT and 5-7 after 8-wk treatment of 100mM NaCl on alternative days to 3-wk old plants. B. Boll formation in NaCl treated WT and transgenic plants at the end of the treatment. Upper panel shows improper development of bolls in WT plant. Lower panel shows the unaffected boll formation 5-7. C. Seed development in WT and 5-7 at the end of NaCl treatment. WT (left) showed the formation of shrunken seeds and transgenic seeds (middle), unaffected by NaCl stress, appear normal as untreated control plants (right). D. Total cellulose content of the salt treated plants indicating means ± SE. Bars represented by different letters differ significantly at $P<0.05$. E. Fiber cross sections (30DPA) of WT (upper panel) and 5-7 (lower panel). Flat ribbon shaped immature cells are prevalent with thin secondary walls (arrows) with lesser theta in WT. In 5-7, showing mature, circular fibers with thick secondary walls and higher theta are prevalent.

When the cross sections of the fibers were analyzed under light microscopy to observe the extent of fiber maturity under NaCl stress, WT plant evidenced a high IFC%, which indicates the immature fiber content with θ-value less than 0.25 compared to the transgenic lines 4-3, 5-7and 11-1 (Fig 13E). The maturity ratio values of the latter were significantly high, showing fewer immature fibers compared to the WT plant, indicating that normal fiber development was maintained in the transgenic under stress compared to the unstressed WT plant (Table 4).

Table 2. Fiber parameters in WT and 5-7 under salt stress. Mean ± SE values followed by different superscripts are significantly different at below mentioned *P* values.

Line	**Secondary wall area (µm^2)**	**Perimeter (µm)**	**Theta**	**Immature fiber content (IFC %)**	**Maturity ratio**
WT	113.4 ± 1.24^a	54.0 ± 1.90^a	0.408 ± 0.011^a	8.65^a	0.72^a
4-3	141.1 ± 2.20^c	55.9 ± 0.92^b	0.492 ± 0.009^b	5.34^c	0.85^b
5-7	148.6 ± 1.18^d	57.2 ± 0.67^b	0.516 ± 0.016^c	4.32^c	0.89^c
11-1	138.2 ± 1.14^b	56.6 ± 1.11^b	0.487 ± 0.011^b	6.01^b	0.84^b
WT (Unstressed)	156.6 ± 2.61^d	60.9 ± 1.24^c	0.541 ± 0.008^c	3.48^c	0.93^c
P value	<0.024	<0.02	<0.01	<0.015	<0.006

6.4 Discussion

Since annexins appear to be involved in fiber development in cotton, and might have a potential role in scavenging free radicals developed under stress because of the peroxidase activity possessed by the protein (Gorecka et al. 2005), we conducted a detailed functional analysis and fiber development studies in transgenic cotton plants expressing *AnnBj1*. An analysis of the stress parameters in transgenic plants indicated that they had enhanced relative water content, dry mass, root length parameters, enhanced total chlorophyll content and reduced TBARS under stress. The established substantial increase in the DW after stress imposition probably reflects the synthetic activity and enhanced carbohydrate metabolism as reported in transgenic plants of tomato expressing the SPS under constitutive promoter (Foyer and Ferrario 1994). Further, to get an insight into the physiological changes that could occur during stress conditions; we analyzed the carbohydrate levels in the leaves of the fully grown plants. A correlation was observed in the transgenic plants between the upregulation of the transcripts of genes for enzymes involved in sucrose and cellulose biosynthesis, cellulose content, and fiber parameters. The cotton transgenics with overexpressed spinach *SPS* maintained higher sucrose to starch ratio under controlled environmental conditions (Haigler et al. 2007).

Enhanced peroxidase activity in the transgenics

Recombinant *AnnAt1* purified from *Escherichia coli* or *Nicotiana benthamiana* expression systems possessed peroxidase activity *in vitro* and this activity could be due to the homology between the N-terminal region of the *AnnAt1* and heme-binding region of the plant peroxidases (Gidrol et al., 1996; Gorecka et al. 2005). The peroxidase activity of annexins is based on the presence of the conserved His40 residue in the first annexin repeat, which has similarity to heme binding domain of plant peroxidases (Clark et al. 2001, Gorecka et al. 2005; Jami et al., 2008; Laohavisit et al. 2009). The presence of His40 residue is identified in the sequence comparison analysis of AnnBj1 with other annexins and horse radish peroxidase.

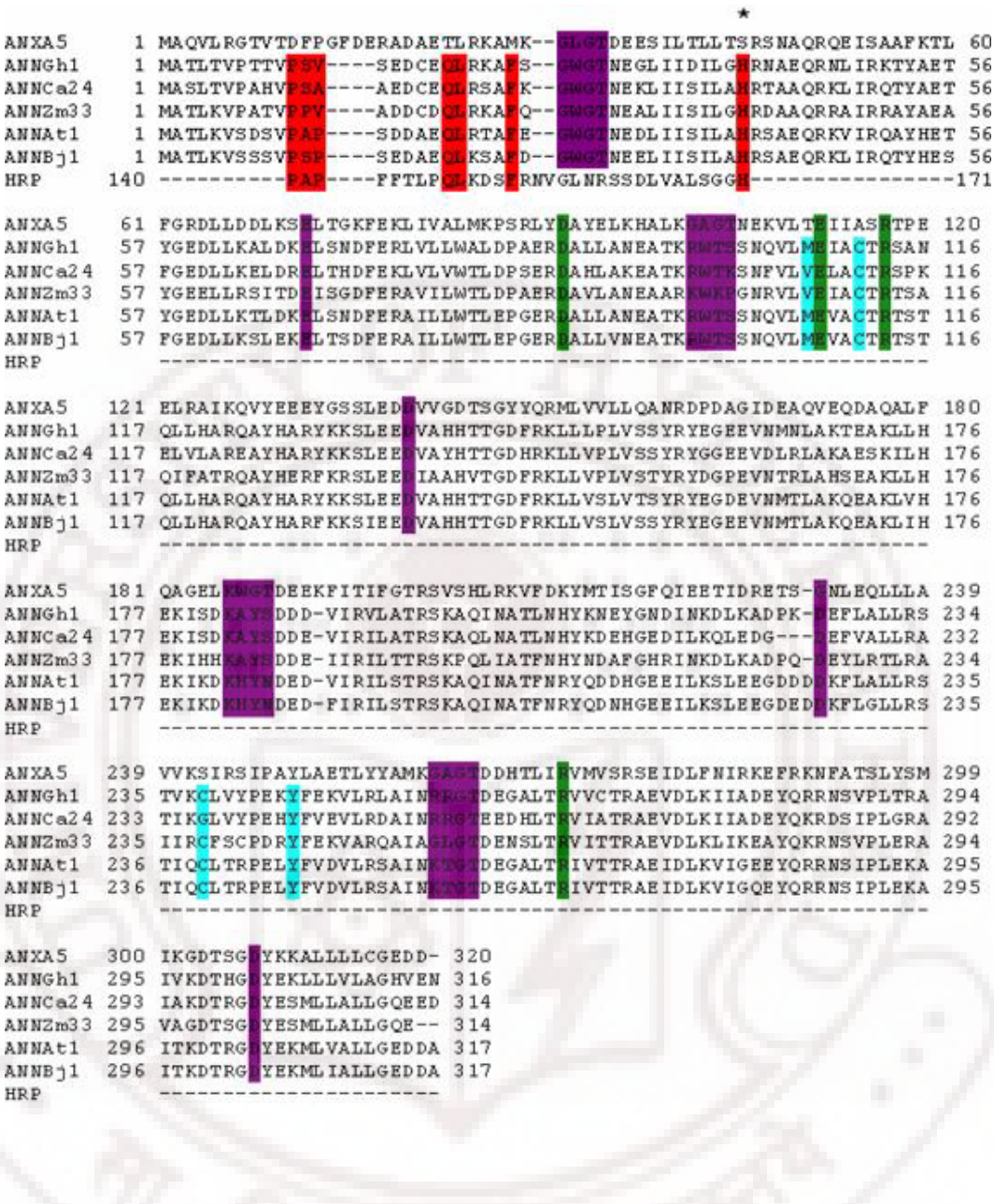


Figure 6.14 Alignment of plant amino acid sequences.
The following sequence data were obtained from GenBank/NCBI/EMBL RefSeq data libraries with the accession numbers: Human annexin 5 Anx5 (U05760) and HRP (*Armoracia rusticana* horseradish peroxidase, CAA00083). Amino acid sequence alignment was performed using ClustalW and edited in JalView (http://www.jalview.org; Clamp et al., 2004). The sequences highlighted are as follows: red, shows the heme binding motif of peroxidase from *Armorecia rusticana* (HRP/140-171) with the N terminus of annexins with identical residues highlighted and the conserved His residue marked (asterisks); blue, the S3 clusters (Hofmann et al., 2003) putatively involved in redox reactions; green, salt bridges involved in channels function of animal annexins; purple, endonexin (type II Ca^{2+} binding) sequences. The annexin sequences are ANN Bj1/1-317; ANX A5/1-320; ANNGh1/1-316; ANN At1/1-317, ANN Ca24/1-314; ANN Zm33/1-314.

The *in vitro* preliminary studies with recombinant AnnCa24 purified from *E. coli* also revealed that it possessed peroxidase activity (Mortimer et al. 2007). More recently, the *in vitro* peroxidase activity of a *Zea Mays* annexin and its calcium channel activity modulating the cytosolic calucium influx have been demonstrated (Laohavisit et al. 2009). The above findings support our studies on the enhanced total peroxidase activity in transgenics and with H_2DCFDA staining after NaCl treatment that revealed teh enhanced ROS accumulation in WT roots. In the study involving alternative oxidase overexpression, enhanced ROS accumulation in KCN treatment in WT plants was reported where as the transgenic plants showed significantly much lower ROS as determined by lipid peroxidation and ROS accumulation in roots (Umbach et al. 2005). Transgenic overexpression of glutathione S-transferease in Arabidopsis also resulted in the reduced ROS accumulation in transgenic roots in NaCl stress (Katsuhara et al. 2005). Further analysis to determine the ROS levels in the leaf samples treated with NaCl showed enhanced H_2O_2 levels in both WT and transgenic plants. The WT plants showed almost double the hydrogen peroxide content when compared to transgenics, which might also be responsible for the enhanced fluorescence in the stomatal guard cells of WT plants. Even though ROS play a central role in many signaling pathways in plants, the enhanced H_2O_2 levels in stress conditions were proved to be deleterious causing cellular damage (Miller et al. 2007). Previous studies with transgenic plants with enhanced stress tolerance displayed lesser H_2O_2 content in stress (Xue et al. 2009). In our studies also we observed a decrease in the H_2O_2 levels in transgenic plants under stress in compared to WT plants, which was in line with the less ROS detection in transgenic plants and tempting to suggest that the mustard annexin possess ROS scavenging activity. But further investigation is needed to know the relation between the stress tolerance and stomatal closure in the guard cells experiments in transgenic plants.

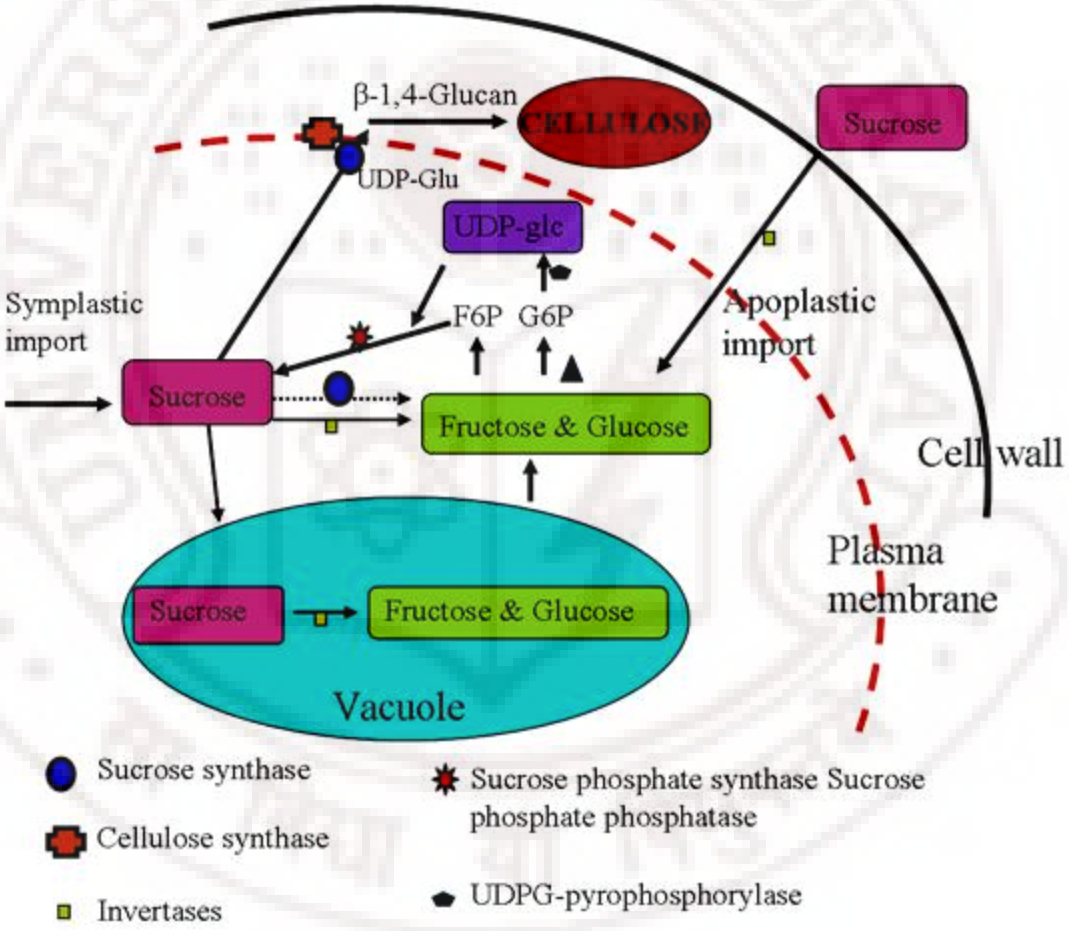
Previous reports showed that the modulation in cytosolic Ca^{2+} during salt stress can regulate the gene expression of P5CS encoding Δ-pyrroline-5-carboxylase synthetase, the first enzyme in the proline biosynthesis pathway (Knight et al. 1997, Parre et al. 2007). Proline has been shown to be a common

osmoprotectant to counter the stress induced by NaCl and other stress treatments (de Ronde et al. 2004, Verbruggen and Hermans 2008). It has also been shown to have hydroxyl radical scavenging property (Smirnoff and Cumbes 1989). Overexpression of genes involved in proline biosynthesis has been shown to confer significant levels of osmotic stress tolerance in transgenic plants. Accumulation of sugars in different parts of plants is enhanced in response to a variety of environmental stresses including salt stress (Prado et al. 2000). The present investigation on heterologous expression is shows the beneficial role of annexins in imparting tolerance to abiotic stresses, particularly osmotic stress and oxidative stress in cotton.

Elevated expression of the SPS, SUS and CelA1 genes in transgenics

In the sucrose synthesis pathway, SPS catalyses a reversible reaction of the formation of sucrose-P from fructose-P and UDP-glucose (Haigler et al. 2007) and its increased activity is correlated with the enhanced cellulose biosynthesis for secondary wall deposition (Babb and Haigler 2001). The elevated expression of SPS might be the cause for enhanced sucrose levels in the transgenic plants in recycling fructose along with UDP-glucose and provides the raw material for the secondary wall cellulose synthesis. We observed elevated expression levels of the three genes, *SPS*, *SUS* and *CelA1* in the annexin transgenics in leaf and fiber samples in transgenic plants when compared to WT plants under salt stress. The enhanced available sucrose content can trigger the reversible expression of *SPS* and subsequently *SUS* and *CelA1* genes in the cellulose biosynthesis pathway (Haigler et al. 2007) suggesting a role for annexin in the activation of the concerned genes, which control cellulose deposition in the fibers. Annexin are suggested to sreve in anchoring the accessory proteins such as sucrose synthase converting sucrose into fructose and UDP-glucose in the cotton fiber plasma membrane (Delmer and Potikha 1997) and an implication of AnnGh1 with cellulose synthase complex has been suggested previously by Hofmann et al. (2003). In the present case, the transgenic plants fared better compared to the WT plants in stress treatments with elevated sucrose content and upregulation of *SPS*,

SUS and *CelA1* and the elevated expression levels of these genes in transgenic plants could be an indirect effect of overexpressed annexin. Shin and Brown (1999) demonstrated that an annexin was highly expressed in the flower and root tissues, and fibers. The expression of *AnnGh1* was very high in cotton fibers up to 18 DPA, which is the active elongation period, and the transcript levels gradually diminished as the cotton fibers enter the secondary phase of development suggesting its role in the fiber cell elongation. However, till now the role of this annexin overexpression in cotton fiber development has not been clearly defined.



