Heterologus expression of *Brassica juncea* Nonexpressor of pathogenesis related genes 1(*BjNPR1*) and *Tephrosia villosa* defensin (*TvD1*) for amelioration of biotic stress tolerance

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Doctor of Philosophy in Plant Sciences

By

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Certificate

This is to certify that I, **S. VIJAYAN** have carried out the research work embodied in the present thesis entitled "**Heterologus expression of *Brassica juncea* Nonexpressor of pathogenesis related genes 1(*BjNPR1*) and *Tephrosia villosa* defensin (*TvD1*) for amelioration of biotic stress tolerance**" 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 &

LORD MANAKULA VINAYAKAR

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S. Vijayan

Abbreviations

µg	Microgram
µM	Micro Molar
µCi	Micro Curie
2,4-D	2,4-Dichlorophenoxyacetic acid
BAP	6-Benzylaminopurine
bp	Base pair
cDNA	Complementary DNA
CIAP	Calf Intestine Alkaline Phosphatase
CTAB	Cetyl/ Hexadecyltrimethyl Ammonium Bromide
Da	Dalton
DDW	Double distilled water
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide
dsDNA	Double stranded DNA
EDTA	Ethylene Diamine Tetra Acetic Acid
EtBr	Ethidium Bromide
hr/hrs	hour/s
IAA	Indole-3-Acetic Acid
IBA	Indole-3-Butyric Acid
IPM	Integrated Pest Management
kDa	Kilo Dalton
Kin	Kinetin/ 6-Furfurylamino purine
NAA	Naphthalene Acetic Acid
ng	Nanogram
nm	Nanometre
OD	Optical Density
PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
pmol	Picomol
PMSF	Phenyl Methane Sulphonyl Fluoride
PVDF	Polyvinylidine Fluoride
PVPP	Polyvinyl Polypyrrolidone
RT	Room Temperature
SDS	Sodium Dodecyl Sulfate
SSC	Sodium Chloride Sodium Citrate
ssDNA	Single stranded DNA
SSPE	Sodium Chloride Sodium Phosphate EDTA
TAE	Tris Acetic acid EDTA
TBS	Tris Buffered Saline
TBST	Tris Buffered Saline Tween
TDZ	Thidiazuran/1-phenyl-3- (1, 2, 3-thiadiazol-5-YL) urea
TE	Tris EDTA
TEMED	N, N, N, N-Tetra Methyl Ethylene Diamine
Tris	Tris (hydroxymethyl) amino methane
β-Merc	β-mercapto ethanol

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Chapter 1

INTRODUCTION

Plants are the source of food and shelter for a wide range of organisms including other plants (parasitic plants). Plants are constantly under heavy stress from various biotic and abiotic sources, which affects their growth and productivity. Stress caused by any living organism is called as **biotic stress**, which is generally affected by pathogens and insects. Unlike their animal counterparts, plants lack a circulating adaptive immune system to protect themselves against the invading pathogens. Plants are resistant to most pathogens in their environment, as they are not hosts for a particular pathogen or they could be, but harbor resistance genes that allows the induction of active defense mechanisms, which are based upon early detection and quick response to the invading organisms. In general, pathogens were classified into three basic types based on their mode of life style. They are necrotrophs (secrete toxins and feed on dead cells), biotrophs (live on living host and derive nutrients through a specialized organ were called haustorium). If it displays both the types of life style they are called hemibiotrophs (**Fig. 1.1**).

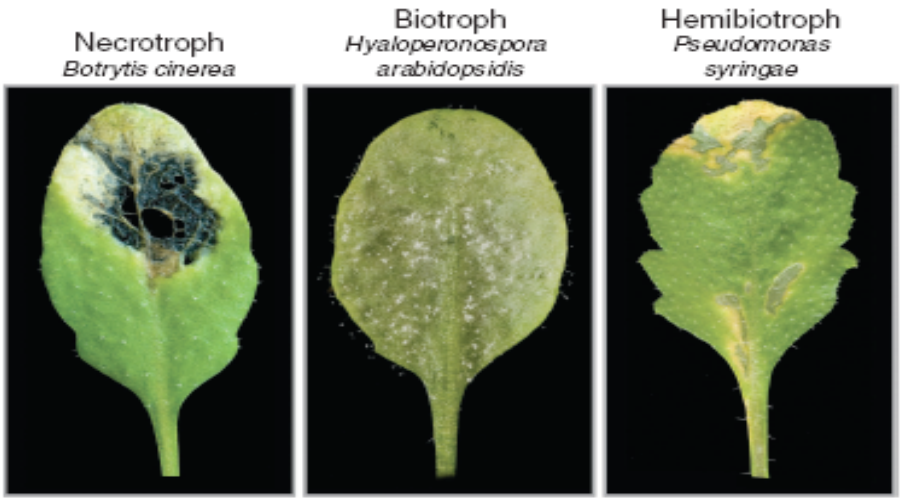


Figure 1.1. Arabidopsis leaf infected with necrotroph (*B. cinerea*), Biotroph (*H. arabidopsidis*) and Hemibiotroph (*P. syringae*) (Pieterse et al. 2009).

In order to respond to the pathogenic invaders, many early signaling events are triggered within the plant as a defensive response. It includes the fortification of cell wall through cellulose synthesis and lignin deposition, production of antimicrobial secondary metabolites such as phytoalexins and the accumulation of PR proteins such as glucanases and chitinases. Recognition of pathogen specific effectors leads to the production of ROS at the site of infection, which

develops a hypersensitive response (HR) that is followed by cell death (Glazebrook et al. 2005). This isolates the pathogens from further spread into the adjoining healthy tissues. This is, in fact, advantageous in the case of necrotrophs but not for biotrophs. Necrotrophic response is triggered by pathogenic toxins or Pathogen-Associated Molecular Patterns (PAMP), such as the breakdown product of plant cell wall by the activity of pathogen derived enzymes, which can stimulate the plant defensive responses (Schwessinger and Zipfel 2008; Nurnberger et al. 2009). Detection of these virulence proteins or other general elicitors triggers signaling events within the host cells that leads to diverse cellular responses, including changes in ion fluxes, synthesis of the stress related hormone such as ethylene, transcriptional reprogramming, production of reactive oxygen species (ROS), and often a localized form of programmed cell death, often referred to as the hypersensitive response (HR).

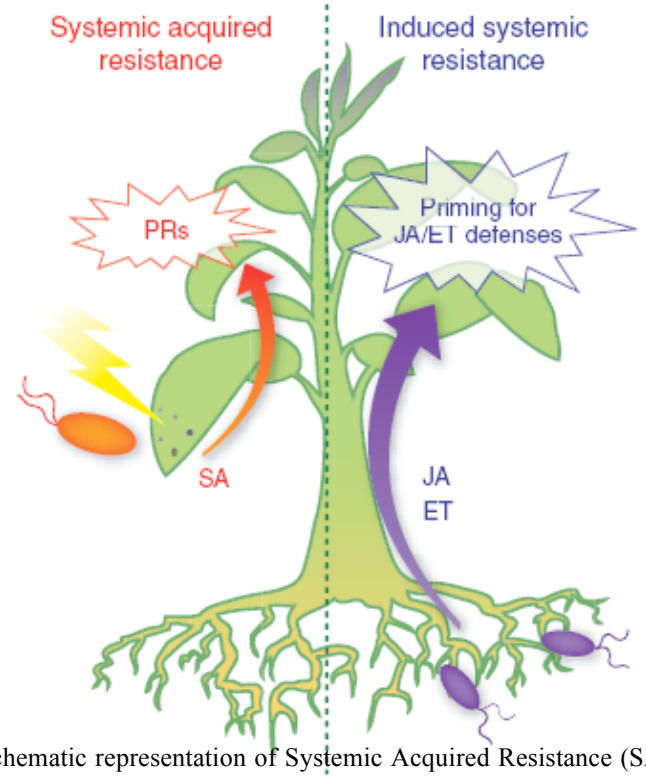


Figure 1.2. Schematic representation of Systemic Acquired Resistance (SAR) and Induced Systemic Resistance (ISR) in plants (Pieterse et al. 2009)