Xu et al., (2007) J. Int. Plant Biol. 49: 69-74.

Figure 6.14.The particulate Sucrose synthase (upper left) shown channels UDP-Glu to the cellulose synthase. All enzymes in the diagram have been found in cotton fibers. The bold arrows indicate reactions that involved in cellulose synthesis, includingnproduction of cytoplasmic UDP-Glc so that fructose released by SUS can be used by SPS to synthesize more sucrose.

Increased expression of genes involved in cellulose synthesis was correlated with the improved fiber quality

In the previous report on the overexpression of Arabidopsis sodium/proton antiporter in cotton, the salt stress imposed on transgenic plants was started at 50mM has been gradually increased up to 200mM NaCl (He et al. 2005). As we determined to see the effect of salt stress on fiber characteristics in both WT and transgenics, we conducted stress treatment with 100mM NaCl for a prolonged period i.e. 8 wks to complete maturation of bolls including seed maturation to facilitate the studies with cotton fiber parameters. The cotton boll formation was severely damaged by the continuous salinity treatment leading to the under developed shrunken seeds, where as the boll and seed size in transgenics were more or less similar to the untreated WT plants. This might be because of the elevated expression levels of the *SUS* gene in annexin transgenics under stress conditions. Similar observations with the overexpression of *SUS* in stress conditions were reported in the studies of Ruan et al. (2005) during the formation of endosperm. The cotton fiber is a very active sink cell, which uses sucrose in expansion and cellulose biosynthesis, and SUS is the key sucrolytic enzyme converting sucrose in to fructose and UDP-glucose. SUS transcripts and protein were localized in the fiber cells at the initiation of fiber development. In the transgenic cotton, suppressed *SUS* expression negatively affected the availability of hexoses and damaged the cell wall integrity due to reduced UDP-glucose supply leading to the formation of fiberless seeds suggesting that SUS can be used as a biochemical marker for the sink strength of fiber cell development (Ruan et al. 2003). The enhanced expression levels of *SUS* improved the fiber length and cellulose content of the transgenics plants under abiotic stress conditions in the present case.

Experimental evidence showed the enhanced sucrose levels in the leaves of the transgenic plants after salt stress, which is the foremost photoassimilate imported apoplastically form leaves to the cotton fiber cells in flower during their development (Ruan et al. 2003). Availability of enhanced sucrose content as a

substrate upregulates the *SPS* gene. Enhanced sucrose pools in the leaves are translocated to the fibers during elongation and secondary wall synthesis leading to enhanced cellulose depositions on the fibers for better fiber quality. Our analysis using the 30 DPA fibers showed that the transgenic plants had a significantly higher proportion of well developed and mature fibers compared to the fibers from the WT, which had a significant proportion of immature fibers, which was represented as IFC % under stress. This is consistent with the chemical analysis estimating the crystalline cellulose in different plants, which was up to 8% higher in transgenics supporting the enhanced secondary wall deposition in fibers. This clearly showed that the quality of cotton fibers in the transgenic plants overexpressing the mustard annexin did not reduce drastically under NaCl stress, which was severely affected by the same. These studies hold a lot of promise for cotton annexin transgenics with high economic importance.

6.5 Summary

In conclusion, the annexin transgenics plants of cotton showed high total chlorophyll content, lower lipid peroxidation levels, increased peroxidase activity and accumulation of protective osmolytes like proline and sucrose. Jami et al. (2008) also concluded the similar results by overexpressing the *AnnBj1* in tobacco in the response towards different stresses, but did not proceed further to identify the variation in the osmolytes levels and study the gene expression changes. Also, we have demonstrated the ROS scavenging activity in the epidermal peels of transgenic plants, which was not shown by Jami et al. (2008) in tobacco transgenic plants. These results were in concurrence with previous studies of various annexins responding to different stress conditions. When the WT and transgenic plants were compared under control conditions without any stress there was no significant difference among these phenotypically, physiologically and in biochemical parameters. These transgenic plants also possessed higher cellulose content in the fibers, which might be the indirect effect of annexin overexpression as enhanced sucrose availability in cotton fibers can lead to and the expression levels of the key genes participating in the cellulose synthesis pathway i.e. sucrose phosphate synthase (*SPS*), sucrose synthase (*SUS*) and cellulose synthase (*CelA1*) were significantly increased under the stress conditions along with enhanced peroxidase activity and enhanced proline content improving abiotic stress tolerance.

Chapter 7

Ethylene inducible E8 promoter driven fruit specific overexpression of tomato anionic peroxidase in ripened tomato fruits and evaluation for fungal resistance

7 Results

7.1 Developmental stage specific expression of *tap1* in tomato fruits

The expression levels of *tap1* gene at different stages of tomato fruit development was determined by northern blotting. The *tap1* is expressed exclusively in unripened stage of the fruit and it is almost undetectable in breaker and red ripened stage of the fruit (Figure 7.1).

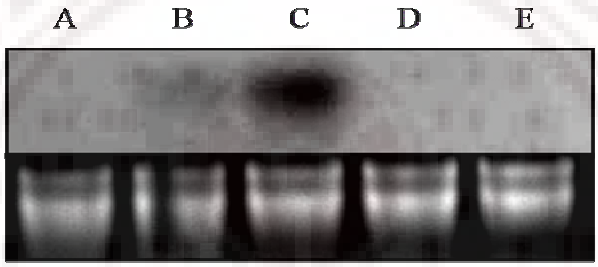


Figure 7.1 Endogenous expression of tap mRNA levels in tomato fruits at progressive stages of development. A. Very immature B. Immature green C. Mature green D. Breaker fruit with even mix of pink and green pigmentation E. Red ripe fruit with soft flesh. Lower panel shows the equal loading of 10 µg of total RNA isolated from different stages of tomato fruit.

In order to achieve the overexpression of wound and pathogen responsive *tap1* in red ripened stage of tomato fruits a construct was made for transformation of *tap1* under fruit specific ethylene inducible promoter E8 (obtained from Kolattukudy's lab) (Figure 7.2). The pBI101 vector containing E8-*tap1* was transformed in to *Agrobacterium* strain LBA4404, which was used in the tomato transformation experiments.

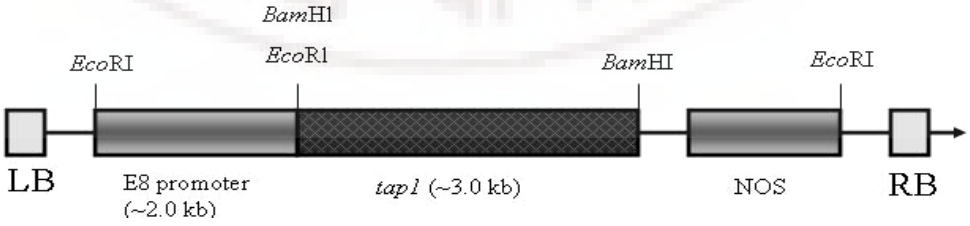


Figure 7.2 T-DNA of pBI101.1 binary vector with E8 promoter and *tap1*. CaMV 35S promoter in the original vector was excised out as an *Eco*RI fragment and E8 promoter was cloned.

7.2 Generation of transgenic tomato lines using cotyledonary explants

The transformation vector contains *nptII* gene encoding kanamycin resistance as a selectable marker. Successful regeneration was obtained from the cotyledonary explants of 8 d old tomato seedlings. The optimized transformation procedure included culture of cotyledonary explants on pre-culture medium (MS + 2 mgl^{-1} BAP + 0.1 ml^{-1} IAA) for 24 h. Explants were dipped in overnight grown *Agrobacterium* culture (LBA4404) and vacuum infiltrated for 10 min. They were blotted on sterile tissue paper to remove excess bacterial culture and continued on a regeneration medium supplemented with cefotaxim and kanamycin.

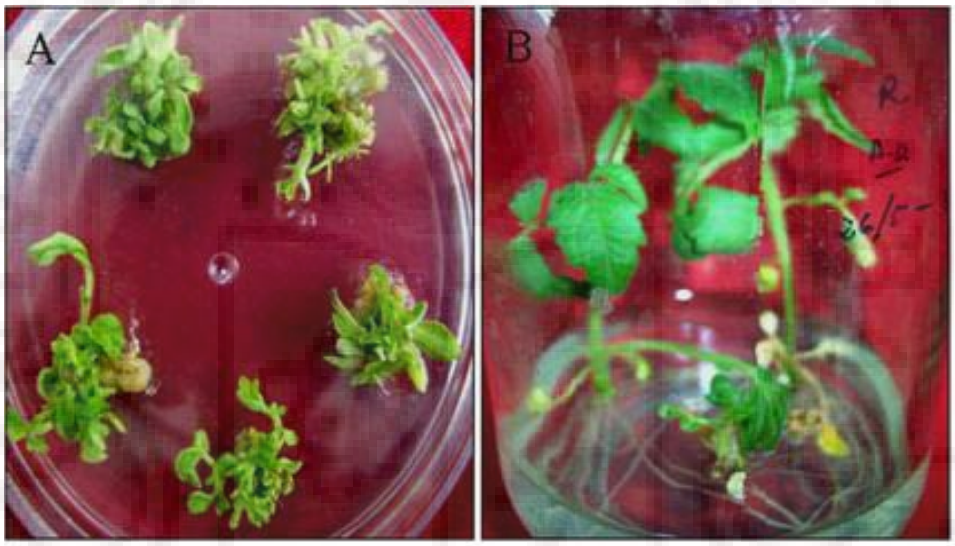


Figure 7.3 Regeneration and development of putative transgenic shoots from cotyledonary explants in tomato. A. Proliferation of green shoot buds from the cotyledonary explants on kanamycin medium. B. Rooting of the putative transgenic shoots on selection medium.

After the initiation of shoot buds, the explants were transferred on to elongation medium (BAP + GA_3, 1 mgl^{-1} each) to facilitate proliferation and elongation of shoots (Fig 7.3A). The putative transgenic shoots which tolerated the kanamycin concentration remained green where as non-transgenic shoots bleached completely. The green shoots grown up to 3 cm were cut at the internodal position and transferred to rooting medium containing IAA (0.1 mgl^{-1}).

Kanamycin ($40mgl^{-1}$). Rooted putative transgenic plantlets were carefully acclimatized to green house conditions (Fig 7.3B).

7.3 Molecular characterization of T_0 plants

Total genomic DNA was extracted from fresh young leaves of 2 wk old putative transgenic plants for preliminary screening by PCR amplifying the marker *nptII* fragment. A total 24 PCR positive plants were obtained further confirmed for the transgene integration by Southern blot analysis. For DNA digestion, *Xba*I was used which has a single site in the T-DNA region of the binary vector. The membrane was probed with the marker *nptII* instead of *tap1* because of the nativity of the latter in tomato genome. Among all the plants, 9 showed the signal when probed with *nptII*. The plants no. 6 is showing single copy integration and 11 is a double copy plant. The plants with three copy integration were 2, 4, 7, 8, 9 and 3, 5 showed four copy integrations (Fig 7.4A).

All these plants were allowed to flower and fruits were grown after self-pollinating the flowers. Seeds were collected from each plant carefully and molecular and physiological assays were performed in the T_1 generation plants. Preliminary analysis of the transgenic seedlings was done using the germination assays on kanamycin containing medium. The kanamycin germination experiment of the T_1 seeds revealed the typical Mendelian segregation pattern of the transgene. The kanamycin resistant seedlings were again transferred to greenhouse and Southern analysis was performed in the T_1 generation plants to identify the segregation pattern and copy number. The T_1 plants 2-a, 8-a, 9-a are the double copy plants and 8-a and 11-a are single copy insertion plants (Fig 7.4B).

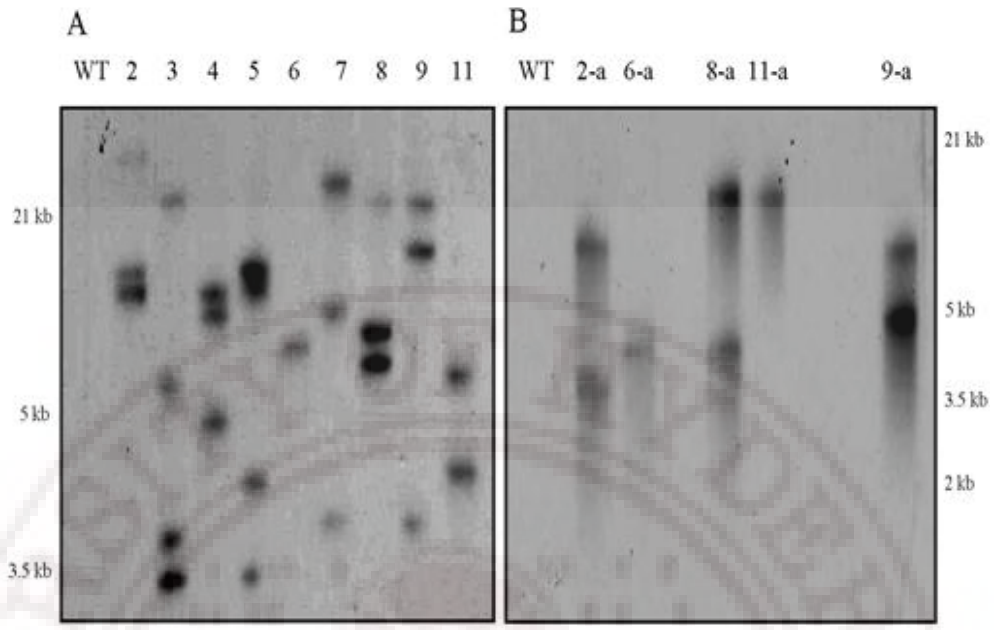


Fig 7.4 Southern blot hybridization in transgenic plants for analyzing copy number. A. Genomic DNA T_0 plants was digested with *Xba*I and probed with PCR amplified gel eluted *nptII* fragment. B. Southern analysis in T_1 plants- DNA was digested with *Xba*I and probed with *nptII* fragment.

After Southern confirmation the T_1 plants were allowed to set the fruits with self-pollination. The overall phenotypic traits and performance of these transgenic lines were similar to the non-transformed WT plants.

7.4 Transgene expression in transgenic lines

Total RNA was isolated from pericarp tissue of the fruits in three stages from transgenic and WT plants. These are A-mature green, B- Breaker and C-Red ripened fruit with the initiation of softening of the fruit flesh (Fig 7.5). The RNA was electrophoresed on the denaturing formaldehyde gel and the membranes were probed with the coding region of the *tap1* gene (~1.5 kb). The *tap1* expression levels in T_1 transgenic lines were analyzed in three different stages of the fruit. Transgenic transcripts were detected in all the T_1 lines with varied levels but they were not detected in WT plants.

In general the WT fruit did not show any expression of the *tap1* in breaker and red ripe fruits whereas the transgenic plants showed the expression of the *tap1* in these two stages. The expression levels of the *tap1* in breaker and red ripe

fruits were also in correlation with the general ethylene levels reported in the tomato fruit. There will be an ethylene outbreak and sudden increase in the breaker stage of the tomato fruit, which decreases subsequently when it passes through the ripening stages. The ethylene inducible *tap1* expression also decreased in the ripened fruits of transgenic plants in similar fashion, while WT plants did not show any message of *tap1* in these fruit stages. Based on the transcripts detected in the transgenic plants, they could be classified into high expression and low expression lines. The Plant 6-a is a low expression line and 11-a is the high expression line with 8-a showing the moderate expression (Fig 7.5).

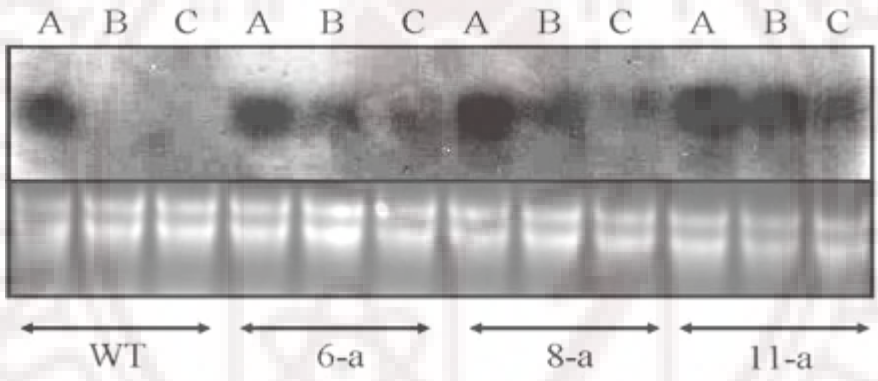


Fig 7.5 Northern blot analysis showing the expression of *tap1* in transgenic tomato fruits in different developmental stages. A - Mature green, B – Breaker fruit, C – Ripened red fruit. Expression levels of *tap1* in WT and T_1 transgenics 6-a, 8-a and 11-a were analyzed where 6-a is a low expression plant and 11-a is a high expression plant. Lower panel shows equal loading of 10 µg of total RNA electrophoresed for blotting on to the membrane.

7.5 Estimation of total peroxidase activity in fruits

The pericarp (100mg) of ripened fruits with very soft flesh was used to prepare the fruit extract (100 µl). The fruit extract (100 µg protein) from WT and transgenic fruits was dot blotted on a nitrocellulose membrane and peroxidase activity was estimated using ECL substrates followed by exposure to autoradiography. Horse radish peroxidase was loaded as a positive control. While the WT sample did not showing any signal, all the transgenic plants showed

peroxidase activity with activity levels highly correlating with the transgene expression levels (Fig 7.6).

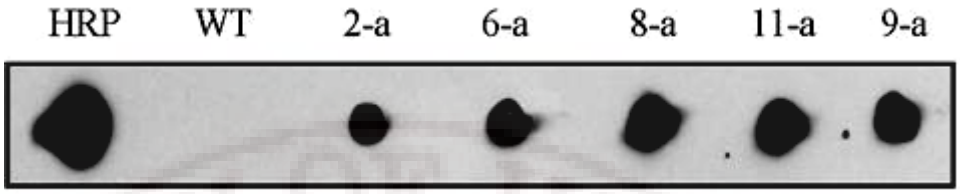


Figure 7.6 Dot blotting of the fruit extract (100 μg) on nitrocellulose membrane and probing with ECL substrate revealed the peroxidase activity in transgenic fruits. The low expression line 6-a showed lower signal when compared to 8-a and 11-a with enhanced signal which are in correlation with the tap1 expression levels.

7.6 Estimation of polyphenols by autofluorescence

Red fruits from both WT and transformed plants were wounded by uniform and slight removal of fruit exocarp and then allowed to heal for another 7 days. The wound healing periderms were excised and fixed overnight in 100mM sodium phosphate buffer containing paraformaldehyde. Samples were cut into thin sections (8-10 μm) using a microtome, UV irradiated and autofluorescence of cell wall polyphenolics was observed under confocal microscope with a magnification of 100X at $\lambda_{(330\text{-}380nm)}$. The microscopic analysis with numerous samples showed that the enhanced autofluorescence originating from the cell wall phenolics was more intense in the transformed fruits. This result suggests that the total phenolic deposition in response to wounding was associated with enhanced TAP activity in transgenic plants (Fig. 7.7).

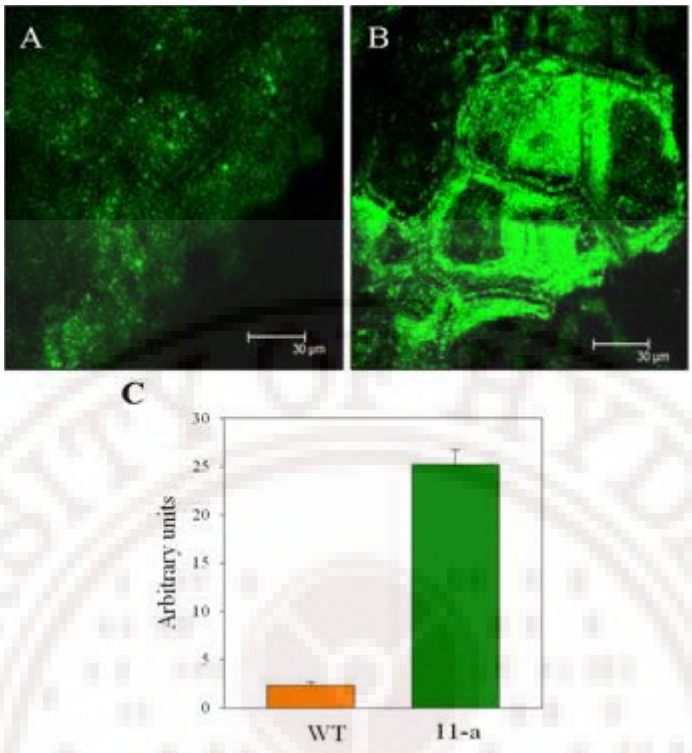


Figure 7.7 Autofluorescence of polyphenolic compounds in the cell walls of wound healed periderm in red fruits of WT and transgenic (11-a) plants. Tissue samples were prepared after 72 h of wounding and observed under a Confocal microscope ($\lambda_{330\text{-}380nm}$).

7.7 Evaluation of transgenic fruits for fungal resistance

7.7.1 Fruit bioassay with *Alternaria alternata* f. sp. *Lycopersici* (Black mold)

Inoculation sites on the ripening tomato fruit were made with pin prick by a needle. Inoculation of a 10 µl sample contained ~1 × 10^5 spores per ml was performed and the fruits were incubated under humid conditions. This insured that 100% of the inoculated sites resulted in infection in control fruits. Lesions developed as blackening circular depressions. Mean lesion diameter of lesions on *tap1* overexpressing fruit was compared with mean lesion diameter on susceptible WT fruit. A total of 10 fruits each from WT and transgenic plants were observed for three days after inoculation for lesion diameter measurements. The lesion size in WT plants was approximately 10 times more (up to 9.5 mm) than transgenic fruits, which showed significant resistance to black mold disease (Fig 7.8).

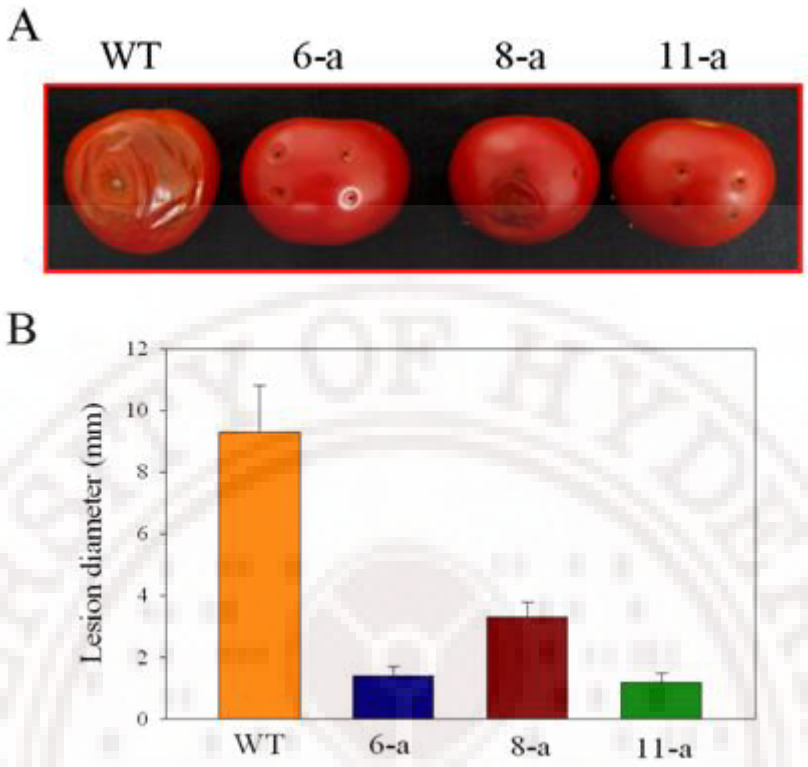


Figure 7.8 Fruit specific overexpression of *tap1* and pathogenecity of *Alternaria alternata* black mold. The area of pericarp for inoculation on green house grown ripened fruits was surface sterilized. Five microliters of *A. alternata* conidia ($1x10^5$ conidia per microliter) was inoculated on wounds made with a needle. High humidity was maintained in incubation of these fruits. Lesions were measured daily for three days for ten fruits each from WT and transgenic plants in three individual experiments. Average values were plotted with the mean ± SD.

7.8 Germination assay of *Fusarium solani*

Fusarium solani is an important rot causing fungal pathogen in tomato fruits after harvesting. The conidia were extracted from the fully grown mycelium plate at a concentration of (10^3 conidia/ µl). The fruit extracts were prepared in phosphate buffer and used to incubate the conidia for germination studies. Two different concentrations of fruit extracts were maintained 50% (Fig7.9 A-D) and 90% (Fig. 7.9 E-H) in sterile distilled water and the growth of the mycelium was monitored in WT and transgenic fruit extracts. The inhibition of spore germination and growth of mycelium was more in 90% extract when compared to

50% dilution. The germination rate was very high in WT samples and mycelial growth was comparatively faster than transgenics.

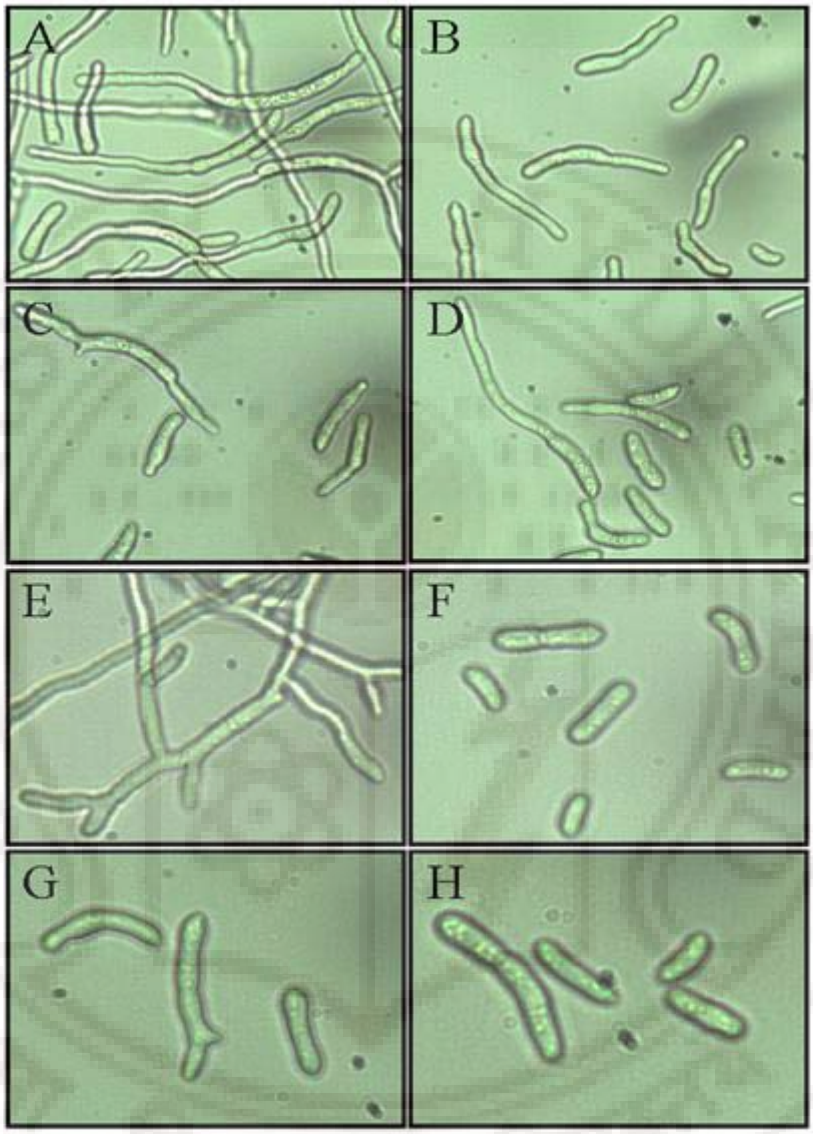


Figure 7.9 The germination of *Fusarium solani* spores in fruit extracts. A-D refer to germination in 50% fruit extract and E-H contains 90% fruit extract.
A and E - WT; B and F - 6-a; C and G - 8-a; D and H – 11-a.

7.9 Discussion

Tomato (*Lycopersicon esculentum* Mill.) is considered to be an important vegetable crop, which needs no introduction about its nutritional value. It is a model species for introduction of agronomically important genes into dicotyledonous crop plants. Insights from this work will contribute to the development of methods for producing attractive, safe and healthy tomato fruit for human consumption During ripening, fruits undergo a crucial developmental transition from being unripe, green, and resistant to or tolerant to the fungal pathogens, to being ripe, red, and being susceptible to damage by the pathogens (Alexander and Grierson, 2002). The natural theory is that green fruits respond uniquely to pathogen, perhaps differently from red fruit, and from the leaf and stem parts of the plant. This response changes in ripe fruit to encourage the fungus to break down the fruit and allow the dispersal of mature seeds. But this natural phenomenon makes ripened fruits vulnerable to pathogen attack causing post-harvest deterioration to fruit crops such as tomato.

Anionic peroxidases are involved in catalyzing polymerization of cinnamoyl groups into lignin, suberization of cell walls, accumulation of phenolic polymers, and binding of extensin to cell wall (Mohan and kolattukudy 1990). Lignification and suberization involve synthesis of phenolic polymers, which act as barriers to prevent the pathogen entry and this polymerization is catalyzed by cell wall associated anionic peroxidase (Kolattukudy et al., 1992). The primary responses of polymerization of cell wall polymers upon pathogen attack is lacking in the ripened fruits. A correlation between sustained anionic peroxidase activity and the ability of the tomato fruit tissue to withstand fungal attack has been previously suggested by Lurie et al. (1997). When tomato has been genetically transformed with tobacco anionic peroxidase, leaf feeding by first instar *Helicoverpa zea* and *Manduca sexta* was significantly reduced on both intact plants and leaf discs when compared to WT plants. Green fruits from transgenic plants also considerably decreased the mortality of first instar larvae (Dowd et al., 1999).

Promoter, as one of foremost transcript regulators, plays a major role in gene expression. The Cauliflower mosaic virus 35S (CaMV 35S) promoter can efficiently drive foreign gene expression in plant cells. However, this promoter does not confer any specificity-neither tissue specificity nor plant developmental stage specificity on exogenous gene expression leading to lower expression levels (Smigocki and Owens 1988). E8 promoter has been employed successfully to drive the expression of exogenous genes in transgenic tomato fruits (Yakoby et al., 2006). The results reported in this study indicate a potential agricultural value for these transgenic tomato plants. In order to explore the gene over-expression profile driven by E8 promoter in tomato fruits, genetic transformation was performed using tomato cultivar Arka Meghali cotyledonary explants. E8 gene expression is temporarily regulated by ethylene during fruit ripening. At the onset of fruit ripening, E8 mRNA showed an increase over the level of ethylene (Deikman and Fischer 1988, Deikman et al., 1998).

Ethylene is a small, readily diffusible hormone that has an important role integrating developmental events with external stimuli. It is a critical component of such diverse developmental processes such as seed germination, fruit ripening, abscission, and senescence. It is also an important stress hormone. Adverse biotic or abiotic stimuli usually lead to ethylene synthesis. This ethylene, in turn, slows down plant growth until the stress is removed. At the level of gene expression, ethylene induces transcription of many genes in response to a multitude of environmental and developmental stimuli.