Additionally, activation of a local defense response can trigger the production of salicylic acid (SA) and the elicitation of broad-spectrum defense responses known as systemic acquired resistance (SAR). It is activated at the site of infection and the defense response is triggered in distal parts that are usually away from the infection site to protect the healthy tissues from the pathogenic

invaders (**Fig. 1.2**) and it is characterized by the co-ordinate activation of set of PR genes (Pathogenesis Related genes), many of which have antimicrobial activity (Van Loon et al. 2006).

Although some of these have been shown to have specific enzymatic activity, i.e., chitinases (PR-3) and ß-(1, 3)-glucanases (PR-2), many have no known catalytic function and their mechanisms of action is not clearly understood (Yun et al. 1997). In some cases, the soil microorganism such as mycorhiza or rhizobacteria activates the defense responses especially against the necrotrophs at distant healthy tissue by producing the signaling components such as JA (Jasmonic acid) or ET (Ethylene). Such type of resistance is termed as Induced Systemic Resistance (ISR) (Pieterse et al. 2009) (**Fig. 1.2**).

Protection of crop from diseases can substantially improve the agricultural production. Although fungicides and pesticides have been used successfully to control diseases and pests, their continued and increasing use might lead to harmful effects on health and environment. In India, the amount of usage of chemical pesticides has increased five times from 1965-66 to 1990-91. Extensive usage instigates additional hazards like resistance to fungicides in fungal pathogens and resurgence in insect pests. So far, over 700 insect pests are reported to have developed resistance worldwide to a variety of insecticides, out of which 33 have been reported from India (Rai 2004).

Use of high-yielding crop varieties can improve the productivity, but carries the risk of devastating epidemics due to genetic uniformity. Conventional breeding utilizing the available gene pool of resistant traits has led to the development of a number of resistant plant varieties. However, there are some inherent problems associated with the conventional approach, such as, long gestation period in developing resistant hybrids. During these long years, even the pathogens might evolve and overcome the resistance, leading to a futile effort. Besides this, because of the differences in the ploidy level of the genome and other reasons, effective introgression of resistance genes into the susceptible cultivar from resistant wild species is often difficult in many crops. Additionally, transfer of desirable traits is often associated with transfer of undesirable genetic material because of linkage, known as linkage drag, which consequently reduces

the potential of the resulting introgressed material in subsequent crop improvement programs.

In this regard, perhaps the most promising field of genetic research has been genetically modified (GM) crops. Several GM crops with different altered traits e.g. herbicide tolerance (in canola, cotton, flax, maize, rice, sugar beet and carnation), improved insect resistance (Bt-cotton, maize, potato and tomato) and higher shelf life (tomato) are already under commercial use. Disease resistance through genetic modification has tremendous potential but requires a lot of patience and perseverance as the phytopathogens being microbial in nature might evolve resistance to the transgenes deployed against them. Transgenic modification also allows the introduction of genes for disease and pest control from unrelated plant species (Boller 1993) or organisms outside the plant kingdom (Rao 1995) to be incorporated into crops. Several current research efforts are aiming the development of disease resistance through recombinant gene technology, for example, transgenic modification, metabolic manipulation, defense pathway manipulation and knock out of genes.

However, the development of diseases and mechanism of disease resistance in plants are governed by the combined genotypes of the host and pathogen, and depends on a complex exchange of signals and responses. During the long process of host-pathogen co-evolution, plants have developed various mechanisms to ward off pathogen attack and the genes governing these intricate defense mechanisms could be used in developing genetically modified disease resistant crops.