The most noticeable characteristics of the transgenic peroxidase plants was the wilting phenotype observed in tobacco (Lagrimini et al., 1990) and tomato (Lagrimini et al., 1992) when overexpressing an anionic tobacco peroxidase gene. This phenotype appeared when the plant reached maturity and apparently was due to the reduced development of the radicular system, as a consequence of indoleacetic acid (IAA) metabolism by the peroxidase (Lagrimini et al., 1997a). However as expected we did not observe any morphological and physiological differences in the non-transformed and transformed tomato plants because of the tissue expression of the *tap1*. The flowering and fruiting in

transgenic plants were similar to WT plants. The transgenic fruits showed enhanced resistance levels in the red ripened stage of the fruit with enhanced ethylene levels. The fruit life was increased after harvesting them from the plants in transgenics under control conditions without any treatment (data was shown).

Peroxidases have indirect antimicrobial activity by catalyzing oxidative crosslinking of proteins and phenolics in the plant cell wall, and thus protect the host from degradation by hydrolytic enzymes produced by the pathogen's. In previous reports many lines of indirect evidences suggested the involvement of anionic peroxidase in suberization. Time period of suberization, expression and localization of anionic peroxidase were exactly correlated upon wounding in tomato fruit and potato tubers; by ABA in potato and tomato tissue cultures; by pathogens in tomato fruits (Roberts and Kolattukudy 1989; Robb et al., 1991). Their expression levels were also induced to high levels by fungal elicitors in tomato cell cultures (Mohan and Kolattukudy, 1990). In our studies, we observed the enhanced fluorescence of polyphenolic compounds in the wounded periderm samples of anionic peroxidase overexpressed transgenic fruits, which is in support of the previous reports of suggested involvement of anionic peroxidases in polymerization of suberin monomers. Wound induced deposition of polyphenol in peroxidase overexpressed transgenic plants was earlier reported by Lagrimini, (1991).However, studies with anti-(tap1/tap2)-expressing transgenic plants with abolished anionic peroxidase activity did not show any reduction in the fluorescence levels of green tomato fruits after wounding suggesting that the multiplicity of the peroxidases in plants is playing a role in the substitution of TAP activity in transgenic plants.

7.10 Summary

It was proved that tomato anionic peroxidase (*tap1*) is strongly expressed in mature green fruits, but it is not detected in red ripened fruits. The diminished *tap* activity in ripened fruits of tomato is in corroboration with their vulnerability to various fungal pathogens when compared to green fruits. In this study, we explored the potential use of E8 promoter in the ethylene inducible fruit specific expression of the *tap1* gene in the ripened tomato fruits. Transgenic tomato plants were developed overexpressing *tap1* gene under fruit specific ethylene inducible promoter E8. The transgenic plants were confirmed by PCR, Southern and northern analyses. The peroxidase activity was detected in the transgenic ripened fruits with very soft flesh, which was not detected in WT ripened fruits. The transgenic fruits overexpressing *tap1* showed enhanced polyphenolic accumulation after 72 h of wounding. They were also resistant to *A. alternata* inoculation. The spore germination and growth of the mycelium *F. solani* were also inhibited in the transgenic fruit extracts.

Overall, the transgenic ripened tomato fruits were more resistant to pathogen attack, which is a promising development towards increasing the life of the tomato fruits by controlling post-harvesting pathogen attacks.

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Efficient regeneration from hypocotyl explants in three cotton cultivars

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Abstract

A high frequency *in vitro* shoot bud differentiation and multiple shoot production protocol from hypocotyl segments of 8 to 10-d-old seedlings of cotton has been developed. Murashige and Skoog (MS) basal medium with Nitsch and Nitsch vitamins was found to be optimal in shoot regeneration. A combination of 2 mg dm^{-3} thidiazuron and 0.05 mg dm^{-3} naphthaleneacetic acid was the most effective for shoot regeneration (76 %) and an average of 10.6 shoots per responding explant. Combination of the cytokinins benzylaminopurine and kinetin induced better regeneration response than their individual treatments. Supplementation of the culture medium with ethylene inhibitor silver nitrate and activated charcoal showed beneficial effects. Optimal rooting was obtained on half-strength MS medium supplemented with 1 mg dm^{-3} indolebutyric acid and activated charcoal. Scanning electron micrographs of *in vitro* cultured explants revealed that shoot primordia were formed *de novo*.

Additional key words: benzylaminopurine, ethylene inhibitor, hyperhydricity, multiple shoot regeneration, thidiazuron.

Introduction

Cotton is a high value commercial crop and its importance needs no introduction. Genetic engineering in cotton has been very painstaking as only cvs. Coker or Acala were amenable to genetic transformation and regeneration. The development of protocols for regeneration in other cultivars started recently (Ali *et al*. 2004, Aydin *et al*. 2004, Jin *et al*. 2006). The proliferation of pre-existing meristems from cotyledonary nodes, primary and tertiary leaf nodes, *etc*., into elongated multiple shoots *in vitro* has been used (Saeed *et al*. 1997, Agrawal *et al*. 1997, Gupta *et al*. 1997, Hemphill *et al.* 1998, Morre *et al*. 1998, Zapata *et al*. 1999, Hazra *et al*. 2000, Caramori *et al*. 2001, Ali *et al*. 2004). However, the development of shoots *de novo* in a short period of time is a more advantageous approach because of the possibility of obtaining non-chimeric transgenic plants. The hypocotyls sections were used as explants for direct shoot organogenesis earlier but the efficiency of intact plantlet regeneration reported was low (Ouma *et al*. 2004) and in fact there was no clear mention about the frequency of regeneration and rooting in the report.

In the present communication, we describe an efficient, rapid, genotype-independent regeneration protocol for obtaining direct shoot organogenesis from hypocotyl explants, with successful rooting of the regenerated shoots.

Materials and methods

Seed germination: Mature de-linted seed of cultivated cotton (*Gossypium hirsutum* L.) cultivars Bharani, Durga and JKCH-99 (provided by *JK Agrigenetics*, Hyderabad, India) were surface sterilized using 70 % (v/v) ethanol for 2 min. Subsequently, the seeds were sterilized by agitation in 4 % sodium hypochlorite (v/v) (*Qualigens*, Mumbai, India) for 15 min followed by 4 - 5 rinses with sterile double distilled water. Seeds were soaked in sterile water for 4 - 5 h and kept for germination in culture bottles with sterile moist tissue paper or cotton with half-

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Abbreviations: AC - activated charcoal, BAP - benzylaminopurine, EM - elongation medium, GA_3 - gibberellic acid, IAA - indoleacetic acid, IBA - indolebutyric acid, KIN - kinetin, LS - Linsmaier and Skoog, MS - Murashige and Skoog, NAA - naphthaleneacetic acid, NN - Nitsch and Nitsch, RIM - rooting medium, SIM - shoot induction medium, TDZ thidiazuron.

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strength MS liquid medium under dark for 48 h. When radicles emerged, seedlings were transferred to 16-h photoperiod (irradiance of 70 µmol m^{-2} s^{-1}) and temperature of 28 ± 1 °C.

Seedlings (8 to 10-d-old) were collected and their cotyledons were aseptically removed. Acropetal dome like apical bud was visible at this time and a proximal cut was made approximately 2.0 mm below the apical meristematic region and 0.5 cm explants of hypocotyls were prepared. These explants were placed horizontally on the medium and maintained in the above-mentioned photoperiod conditions.

Culture medium: The morphogenic potential of hypocotyl segments of the three cultivars was tested on MS basal medium with 3 % glucose (*Himedia*) and with vitamins according to Murashige and Skoog (1962; MS), Gamborg *et al*. (1968; B_5), Nitsch and Nitsch (1969; NN), Linsmaier and Skoog (1965; LS), or Lloyd and McCown (1980; McCown) and these media were designated as MS_1, MS_2, MS_3, MS_4 and MS_5, respectively. The culture medium was solidified using 2 g dm^{-3} *Phytagel* (*Sigma-Aldrich*, St. Louis, USA). Initial factorial experiments were performed with the above mentioned vitamin combinations with MS basal media, and different concentrations of cytokinins, thidiazuron (TDZ; 0.5 - 3.0 mg dm^{-3}), benzylaminopurine (BAP; 0.5 - 5.0 mg dm^{-3}) and kinetin (KIN; 1.0 - 5.0 mg dm^{-3}) each in combination with naphthalene acetic acid (NAA; 0.01 - 0.1 mg dm^{-3}) were tested. The experiment was performed following a randomized block design. Later synergistic effect of cytokinins TDZ, BAP, KIN at concentrations 0.5 - 3.0 mg dm^{-3} each was examined. In another experiment, varying concentrations of silver nitrate (1 - 9 mg dm^{-3}) were added to MS_3 with TDZ and NAA. Control experiments were performed in parallel with these trials. The third experiment included 0.1 % activated charcoal (AC) in MS_3 with TDZ, NAA and silver nitrate. Different auxin concentrations like indole butyric acid (IBA), NAA and indole acetic acid (IAA) (0.01 - 1.0 mg dm^{-3}) were tried for root induction. Rooting medium comprised half-strength MS basal medium with 3 % sucrose and 1 g dm^{-3} acid washed neutralized AC. The pH of the culture medium was adjusted to 5.8 prior to adding AC and *Phytagel* and sterilized by autoclaving at a pressure of 1.1 kg cm^{-2} and a temperature of 121 ºC for 20 min. Filter sterilized silver nitrate was added to the culture medium before dispensing into the Petri dishes. Further, experiments were conducted by culturing hypocotyls on the shoot induction medium (SIM) comprising optimized concentrations of growth regulators.

Optimization of activated charcoal and silver nitrate: Concentration of activated charcoal was maintained at 1.0 g dm^{-3} in all the experiments and silver nitrate concentration was optimized using various concentrations of 1.0, 3.0, 5.0, 7.0 and 9.0 mg dm^{-3} with MS_3 containing 2.0 mg dm^{-3} TDZ and 0.05 mg dm^{-3} NAA. The experiments were conducted simultaneously with or without AC and silver nitrate to observe the additive effects of the same on the morphogenic potential of explants.

Elongation of shoot buds: Explants showing visible shoot primordia after two subcultures of 10 d each on a culture medium containing TDZ were transferred and maintained on a medium with 2.0 mg dm^{-3} BAP and 0.1 mg dm^{-3} NAA to obtain full growth of individual shoots (SIM). After 3 - 4 subcultures on SIM, shoots that reach 1.0 cm in height were cut at the internodal region and were cultured on elongation medium (EM) fortified with 1.0 mg dm^{-3} BAP and 2.0 mg dm^{-3} GA_3. From each organogenic clump, 3 - 5 shoots were excised and the explants with developing buds were transferred back to SIM. After two subcultures of 10 d duration each, shoot primordia were grown to individual shoots and the shoots on elongation medium were ready (2 - 3 cm) for root induction.

Rooting and transfer to soil: Elongated and well-developed shoots (> 2.0 cm) were excised and transferred to half-strength MS medium (RIM) augmented with different concentrations of NAA, IBA and IAA (0.01, 0.1 or 1.0 mg dm^{-3} each) individually for root initiation. In subsequent experiments, shoots were cultured on MS_3 with 1.0 mg dm^{-3} IBA, which has been observed as the optimal concentration for rooting. Well-rooted plantlets were transferred to small pots containing soil and *Vermiculite* (1:1). Later, these were transferred to bigger pots and maintained under greenhouse conditions.

Statistical analysis: Cultures were observed on visual basis and the shoot primordia were counted microscopically. The regeneration percentage was calculated as the percent of responding explants with a minimum two shoots each out of the total cultured explants. The data were analyzed using *ANOVA* for a completely randomized design and the treatment means were compared using *MSTAT* software and Duncan's multiple range test.

Scanning electron microscope studies: The proximal parts of the hypocotyls were fixed in 2.5 % glutaraldehyde at 4 ºC for 4 h. These tissues were removed, washed with double distilled water and again fixed in 1% OsO_4 for 2 h at 4 ºC. After a few washes in double distilled water, the specimens were dehydrated in graded ethanol solutions. Tissues were mounted on specimen stub Critical point drying was done in *Palaron Jumbo Critical Dryer*. Gold sputter coating was applied on the samples under reduced pressure After coating, the proximal region of the tissues were examined under scanning electron microscope (*FEI XL 30 ESEM*) at University of Hyderabad.

Results

Germination frequencies in cotton seeds was 94, 88, and 72 % in Bharani, Durga, and JKCH-99, respectively. The germination percentage was distinctly higher, if seeds were incubated in culture bottles containing filter papers moistened with half-strength MS liquid medium, when compared to germination on MS agar medium.

Among different explants tried for *de novo* regeneration, hypocotyls gave a better response. Age of the seedlings from which explants were prepared influenced the regeneration frequency considerably. In the beginning, 2 to 20-d-old seedlings were tested and explants from 8 - 10-d-old seedlings were chosen for further analysis because of their better morphogenic ability (data not shown). When hypocotyl sections (0.5 - 1 cm) with apical bud removed were placed horizontally on the medium (Fig. 3*A*), both ends of the hypocotyls bulged and proximal ends differentiated shoot buds by the end of second week of culture. These microscopic buds developed into individual shoots at the proximal region whereas, it was a simple cell division and minor callus formation at the distal end of the explant.

Among all vitamin combinations added to MS basal medium along with 2.0 mg dm^{-3} TDZ and 0.05 mg dm^{-3} NAA, MS_3 (MS salts with NN vitamins) was most responsive in producing highest regeneration (76 %) at the end of 5th week. Therefore this medium was selected for further studies (Fig. 1). The order of vitamin combinations influencing the regeneration was NN > Mc Cown > MS >LS > B_5 and the least response was recorded in MS_2.

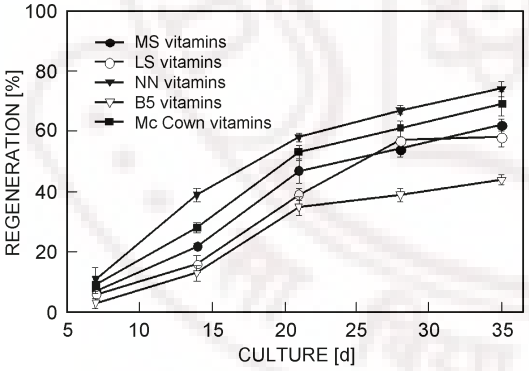

Fig. 1. Regeneration of hypocotyl explants using different vitamin (according to media MS, LS, NN, B5 and Mc Cown) added to MS basal medium comprising 2 mg dm^{-3} TDZ, 0.05 mg dm^{-3} NAA, 5 mg dm^{-3} $AgNO_3$ and 1 g dm^{-3} AC as constant components. Regeneration percentage has been calculated as the percentage of the responding explants out of the total explants cultured. *Vertical bars* represent SE of the means; $n = 20$.

Experiments with TDZ (0.5 - 3.0 mg dm^{-3}), BAP and kinetin (1.0 - 5.0 mg dm^{-3} each) and NAA (0.01 - 0.1 mg dm^{-3}) resulted in good regeneration response. Besides attaining a maximum number of responding explants, the highest number of shoot primordia (19.2 per responding explant) (Table 1) and a maximum number of shoots per explant (10.6) with high regeneration percentage up to 76 % was attained on 2.0 mg dm^{-3} TDZ and 0.05 mg dm^{-3} NAA (Table 2, Fig. 2). This was followed by the combination of BAP and KIN (12.3 shoot buds per responding explant). Lower concentrations of TDZ induced the formation of high number of shoot primordia (Fig. 3*B*) and a many of them were converted into individual shoots, whereas numerous shoot primordia appeared at higher TDZ concentrations developed into hyperhydric shoots associated with fasciated water soaked callus. Hence, explants with shoot primordia from TDZ medium were transferred to 2.0 mg dm^{-3} BAP and 0.1 mg dm^{-3} NAA after two subcultures to obtain optimal growth of shoot buds into individual shoots. Shoots cultured on TDZ medium also showed delayed rooting by more than eight weeks with 1 - 2 roots per shoot only.

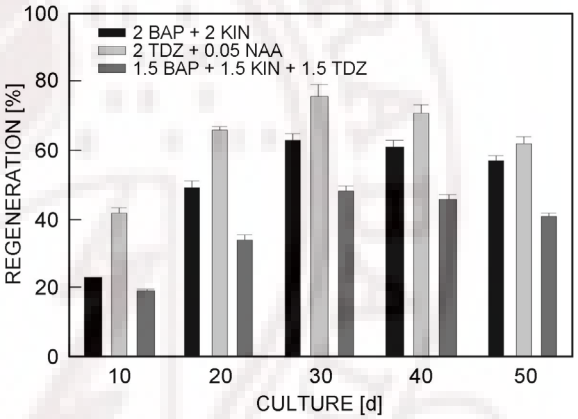

Fig. 2. Regeneration of hypocotyl sections in MS basal medium augmented with NN vitamins, 5 mg dm^{-3} $AgNO_3$ and 1 g dm^{-3} AC with different growth regulator combinations [mg dm^{-3}]. *Vertical bars* represent SE of the means; $n = 20$.

The shoot elongation was observed on EM with 1.0 mg dm^{-3} BAP and 2.0 mg dm^{-3} GA_3 (Fig. 3*C*) From different media tried for rooting of well-developed shoots, highest rooting percentage (86 %) was obtained on medium with 1.0 mg dm^{-3} IBA (Table 3, Fig. 3*D*). Though higher levels up to 10.0 mg dm^{-3} IBA induced rooting on shoots, it was associated with callus at the cut end. The rooting of shoots on RIM with NAA was 75 % but with less number of roots per shoot than on RIM with IBA. Rooting on RIM with IAA was very poor. The survival rate of plantlets rooted on IAA appeared to be less because of the callus formation.

Activated charcoal (AC) enhanced the recovery of shoot primordia into elongated shoots with good internodal length. Leaves were large in size and dark green in colour. Activated charcoal in combination with silver nitrate greatly enhanced regeneration capacity and

Table 1. Effect of various combinations of growth regulators on regeneration in hypocotyl explants of cotton. Data represents means from three replicates ± SE. Means denoted by different letters differ significantly at $P = 0.05$. Number of explants forming shoot primordia (SP) and number of shoots per explant were scored in 4-week-old cultures.

Growth regulator [mg dm^{-3}]				Bharani		Durga		JKCH-99	
TDZ	BAP	KIN	NAA	SP	[explant^{-1}]	SP	[explant^{-1}]	SP	[explant^{-1}]
0.5	-	-	0.01	3.7 ± 0.0kl	5.7 ± 0.8	3.3 ± 1.2lm	3.9 ± 0.1	3.8 ± 0.2kl	3.0 ± 1.1
1.0	-	-	0.01	5.6 ±.0.6jk	9.8 ± 1.9	4.6 ± 2.0jk	6.9 ± 2.1	4.0 ± 0.4kl	4.8 ± 2.3
1.5	-	-	0.01	11.8 ± 0.6e	12.5 ± 0.5	11.1 ± 2.2de	8.8 ± 1.7	9.2 ± 0.1fg	8.1 ± 2.1
2.0	-	-	0.01	14.2 ± 0.8ab	16.9 ± 0.1	11.6 ± 2.0de	12.1 ± 0.0	10.9 ± 0.0de	9.7 ± 0.6
2.5	-	-	0.01	10.8 ± 1.7de	10.0 ± 0.8	9.3 ± 1.6fg	9.6 ± 0.5	9.1 ± 0.3fg	5.8 ± 0.2
3.0	-	-	0.01	5.7 ± 0.1ij	7.8 ± 1.2	6.2 ± 0.2k	4.2 ± 0.8	5.0 ± 1.6jk	3.8 ± 0.0
0.5	-	-	0.05	4.6 ± 1.0k	7.1 ± 1.4	4.5 ± 0.0k	5.6 ± 1.8	4.0 ± 2.0kl	3.2 ± 2.1
1.0	-	-	0.05	8.4 ± 1.2g	12.5 ± 0.1	8.3 ± 0.2g	8.8 ± 0.1	5.3 ± 1.1jk	5.6 ± 1.2
1.5	-	-	0.05	11.6 ± 0.3cd	14.8 ± 0.4	11.3 ± 0.9cd	10.4 ± 0.8	9.1 ± 1.8f	8.8 ± 0.6
2.0	-	-	0.05	15.5 ± 0.4a	19.2 ± 0.7	12.2 ± 1.3bc	14.7 ± 0.4	12.0 ± 1.0cd	9.8 ± 1.9
2.5	-	-	0.05	13.7 ± 0.5bc	13.0 ± 0.2	11.9 ± 1.1bc	10.2 ± 0.6	9.2 ± 0.2fg	6.7 ± 2.0
3.0	-	-	0.05	8.0 ± 0.1gh	9.6 ± 0.5	7.8 ± 1.9g	6.8 ± 1.3	5.4 ± 0.7k	4.9 ± 1.8
-	1.0	-	0.10	2.8 ± 0.8lm	3.2 ± 0.2	2.8 ± 1.0m	2.9 ± 1.9	2.0 ± 0.1m	2.4 ± 0.4
-	2.0	-	0.10	5.2 ± 1.0k	6.0 ± 1.1	4.5 ± 1.4k	5.1 ± 1.2	3.4 ± 1.2l	4.6 ± 0.5
-	3.0	-	0.10	8.7 ± 0.7fg	9.5 ± 0.4	8.1 ± 0.2gh	8.2 ± 0.4	6.9 ± 1.6ij	7.7 ± 0.8
-	4.0	-	0.10	7.4 ± 1.2ghi	8.0 ± 0.2	6.8 ± 0.8ijk	6.4 ± 0.9	6.3 ± 0.1ij	5.4 ± 0.1
-	5.0	-	0.10	4.6 ± 2.2k	5.2 ± 0.3	3.8 ± 1.7kl	4.7 ± 0.1	3.5 ± 2.0kl	3.2 ± 0.0
-	-	1.0	0.10	0.0 n	-	0.0 n	-	0.0 n	-
-	-	2.0	0.10	1.9 ± 0.7m	3.8 ± 0.6	1.8 ± 0.2m	2.6 ± 0.8	0.0 n	-
-	-	3.0	0.10	2.6 ± 1.9m	6.4 ± 1.3	2.6 ± 0.8m	5.7 ± 1.0	2.2 ± 0.8m	5.3 ± 0.4
-	-	4.0	0.10	1.7 ± 0.4m	4.3 ± 1.4	1.7 ± 1.4m	3.4 ± 0.2	1.6 ± 1.8m	3.0 ± 0.4
-	-	5.0	0.10	1.2 ± 0.4m	1.3 ± 0.9	1.2 ± 1.8m	2.2 ± 0.8	1.3 ± 0.4m	1.8 ± 1.0
-	0.5	0.5	-	4.0 ± 1.8kl	3.2 ± 0.2	3.5 ± 0.8l	2.4 ± 0.5	2.8 ± 0.7m	2.0 ± 2.2
-	1.0	1.0	-	4.9 ± 0.1k	5.7 ± 0.3	3.8 ± 1.5kl	5.1 ± 0.9	3.7 ± 0.4kl	4.4 ± 0.9
-	1.5	1.5	-	7.9 ± 0.5fg	9.8 ± 0.6	7.5 ± 1.4fg	9.6 ± 1.0	7.6 ± 0.1gh	8.8 ± 1.3
-	2.0	2.0	-	12.3 ± 0.8bc	14.5 ± 1.2	11.8 ± 0.0cd	12.6 ± 0.9	9.4 ± 1.6fg	10.8 ± 0.8
-	2.5	2.5	-	9.4 ± 0.8ef	11.4 ± 1.3	8.7 ± 1.8fg	9.9 ± 2.0	8.6 ± 0.4fg	7.1 ± 1.1
-	3.0	3.0	-	5.8 ± 1.4k	7.3 ± 1.0	3.9 ± 0.3kl	7.2 ± 0.4	3.6 ± 1.5kl	6.5 ± 1.2
0.5	0.5	0.5	-	3.0 ± 0.2lm	4.2 ± 0.7	2.3 ± 0.8m	2.9 ± 0.1	2.2 ± 1.2m	2.0 ± 1.2
1.0	1.0	1.0	-	4.8 ± 1.6k	7.7 ± 1.3	4.7 ± 0.8k	6.5 ± 0.7	4.0 ± 1.6kl	6.1 ± 0.3
1.5	1.5	1.5	-	9.4 ± 0.9fg	11.9 ± 1.4	8.4 ± 1.3fg	10.3 ± 0.5	5.5 ±0.2k	8.6 ± 0.2
2.0	2.0	2.0	-	7.1 ± 1.1hij	9.6 ± 0.6	6.4 ± 1.7k	9.2 ± 1.1	4.2 ± 1.5k	7.0 ± 1.8
2.5	2.5	2.5	-	6.6 ± 0.9k	5.4 ± 0.9	3.8 ± 0.1kl	3.6 ± 0.8	3.1 ± 1.0m	3.6 ± 2.0
3.0	3.0	3.0	-	2.4 ± 0.4m	3.9 ± 0.8	2.1 ± 1.2m	3.2 ± 1.8	1.8 ± 1.4m	2.4 ± 0.7

shoot yield with almost similar response in all the three varieties. It was observed that inclusion of AC in the culture medium promoted root initiation and elongation that may be due to the immediate adsorption of phenolics at the cut end. Complete plantlets with root system having well developed lateral roots survived upon transfer to pots in the greenhouse and appeared morphologically normal (Fig. 3*E*,*F*).

Silver nitrate had a marked influence on the growth and differentiation from hypocotyl. An optimal response was noticed at 3 - 5 mg dm^{-3} (Fig. 4). The hyperhydricity decreased in the presence of silver nitrate and the quality of shoot primordia as well as elongated shoots improved when compared to shoots regenerated on media without silver nitrate. The effect of silver nitrate was genotype dependent. Increase in concentration of silver nitrate beyond 5.0 mg dm^{-3} disturbed the organization of shoot primordia leading to the formation of friable callus. Visual observations indicated that the shoots appeared healthy and well elongated with inclusion of silver nitrate in the culture medium. Though the number of shoot primordia has not increased significantly by silver nitrate, their conversion rate into individual shoots improved significantly.

Scanning electron micrographs of control explants grown on MS medium without growth regulators did not show any developing shoot buds (Fig. 5*A*), whereas, the proximal parts of *in vitro* cultured hypocotyls showed clusters of shoot primordia obtained *de novo* on SIM (Fig. 5*B*).

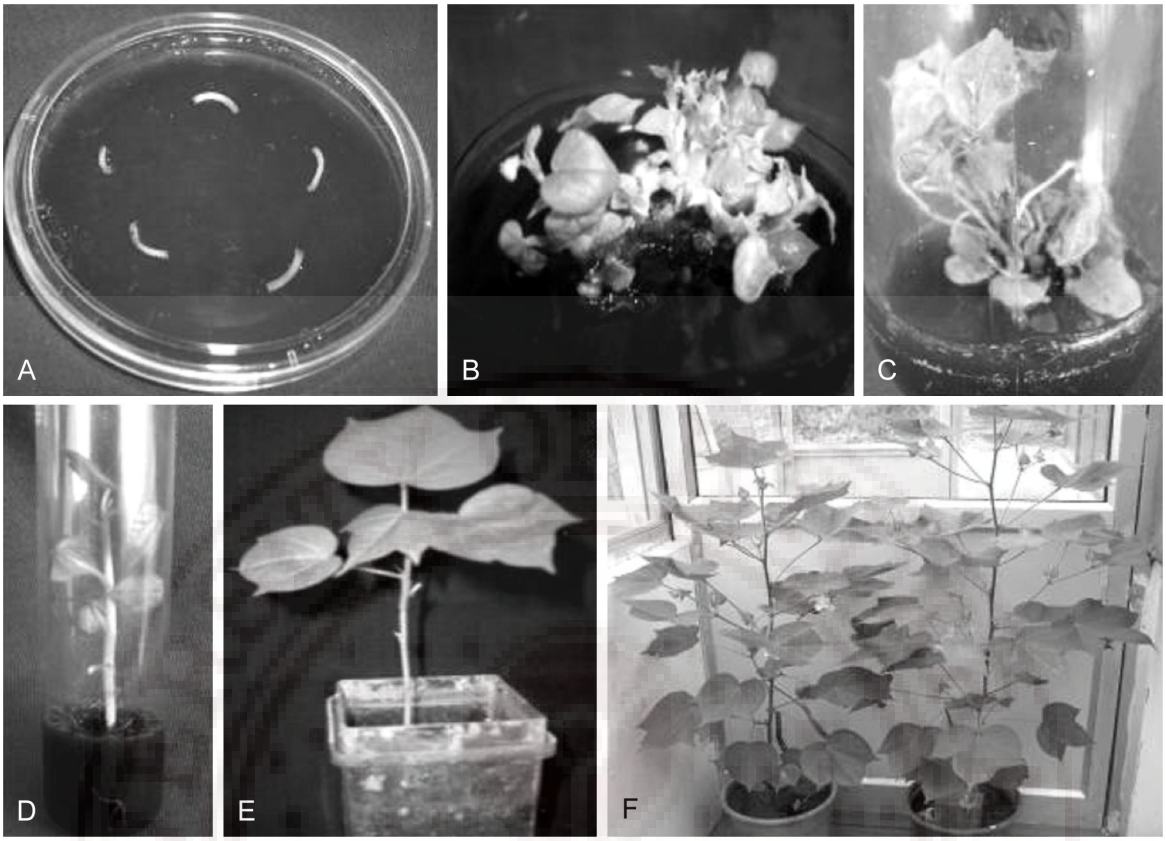

Fig. 3. Plant regeneration by organogenesis from hypocotyl segments in cotton (*Gossypium hirsutum* L.). *A* - Hypocotyl explants from 8 to 10-d-old seedlings on SIM; *B* - Shoot initiation from proximal ends of hypocotyls at the end of 3 weeks on SIM; *C* - Multiple shoot production and elongation on EM; *D* - Isolated and elongated shoot with developing roots when cultured on RIM; *E* - Well-rooted plant transferred to greenhouse; *F* - Regenerated plants showing flowers and bolls.

Discussion

An efficient protocol for multiple shoot regeneration in a genotype independent manner is a pre-requisite for genetically manipulation of crop plants. Though there is a large body of literature available on regeneration in cotton cv. Coker through somatic embryogenesis, efforts on the establishment of *de novo* shoot regeneration protocols in commercial cotton cultivars were rare (Ouma *et al*. 2004).

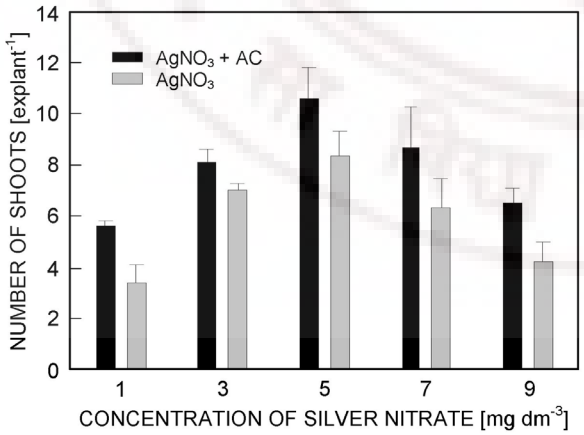

Fig. 4. Effect of different concentrations of $AgNO_3$ with or without AC on multiple shoots induction on TDZ medium. *Vertical bars* represent SE of the means.

Our studies showed that TDZ induced high frequency production of shoot primordia from hypocotyls sections. TDZ has been shown to regulate plant morphogenesis by possessing a high cytokinin like activity (Murthy *et al*. 1998). Lower concentrations of TDZ yielded more shoot primordia compared to higher concentrations, and resulted in hyperhydricity of shoots associated with water soaked callus (Huetteman and Preece 1993, Ouma *et al*. 2004). Ouma *et al*. (2004) reported that TDZ concentrations higher than 0.5 mg dm^{-3} resulted in callusing in contrast to our observations where TDZ concentrations up to 2 mg dm^{-3} appeared to be optimal and did not induce significant amounts of callus. This was probably due to the different genotypes used. TDZ played a vital role in enhancing the *de novo* regeneration potential in pea and lentil (Malik and Saxena 1992), chickpea (Murthy *et al*. 1996) and many other species. However, shoot elongation was greatly hindered when

the cultures were maintained for a long period on TDZ medium as reported in pepper (Hyde and Phillips 1996) and cotton (Caramori *et al*. 2001). Similarly, TDZ inhibited shoot elongation in our cultures. The induction of roots on shoots obtained from TDZ containing media was delayed and they did not elongate further as reported by (Nielson *et al*. 1993, Ouma *et al*. 2004). To avoid these negative effects of TDZ, subculture of explants was carried out on a medium containing 2.0 mg dm^{-3} BAP and 0.1 mg dm^{-3} NAA after shoot primordia formed. Even though shoot bud differentiation was effective in all the tested vitamin combinations with MS basal medium, better response was achieved in MS_3 medium along with TDZ (upto 76 % regeneration frequency with a maximum of 10.6 shoots per explant) followed by 62 % obtained on 2.0 mg dm^{-3} each of BAP and KIN. The regeneration response and shoot yield per explant decreased considerably after 30 d of culture because of the excessive production of callus. To avoid excessive callusing on SIM, cultures were transferred to a medium with lower cytokinin concentration. Addition of GA_3 in the EM promoted shoot elongation but only one or two shoots per cluster elongated at a time and the clumps with shoot buds were again cultured on SIM for further elongation of shoots.

Ethylene, a gaseous hormone released by tissues was reported to accumulate in the intercellular spaces forming aerenchyma thus blocking the differentiating ability of tissues (Topa and McLeod 1988, Biddington 1992, Kong and Yeung 1994). To counteract this, silver nitrate, an anti-ethylene compound, was used to enhance regeneration in many recalcitrant species including cotton (Ouma *et al*. 2004). Silver nitrate incorporation was beneficial in increasing regeneration response by lowering hyperhydricity, as reported in sunflower (Mayor *et al*. 2003) and potato (Turhan 2004) and resulted in better quality shoots.

Table 2. Effect of growth regulators [mg dm^{-3}] on number of regenerated shoots from responding explant and the shoot regeneration response [%]. Means from three replicates ± SE. Data were scored after 4 weeks of culture.

TDZ	BAP	KIN	NAA	Number of shoots [$explant^{-1}$]	Regeneration frequency [%]
0.5			0.05	4.5 ± 0.5	59.0
1.0			0.05	7.2 ± 1.4	65.2
1.5			0.05	10.1 ± 0.2	73.3
2.0			0.05	10.6 ± 0.3	76.7
2.5			0.05	9.2 ± 1.0	69.5
3.0			0.05	6.0 ± 1.2	66.0
	1.0		0.1	2.2 ± 1.1	28.0
	2.0		0.1	4.1 ± 0.3	40.0
	3.0		0.1	5.6 ± 0.5	55.1
	4.0		0.1	5.0 ± 1.0	53.0
	5.0		0.1	3.0 ± 0.2	42.4
		1.0	0.1	0.0 ± 0.0	0.0
		2.0	0.1	1.8 ± 0.2	21.6
		3.0	0.1	3.4 ± 1.6	32.8
		4.0	0.1	2.8 ± 0.8	26.5
		5.0	0.1	2.2 ± 0.8	25.4
	0.5	0.5		3.0 ± 1.6	44.0
	1.0	1.0		6.8 ± 2.0	50.5
	1.5	1.5		8.2 ± 0.8	62.4
	2.0	2.0		8.8 ± 0.6	70.5
	2.5	2.5		7.5 ± 1.0	68.0
	3.0	3.0		5.2 ± 1.1	55.5
0.5	0.5	0.5		3.0 ± 0.5	28.5
1.0	1.0	1.0		3.2 ± 0.5	41.0
1.5	1.5	1.5		6.2 ± 0.1	58.0
2.0	2.0	2.0		5.8 ± 1.0	48.8
2.5	2.5	2.5		3.6 ± 0.8	39.0
3.0	3.0	3.0		1.2 ± 1.4	16.5

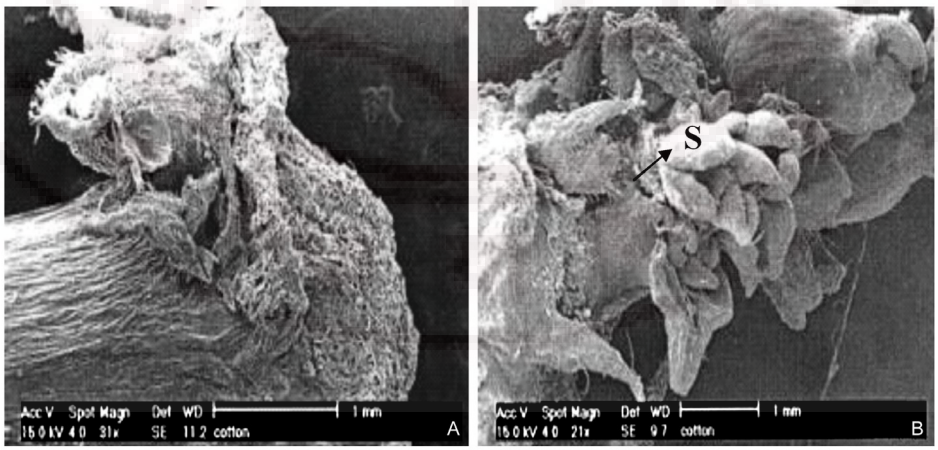

Fig. 5. SEM studies of the *in vitro* cultured explants. *A* - Control explant grown on MS medium without growth regulators, $AgNO_3$ and AC showing callus formation; *B* - Acropetal end of hypocotyl with clusters of shoot primordia when cultured on MS medium supplemented with growth regulators and AC; S - shoot bud

Table 3. Root induction in *in vitro* regenerated shoots of cotton by different auxin concentrations. Means from three replicates ± SE; $n = 30$. Means denoted by different letters differ significantly at $P = 0.05$. Data were scored after 4 weeks.

Growth regulators	Conc. [mg dm^{-3}]	Bharani number of responding shoots	number of roots [shoot^{-1}]	rooting [%]	Durga number of responding shoots	number of roots [shoot^{-1}]	rooting [%]
IBA	0.01	20.2 ± 2.2cd	3.1 ± 0.9	70	18.9 ± 1.8d	2.2 ± 1.2	60
	0.10	22.9 ± 3.0ab	5.0 ± 0.5	83	22.5 ± 2.4bc	4.4 ± 0.8	73
	1.00	26.0 ± 3.2a	6.4 ± 0.6	86	24.2 ± 3.0ab	5.5 ± 0.2	80
NAA	0.01	16.9 ± 3.1e	2.8 ± 1.2	53	13.7 ± 2.6ef	1.9 ± 1.0	43
	0.10	19.3 ± 1.8d	4.4 ± 1.4	63	17.0 ± 2.4e	3.4 ± 0.4	56
	1.00	23.7 ± 1.8bc	5.3 ± 0.8	75	20.1 ± 1.6cd	4.3 ± 0.7	66
IAA	0.01	9.5 ± 1.6g	1.2 ± 0.2	30	6.1 ± 2.0g	2.4 ± 1.5	20
	0.10	11.2 ± 2.4f	3.8 ± 0.6	36	10.2 ± 1.2fg	3.1 ± 0.9	33
	1.00	15.8 ± 2.2e	4.2 ± 0.8	50	14.3 ± 2.2e	3.8 ± 1.2	46

Activated charcoal has been used in *in vitro* tissue culture for adsorbing inhibitory compounds such as phenolics, excessive growth hormones and release of growth promoting substances (Van Winkle *et al*. 2003). It was reported that there would be an equilibrium established with adsorbed and desorbed molecules between explant and AC, thereby making growth hormones available to the explant (Pan and Van Staden 1998). Usage of tissue culture grade AC was effective in reducing browning and subsequent necrosis of the explant due to excessive production of phenolic compounds and promoting regeneration response of hypocotyl explants in our experiments. The addition of AC to the RIM enhanced number of robust roots and establishment of tissue culture raised plants (Dumas and Monteuuis 1995, Pan and Van Staden 1998).

In summary, we have established a fast and genotype-independent efficient regeneration system for *de novo* shoot regeneration from hypocotyl explants of three commercial cotton cultivars including their *ex vitro* transfer. The time taken from explanting to the establishment of plants in the greenhouse was about 16 - 20 weeks compared to the longer periods as reported in published regeneration protocols using somatic embryogenesis. Regenerated plants were phenotypically normal and flowered well. Present experiments are aimed at utilizing this simple regeneration protocol in the future development of transgenic cotton.

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BIOTIC AND ABIOTIC STRESS

Transgenic tobacco and peanut plants expressing a mustard defensin show resistance to fungal pathogens

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Abstract Defensins are small positively charged, antimicrobial peptides (~5 kDa in size) and some of them exhibit potent antifungal activity. We have cloned the complete cDNA containing an ORF of 243 bp of a defensin of mustard. The deduced amino acid sequence of the peptide showed more than 90% identity to the amino acid sequence of the well-characterized defensins, RsAFP-1 and RsAFP-2 of *Raphanus sativus*. We have generated and characterized transgenic tobacco and peanut plants constitutively expressing the mustard defensin. Transgenic tobacco plants were resistant to the fungal pathogens, *Fusarium moniliforme* and *Phytophthora parasitica* pv. *nicotianae*. Transgenic peanut plants showed enhanced resistance against the pathogens, *Pheaoisariopsis personata* and *Cercospora arachidicola*, which jointly cause serious late leaf spot disease. These observations indicate that the mustard defensin gene can be deployed for deriving fungal disease resistance in transgenic crops.

Keywords Mustard defensin · Transgenic tobacco · Transgenic peanut · *Fusarium moniliforme* · *Phytophthora parasitica* pv. *nicotianae* · Leaf spot disease

Introduction

In the innate immunity response, only the defensins are a class of peptides that are conserved in plants, invertebrates and vertebrates (Thomma et al. 2002). Although they share common chemical elements and 3-D structures, the plant defensin family is quite diverse in amino acid composition and biological activity (Lay and Anderson 2005; Thomma et al. 2002). Defensins have been found to display antimicrobial activity not only against plant and insect pathogens, but also against human fungal pathogens including *Candida* and *Aspergillus* sps, and they are employed as novel leads in antifungal therapeutics (Thevissen et al. 2007). The antifungal activity of SPE10, a dimeric defensin isolated from *Pachyrhizus erosus* was assessed on three fungal species and the antifungal activity depended on the test fungus (Song et al. 2004, 2005). In vitro antifungal activity of a defensin from *Trigonella foenum graecum* was tested against some fungal pathogens (Olli and Kirti 2006).

Peanut is a crop of high commercial value and its cultivation is hampered by the attack from several diseases. Leaf spot disease, jointly caused by *Phaeosariopsis personata* and *Cercospora arachidicola*, is a very serious problem affecting peanut cultivation in various countries. This disease predominates in the tropics and subtropics, and is reported to be the one of the major diseases of peanut causing high yield losses. The genus *Cercospora* belongs to the class of Fungi Imperfecti and the pathogen survives in the form of conidia, perithecia and mycelia in the soil. Symptoms first appear on the leaves as small brown flecks on both sides of the leaves; early leaf spots caused by *C. arachidicola* appear on the adaxial surface of the leaflets, where as the late leaf spots caused by *P. personata* appear on the abaxial surface and the lesion diameter increases to >2 mm within 10–15 days. If not controlled, the leaf spots reduce yields of typical peanut cultivars up to 90% (Austin Hagan, http://www.aces.edu/pubs/docs/A/ANR-0369/).

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Plant growth promoting rhizobacteria (PGPR), which were earlier reported as elicitors in induced systemic resistance (ISR) in several crop species, did not provide significant protection in peanut against late leaf spot pathogen (Zhang et al. 2001). Other biological/chemical strategies include the application of *Serratia marcescens* (GPSS5), which was reported to provide an improved control of late leaf spot disease of peanut (Kishore et al. 2005). However, these strategies need repeated application on the plants during the growth phase to be effective. Hence, the genetic transformation for transferring suitable candidate genes to peanut offers a more effective solution for developing transgenic varieties with enhanced disease resistance.

Expression of genes for suitable pathogenesis related proteins and defensins offers a suitable approach for controlling diseases that affect crop productivity. Over-expression of pepper pathogen induced genes *CAPIP2*, *CASAR82A* and *RAV1* in transgenic plants resulted in disease resistance and abiotic stress tolerance (Lee and Hwang 2006; Lee et al. 2006; Sohn et al. 2006). The pathogenesis related proteins, that have been shown to have potent antifungal activities, have also been implicated in abiotic stress tolerance. Sarowar et al. (2005) showed that the overexpression of a pepper basic pathogenesis related protein-1 in transgenic tobacco plants could be correlated with disease resistance and enhanced heavy metal tolerance, whereas the same protein conferred oxidative stress tolerance and resistance to *Pseudomonas syringae* in *Arabidopsis* (Hong and Hwang 2005).

There were very few reports on transforming peanut for introduction of genes for fungal disease resistance. Transgenic peanut expressing a tobacco chitinase gene was shown to possess enhanced resistance to the late leaf spot causing organism *P. personata* (Rohini and Sankara Rao 2001). However, the analysis of resistance in this study was limited to detached leaf assay only. Chenault et al. (2005) expressed a rice chitinase and an alfalfa glucanase in transgenic peanut and observed enhanced resistance against Slerotinia blight in the transgenic plants. Expression of a barley oxalate oxidase in transgenic peanut also enhanced resistance to *Sclerotinia minor* (Livingstone et al. 2005). These observations indicate that the introduction of suitable genes for resistance through genetic transformation is a feasible option in combating the diseases in peanut.

In the present investigation, we have shown that a mustard defensin confers resistance to fungal pathogens, *Phytophthora parasitica* pv. *nicotianae*, *Fusarium moniliforme* in transgenic tobacco and against pathogens causing the leaf spot disease in peanut.

Materials and methods

Plasmid construction and *Agrobacterium* transformation

The ORF of the cDNA of the mustard defenisn (*BjD*) was cloned by RT-PCR (Swathi Anuradha et al., unpublished) of the total RNA extracted from leaves of 1-month-old mustard (*Brassica juncea* cv. Pusa Jai Kisan) plants using TRI-reagent (Sigma, USA) using the forward and reverse primers (OD′F′:5′GGG TAC CAT GGC TAA GGT TGA TTC CATC 3′, OD′R: 5′GGG ATC CTT AAC AAG GGA AGT AGC AGA 3′) designed on the basis of sequence information available for a *Brassica oleracea* defensin (NCBI Accession No. CAC37558). The open reading frame of *BjD* was cloned as an *Nco*I–*Bam*HI fragment into a plant expression cassette containing vector pRT100. The entire defensin cassette along with 5′ CaMV 35S promoter and 3′ Nos terminator was cloned as a *Pst*I fragment in the binary vector pCAMBIA2300, which was mobilized into a super virulent *Agrobacterium tumefaciens* strain EHA105 by the freeze thaw method. A fresh overnight culture of *Agrobacterium* was obtained by inoculating a single colony and the culture with bacterial OD 0.6–0.8 was used in leaf disc transformation of tobacco, *Nicotiana tabacum* cv. Petit Havana following the method of Horsch et al. (1985). A peanut cultivar, JL-24 was used in transformations experiments for expressing the mustard defensin. *Agrobacterium* mediated transformation of peanut, selection and regeneration were carried out following the procedures detailed out earlier using embryo axis explants (Swathi Anuradha et al. 2008). Putative transgenic plants were transferred to the glass house, seed collected and the T_1 generation plants were characterized further using Southern and northern analyses.

Molecular analysis of the transformants

DNA isolation, southern blotting, hybridization and washing were performed according to Swathi Anuradha et al. (2006). RNA was isolated from the young leaves frozen in liquid nitrogen using the TRI-Reagent (Sigma–Aldrich, USA) following the manufacturer's instructions in molecular analyses. Radiolabelled probes for *BjD* and neomycin phosphotransferase (*npt*II) were used in the nucleic acid hybridization reactions. Standard protocols were applied in molecular analyses (Sambrook et al. 1989).

Evaluation of transgenic tobacco plants for resistance against fungal pathogens

Detached leaf assays were conducted on transgenic tobacco plants in T_1 generation, which were characterized for the

expression of *BjD*. The oomycete pathogen *Phytophthora parasitica* pv. *nicotianae* was cultured on PDA medium (potato 200 g/l, sucrose 20 g/l, agar 15 g/l, pH 6.5) at 24°C for 5–7 days. *Fusarium moniliforme* was grown on PDA medium at 37°C for 3–4 days. For the detached leaf assay, fully expanded leaves of 2-month-old plants (Control, T_1 progeny of tobacco transgenic plants 8 and 10) were used for inoculation. A small plug of the mycelium was placed at the center of the adaxial leaf surface. The inoculated leaves were then placed on two layers of filter paper saturated with sterilized double distilled water in a Petri dish at a 16 h light/8 h dark photoperiod in a BOD incubator at 24°C and symptoms were evaluated 10 days after inoculation.

Evaluation of peanut transgenic plants expressing the mustard defensin

For resistance studies against *P. personata* and *C. arachidicola*, the causal agents of leaf spot disease (Tikka disease) in peanut, conidia collected from infected leaf samples were allowed to germinate in sterile double distilled water and sucrose at a concentration of 2.0% (w/v) was added to aqueous spore suspension for improving conidial germination. The suspension consisted mostly of conidia of *P. personata* ($\sim$90%) with conidia belonging to *C arachidicola*; conidia of both the pathogens could be very easily identified by observing the morphology microscopically. The concentration of conidia was adjusted to 5×10^4/ml using a haemocytomer and plant assays were carried out using the conidial suspension with 0.1% of Triton X-100 (v/v) as a wetting agent. One-month-old transgenic plants growing in glass house were inoculated with conidial suspension using an atomizer and high humidity was provided to the plants before and after inoculation of spores. The plants were covered with polythene bags to maintain $\geq$95% RH for 48 h. After this treatment, the bags were removed and the plants were monitored regularly for disease development. Data was scored after 28 days of inoculation and plants were assessed for leaf spots based on a 9-point disease scale developed at ICRISAT (Subrahmanyam et al. 1982).

Detached leaf assays on peanut transgenics

For detached leaf assays, the same spore suspension was painted on the surface of the leaflets and $\geq$95% RH was maintained by placing them on filter papers inside 110 mm Petri dishes, sealed with parafilm. The number and average diameter of the lesions were scored after 21 and 28 days of inoculation on control and transgenic leaves. All these experiments were carried out thrice and the data obtained is an average of three independent experiments.

Results

Mustard defensin and its characterization

The cDNA of a defensin (*BjD*) with an open reading frame of 243 bp was cloned by RT-PCR by using the primers designed on the sequence of *Brassica oleracea* defensin (Swathi Anuradha et al., unpublished). PCR amplification of the genomic DNA with the same primers produced the genomic clone, which showed a 91 bp intron interrupting the coding region (NCBI accession numbers DQ191751, DQ191752). RACE has resulted in cloning a 5′-untranslated region (UTR) of 32 bp upstream to the start codon and a 3′-UTR, which was 185 bp long downstream of the stop codon (Swathi Anuradha et al., unpublished).

A NCBI database search with BLAST and the multiple alignment of the amino acid sequence of the present defensin with other defensins showed that BjD had considerable homology to known Crucifer defensins. It showed 98% amino acid sequence identity with RsAFP-1, 90% with the well-characterized defensin, RsAFP-2 with antifungal activity, and 95% with the defensin from *B. oleracea*. The alignment of the deduced mature polypeptide sequences of BjD and plant defensins from several Crucifer species is shown in Fig. 1.

Tobacco transformation and molecular analysis

Agrobacterium strain EHA105, harboring the binary vector pCAMBIA2300 carrying the *BjD* expressed under the CaMV 35S promoter (Fig. 2), was used in the genetic transformation of tobacco using the leaf disc method. This has resulted in the production of several putative transgenic plants. However, molecular characterization was carried out for 14 T_0 plants obtained from leaf disc transformation of tobacco. These plants were acclimatized under culture room conditions, transferred to pots, grown in the glass house, and confirmed through Southern hybridization for copy number. The hybridization pattern indicated that they carried independent integration events for the transgenes and some of them (Plants 7, 8, 10, 13) are single copy carrying transgenic plants (Fig. 3).

The expression of the transgene *BjD* in the T_0 transgenic plants was analyzed by northern hybridization using the PCR amplified fragment as a probe. Out of 14 T_0 plants that were analyzed, 12 plants exhibited varied levels of expression of *BjD* mRNA. The presence of $\sim$250 bp band in the transgenic plants indicated the expression of *BjD* in the T_0 tobacco plants. Transgenic plants 8 and 10 represented the primary transgenics carrying single copy insertion with relatively high expression level of the introduced *BjD*. The plants 2, 5 and 6 showed relatively low-level expression of *BjD* (Fig. 4a). The progeny of

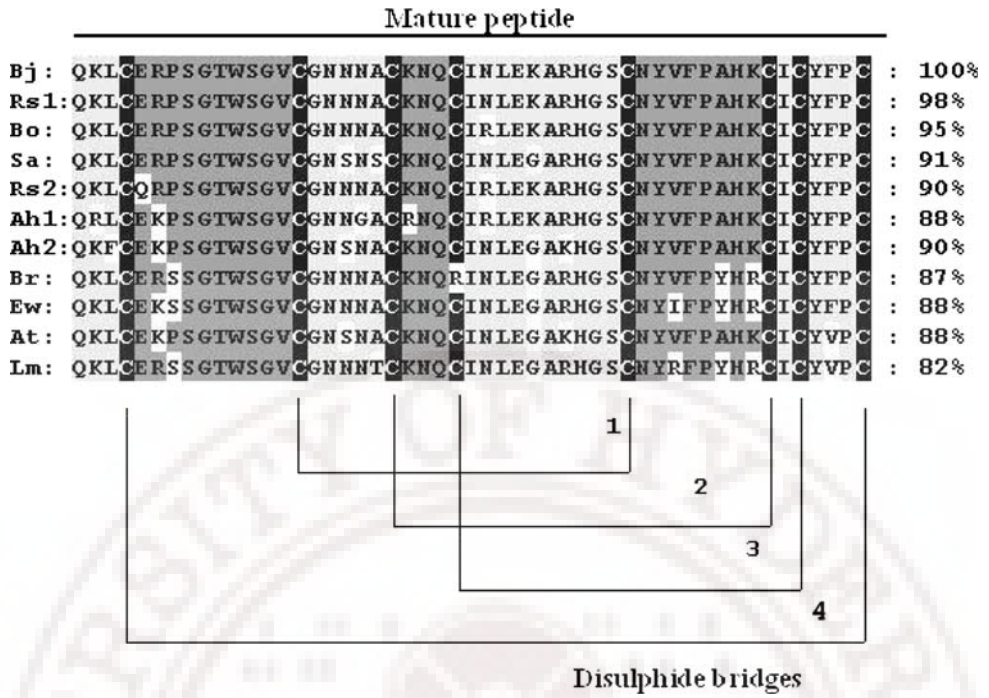

Fig. 1 Bj: *Brassica juncea*, Sa: *Sinapis alba*, Rs: *Raphanus sativus*, Bo: *Brassica oleracea*, Ah: *Arabidopsis halleri*, Br: *Brassica rapa*, Ew: *Eutrema wasabi*, At: *Arabidopsis thaliana* and Lm: *Lepidium meyenii*. The protein sequence shared maximum identity (only the mature part of the peptide from different accession has been indicated here) of 98% to Rs AFP1 of *Raphanus sativus* (P69241), 95% to Bo of *Brassica oleracea* (CAC37558), 91% to *Sinapis alba* (AAY15221), 90% to Rs AFP2 of *Raphanus sativus* (P30230) and Ah2 of *Arabidopsis halleri* (AAY27736). The BjD protein also showed significant sequence identity with other defensins like Br of *Brassica rapa* (AAQ92328), Ew of *Eutrema wasabi* (BAB19054), At of *Arabidopsis thaliana* (NP_199256) and Lm of *Lepidium meyenii* (AAV85992)

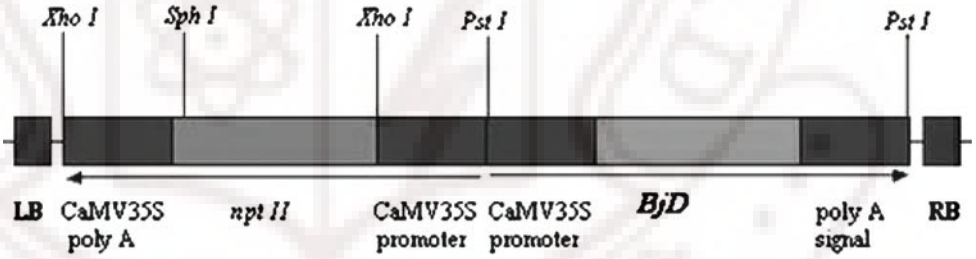

Fig. 2 Representation of the T-DNA in the vector pCAMBIA2300 carrying the mustard defensin

plants 8 and 10 were analyzed by Northern hybridization to detect the stability of expression of *BjD* mRNA level. Progeny plants of the transgenic plants that did not carry the transgene due to segregation also did not show *BjD* expression (Fig. 4b).

Genetic transformation and Southern blot analysis of peanut transgenic plants

By employing pCAMBIA2300 harboring *BjD* and the same *Agrobacterium* strain for transformation, we have generated transgenic peanut plants in the cultivar JL-24. Overall, 17 putative transformants were obtained from six independent transformation experiments using embryo axis explants. Out of these, 13 survived under the glass house conditions, 7 of which were found to be positive in a preliminary PCR analysis. We collected seeds from 11 T_0 plants and the Southern analysis was done with PCR positive T_1 plants. The genomic DNA of transgenic plants was digested with enzyme *Eco*R1, which has a single site on the T-DNA between the *npt*II and defensin expression cassette, and the Southern blot was probed with the PCR amplified *npt*II fragment as probe. The varying length of hybridizing bands indicated that at least three of these primary transgenic plants represented single copy containing independent transformation events. Transgene integration was evident in the progeny of five plants out of eleven T_0 plants that survived the transfer to the glass house (Fig. 5).

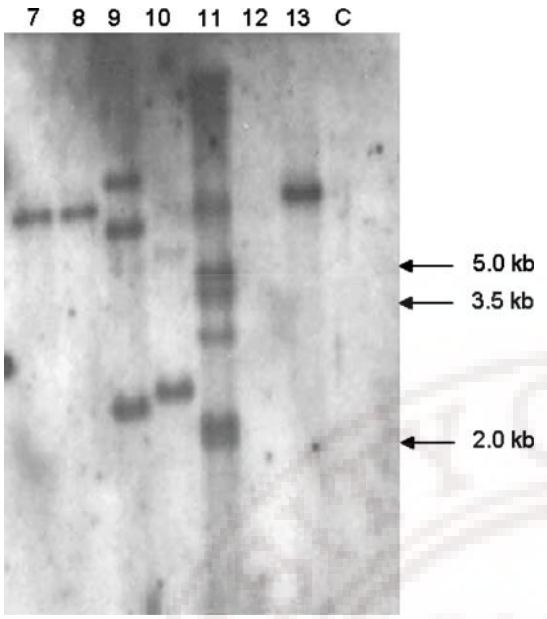

Fig. 3 Southern hybridization analysis of tobacco transgenic plants expressing the mustard defensin. DNA samples were digested by *Eco*RI, which has a single site on the T-DNA, and blots were probed by a PCR amplified fragment of *npt*II. Plants 7, 8, 10, 13 represent single copy integration

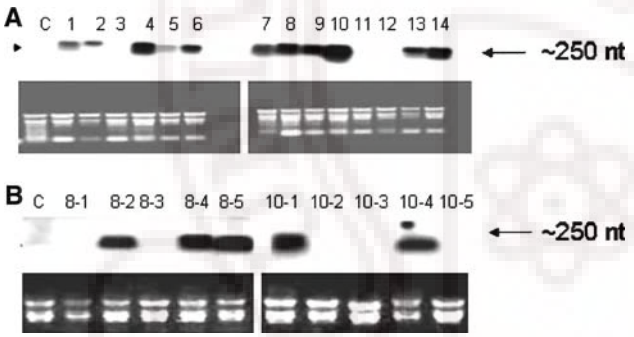

Fig. 4 **a** Northern analysis of tobacco transgenic plants in T_0 generation using the PCR amplified mustard defensin as a probe. Plants 4, 8, 9, 10, 14 represent relatively high expression of mustard defensin. **b** Confirmation of defensin gene expression in T_1 generation plants of the transgenics 8 and 10 and identification of transgene containing progeny plants and segregants without transgenes for resistance analysis

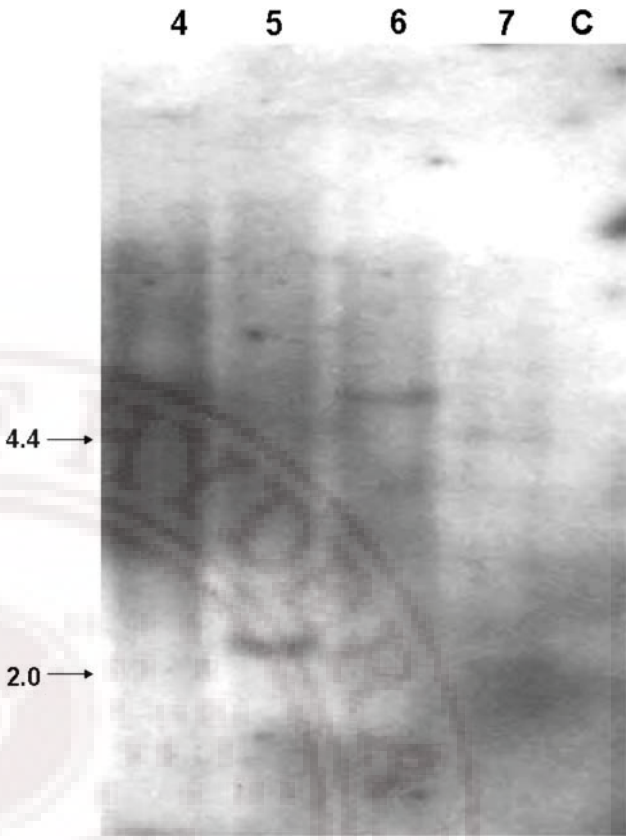

Fig. 5 Southern blot analysis of peanut transgenic plants in T_1 generation. DNA samples were digested with *Eco*RI and the blots probed with *npt*II. Please note single copy insertion in the transgenic plants 4, 5, 6, 7

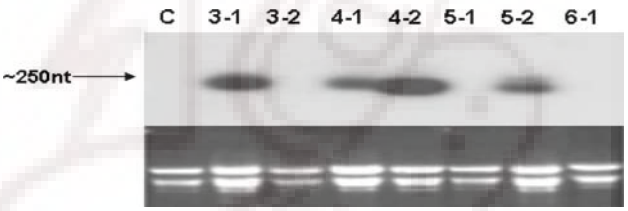

Fig. 6 Defensin gene expression in peanut transgenic plants in T_1 generation with defensin as a probe in the northern analysis and identification of plants for disease resistance analysis

Analysis of constitutive expression of mustard defensin in peanut T_1 plants

Northern hybridization analysis on total RNA isolated from the leaves of T_1 transgenic peanut plants confirmed the constitutive expression of *BjD* (Fig. 6). The expression levels varied among individual plants and hybridization signals corresponding to the expected transcript size of ~250 nt for defensin were detected in transgenic plants. High level expression was evident in the progeny of transgenic plants 3 and 4. No hybridization signals were observed in the control plant. Progeny plants of the transgenic plants that did not carry the transgene due to segregation also did not show *BjD* expression.

Enhanced disease resistance in transgenic tobacco against *Phytophthora parasitica* pv. *nicotianae* and *Fusarium moniliforme*

Phytophthora parasitica pv. *nicotianae* causes a serious disease called black shank disease in tobacco. To determine whether the constitutive expression of mustard

defensin conferred resistance against this pathogen, a detached leaf assay was performed with the leaves of *BjD* overexpressing tobacco T_1 plants. This experiment was carried out to correlate the expression of the *BjD* and resistance against pathogen invasion as the transgenes segregate in a Mendelian fashion from T_0 to the T_1 generation. This results in transgenic segregants expressing the transgene and non-transgenic segregants lacking the transgene. Randomly selected T_1 plants in the greenhouse were checked for the presence of transgenes using PCR for *npt*II and *BjD* (data not shown). Subsequently, the selected plants carrying the transgenes and their non-transgenic counterparts were analyzed for the expression of the defensin through northern analysis and fungal resistance. Disease symptoms started appearing after three days of inoculation and they were scored after 10 days of inoculation. Control plants showed browning of entire leaves with necrotic lesions all over the leaves. T_1 plants (8-1, 8-3, 10-2, 10-3 and 10-5), which did not carry the transgene due to segregation (as shown in the northern analysis for the expression of the mustard defensin, Fig. 4b), also did not show resistance to the pathogen infection like the control plants (Fig. 7), whereas the T_1 transgenic plants carrying stably inherited transgene (8-2, 8-4, 8-5, 10-1 and 10-4) showed significantly enhanced resistance to the pathogen. On these plants, symptoms were not detected even after 15 days post-inoculation and the leaves remained fresh without any sign of disease. In some of the plants, lesions formed, but lesion size did not increase further.

Fusarium moniliforme attacks young seedlings causing vein clearing, wilting, chlorosis and necrosis in many crop plants including tobacco. Plants were affected at later stages also. Transgenic plants were tested for their ability to resist this pathogen infection. Detached leaf assays were performed and symptoms were observed after 1 week. Vein clearing was evident in the detached control leaves, which was not evident in transgenic leaves (Fig. 8).

The above results suggest that transgenic plants expressing the mustard defensin constitutively exhibited enhanced resistance to the fungal pathogen infection.

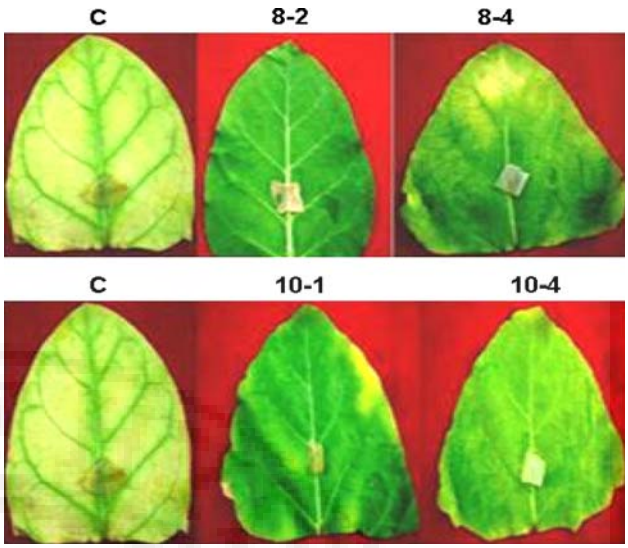

Fig. 8 Analysis of the tobacco transgenic plants with the challenge from *Fusarium moniliforme*. Please note severe vein clearing and necrosis in the control. Transgenic plants resisted the infection

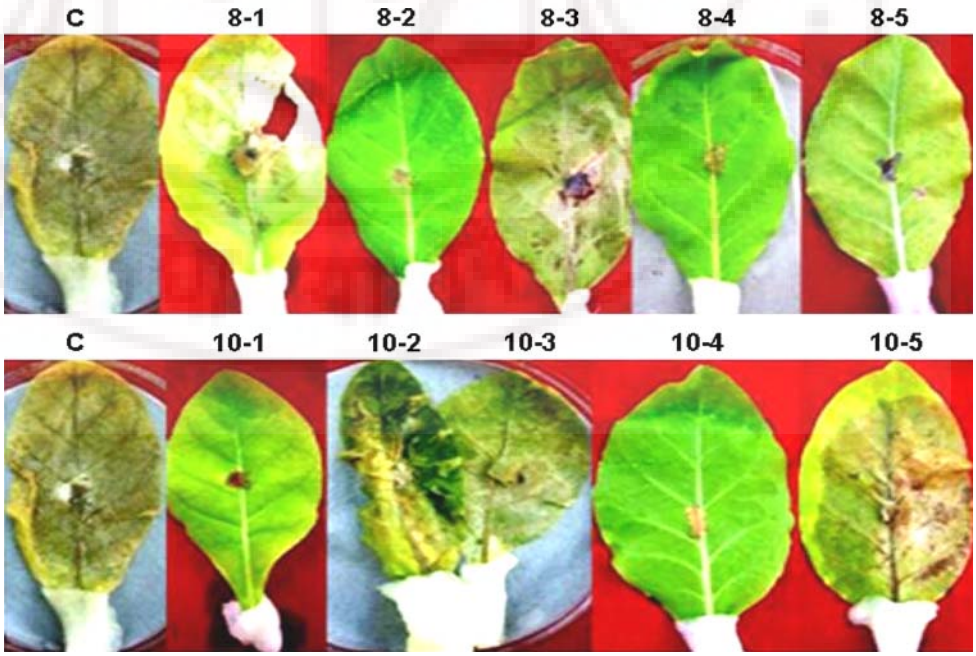

Fig. 7 Analysis of tobacco transgenic plants with the fungus *Phytophthora parasitica* pv. *nicotianae*. Note the development of disease on control and segregants that did not express mustard defensin (8-1, 8-3, 8-5, 10-2, 10-3 and 10-5) and the control plants as shown in the northern analysis in Fig 4b. The transgene expressing segregants resisted the infection

Evaluation of the transgenic peanut plants for resistance to tikka or late leaf spot disease-whole plant assay

The Southern confirmed peanut transgenic plants were assayed for their ability to resist infection from the leaf spot causing pathogens in whole plant analysis using conidial spray. The disease symptoms started appearing on control plants after 10–12 days as small specks, which later developed into lesions. Data was scored after 28 days of inoculation and plants were assessed for leaf spots. On control plants the numbers of spots were high in number and the lesion size also increased within 10–15 days, whereas the size and number of lesions on resistant transgenic plants did not increase even after 28 days. When the total number of intact leaflets (four leaflets per leaf) and the number of diseased leaves on the main stem were counted at the glass house level, the control plants were observed to have a disease severity of 6–7 out of 9 against the transgenic plants, which showed a reaction of 1–3 in the scale (Fig. 9).

Detached leaf assay

We have carried out detached leaf assay to further confirm our results on the analysis in the glass house and the data obtained was consistent with our data on the whole plant assay. Disease symptoms started appearing after 15 days as brown color specks on these leaves. All the transgenic lines showed increased resistance *to P. personata* as measured by the average number of lesions and a reduction in lesion area (Fig. 10). Some of the lesions observed on these leaves belonged to the early leaf spots caused by *Cercospora arachidicola*.

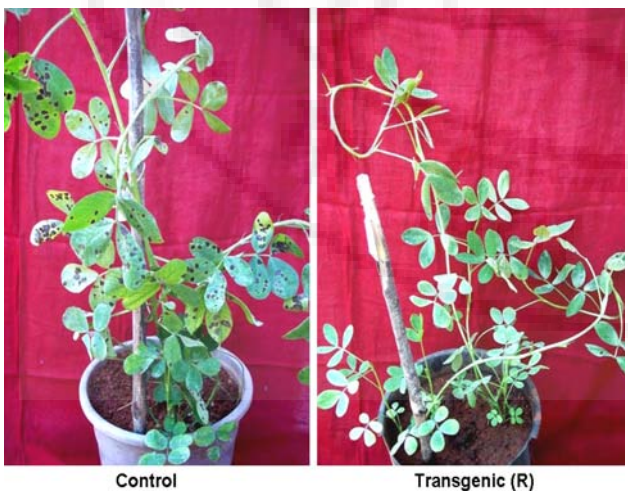

Fig. 9 Field analysis of late leaf spot infection (in the green house) after conidial spray. Note the development of leaf spots in the control plant. Transgenic plant 4-2 resisted the infection. Photograph taken after 28 days of treatment

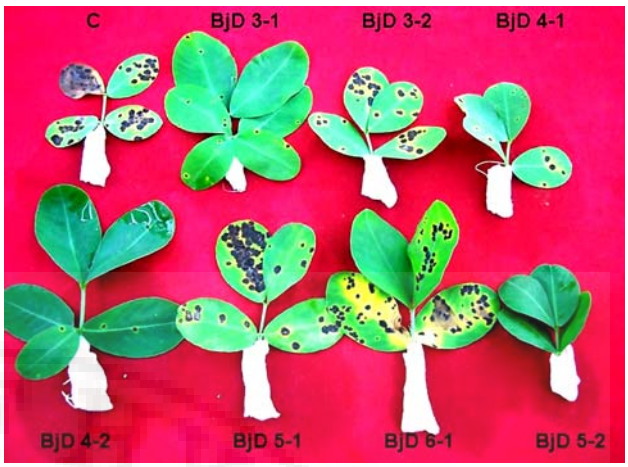

Fig. 10 Detached leaf assay for the leaf spot using conidial spray in Petri dishes. Please note the disease development on control and segregants in the progenies of transgenic plants that did not receive the transgene. Lesions were of a mixture of early and late leaf spots

The average number of lesions was found to be less in transgenic plants compared to the controls (Fig. 11a). The decrease in the number of lesions was found to be significantly lower in transgenics ($P \geq 0.05$). The type of lesion also varied between transgenic and control lines. Controls showed large, continuous, black colored lesions; whereas the transgenic lines typically exhibited smaller lesions resembling a hypersensitive response. The lesion size was found to be significantly less in transgenics ($P \geq 0.05$). The lesion frequency has further decreased to 90.91% in *BjD*-3-1 and 92.73 in *BjD*-7-2. In the same set of plants, the lesion size also decreased to 85.72% in *BjD*-10-2 and 88.58% in *BjD*-7-2 (Fig. 11b). Though the lesion frequency was high in *BjD*10-2, the size of the lesions did not grow even after continued incubation of leaves.

The estimation of spore/conidial germination or growth using crude protein extracts from plants is also commonly used in determining the antifungal activity. We performed MTT assay to check the germination of conidia of the late leaf spot fungus in plant protein crude extracts of both control and transgenic plants. We observed that 50 μg of crude protein extracts of transgenic plants *BjD* 4-2 was able to inhibit 18% of the spore germination. Hence, this concentration can be considered as minimal inhibitory concentration (MIC). Crude protein (100 μg) was able to inhibit 50% of spore germination and this concentration was considered to be lethal for spore germination. Spore germination was not inhibited in control plant protein extracts. This assay showed that transgenic plant protein extracts inhibited fungal spore germination considerably (data not shown).

These results showed that the constitutive expression of *BjD* in peanut confers enhanced resistance against late leaf spot pathogen also.

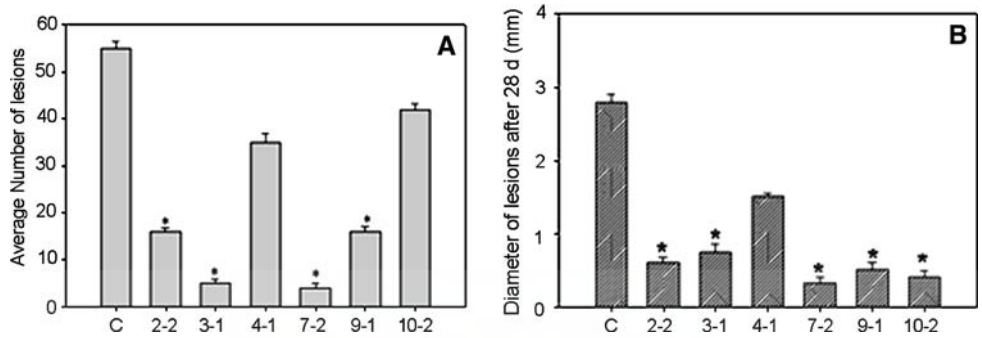

Fig. 11 **a** Analysis of lesion number in control and transgenic plants after conidial spray in detached leaf assays. There is a significant difference in the number of lesions on transgenic plants and the control ($P \geq 0.05$). **b** Analysis of lesion diameter in control and transgenic plants in detached leaf assay. Note the significant reduction in lesion diameter in the transgenic plants ($P \geq 0.05$)

Discussion

Transgenic expression of plant defensins has been reported to enhance protection in vegetative tissues against pathogen attack. Constitutive expression of RsAFP-2 enhanced resistance of tobacco plants to the leaf pathogen *Alternaria longipes* (Terras et al. 1995) and tomato to *Alternaria solani* (Parashina et al. 2000). Canola expressing a pea defensin showed enhanced resistance against blackleg disease caused by *Leptosphaeria maculans* (Wang et al. 1999). The constitutive expression of an alfalfa defensin in potato provided robust resistance against the agronomically important fungus *Verticillium dahliae* under field conditions (Gao et al. 2000). Overexpression of BSD1 (stamen specific defensin) in transgenic tobacco plants enhanced their tolerance against the pathogen *Phytophthora parasitica* (Park et al. 2002). Expression of a defensin gene along with a glucanase gene has been shown to enhance tomato resistance against *Ralstonia solanacearum* (Chen et al. 2006). More recently, Aerts et al. (2007) have reported that the expression of human beta defensin-2 in *Arabidopsis* resulted in enhanced resistance against *Botrytis cinerea* demonstrating the functional homology of defensins across Kingdoms.

We have cloned a defensin from mustard using the sequence information available for a *B. oleracea* defensin and it had high amino acid sequence identity to the *B. oleracea* defensin as well as the well-characterized defensins RsAFP-1 and 2 of *Raphanus sativus*. RsAFP-2 has been shown to possess antifungal activity (Terras et al. 1995). We have generated transgenic tobacco and peanut plants expressing the mustard defensin constitutively. Transgenic tobacco plants expressing mustard defensin showed enhanced resistance against two fungal pathogens, *Fusarium moniliforme* and *Phytophthora parasitica* pv. *nicotianae* in detached leaf assays showing the efficacy of the mustard defensin in imparting disease resistance in tobacco.

Peanut transgenic plants also displayed substantially increased levels of leaf spot disease resistance in detached assay and green house test using the conidial suspension mixture of *P. personata* and *C. arachidicola*. The T_1 progeny plants, segregating for the transgene, were evaluated for resistance against the leaf spot disease and they exhibited varied resistance as indicated by a range of 1–7 on the 9 point scale (Subrahmanyam et al. 1982); those progeny plants that received the transgene showed a reaction 1–2 as against the non-transgenic segregants, which behaved like the wild type control plants. All the control plants and the segregants from the transgenic plants that did not receive the transgene showed extensive disease symptoms leading to defoliation and severe necrosis. Highest percent reduction in lesion frequency was noticed on the transgenic plant *BjD*-7-2. Transgenic plants expressing mustard defensin consistently showed higher levels of resistance.

Our results correlated well with previously published reports in other plants. Lesion size resulting from inoculation with *Sclerotinia minor* was reduced to 97% in transgenic peanut plants over expressing a barley oxalate oxidase gene (Livingstone et al. 2005). Similarly, the lesion size was reduced by 63% in poplar expressing a wheat oxalate oxidase gene (Liang et al. 2001). Oxalate decarboxylase was shown to be produced by the resistant host plants to degrade oxalic acid produced in some plant pathogen attacks and the expression of the gene for this enzyme decreased the lesion size approximately by 89% in transgenic tomato plants inoculated with *S. sclerotiarum* (Kesarwani et al. 2000). Transgenic rice plants overexpressing a wasabi defensin also showed enhanced resistance to *Magnaporthe grisea* and the average size of disease lesions was reduced to about half of that in the non-transformed plants (Kanzaki et al. 2002). The lesion size decreased significantly in grapevine transgenic plants inoculated with anthracnose fungus (Yamamoto et al. 2000). Transgenic tobacco plants expressing a magainin analog MYP30 were evaluated for resistance to *Peronospora*

tabacina infection and significant reduction in sporulation and lesion size were observed (Li et al. 2001). Resistance to *Alternaria brassisicola* in transgenic broccoli expressing a *Trichoderma harzianum* endochitinase gene was assessed by a detached leaf assay of T_0 plants inoculated with *Alternaria brassisicola* and the lesion size showed a negative correlation with endochitinase levels (Mora et al. 2001).

The present investigation shows that the expression of the mustard defensin in transgenic tobacco and peanut plants also conferred enhanced resistance against various pathogens amply demonstrating the efficacy of defensins in imparting disease resistance in crop plants and indicating that they are good candidate genes in crop improvement.

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