In the past 20 years, genetic research has identified several plant genes involved in the defense signaling pathways and disease resistance. Antifungal genes like defensins, chitinases, glucanase, osmotin are a few potential genes that have been used in genetic transformation of important crop species by different research groups for achieving fungal disease resistance.

In plants, several signaling cascades get activated during pathogen infection and Salicylic acid (SA), Jasmonic acid (JA) and ethylene (ET) play a major role in activating the defense response genes. When a biotroph approaches the host, SA signaling cascade gets activated leading to the development of resistance by

expressing the PR genes such as PR-1, PR-2 and PR-5. Here, NPR1 (Nonexpressor of pathogenesis related proteins) plays a crucial role and acts as a positive activator for the expression of PR-1 gene. In contrast, any necrotroph that approaches a host, activates the JA and ET signaling cascade leading to the development of resistance by expressing the genes for defensin (PDF1.2), VSP2 (vacuolar sorting protein2), etc. Hence in the present study, we opted for two genes such as *BjNPR1* from mustard (*Brassica juncea*) and a defensin (*TvD1*) gene from the weedy legume *Tephrosia villosa*. The gene *BjNPR1* was characterized in our lab and identified as one of the candidate genes for plant transformation especially in crop plants. Hence, it has been utilized for mungbean transformation in the present study, and *TvD1* was isolated and functionally characterized for biotic stress tolerance.

Thus, the thesis has been largely divided into two parts. They are as follows

Part A (Chapter 4). Heterologus expression of *BjNPR1* in mungbean for amelioration of biotic stress tolerance.

Part B (Chapter 5). Characterization and heterologus expression of a legume defensin, *TvD1* in tobacco for amelioration of biotic stress tolerance.

Chapter 2

review of literature

Review of literature

Legumes are extensively grown in the rainfed and dryland areas of India. These crops were used as the source of dietary protein, especially for the large vegetarian population of sub-tropical countries like India. Even though they were grown in large acreage, overall productivity of the crops is very less. The plants are being susceptible to various stresses including biotic and abiotic stresses, its suffer leads to yield losses and at increasing severity, the plants might die. In order to improve the crop productivity as well as the nutritional quality and quantity, transgenic technology is of significant importance as the conventional breeding is time consuming and laborious. Among legumes, soybean is the only transgenic crop cultivated worldwide and is about 63% of its total area is under transgenic cultivation (James 2001). The legumes are mostly recalcitrant in nature with poor regeneration ability in tissue culture which is coupled with the lack of appropriate gene delivery system making it difficult to obtain stable transgenic plants.

Mungbean:

Among the pulse crop cultivation and production, mungbean (*Vigna radiata* (L.) Wilczek) (2n=22) stands third after pigeon pea and peanut in India. It is a grain legume widely cultivated in Southeast Asia, Africa, South America and Australia and grown for human consumption as a major source of dietary proteins in developing countries, particularly in India. It is also referred to as green gram, golden gram and chop suey bean. It is grown mostly for use as a human food (as dry beans or fresh sprouts), but can also be used as a green manure crop and as forage feed for the livestock. Mungbean seeds are sprouted for fresh use or canned for shipment to restaurants. Sprouts are high in proteins (21%–28%), calcium, phosphorus and certain vitamins. Because of easy digestibility, it replaces the scarce animal protein in human diets in tropical areas of the world. If the mungbean seed does not meet sprouting standards, it can be used as a livestock food with about 1.5 ton of mungbean being equivalent to 1.0 ton of soybean meal for protein content. Mungbeans are susceptible to diseases caused by pathogens such as white mold, *Phytophthora sp*, mildew, bacterial

rots, *Rhizoctonia sp*, etc. *Cercospora canescens* causes leaf spot diseases in mungbean and crop loss could be upto 47% (AVRDC, 1984). Similarly, powdery mildew caused by the fungus *Erysiphae polygoni* and the corresponding crop loss was upto 40% (AVRDC, 1984). Among the insect predators, seed corn maggots, wireworms and also some grasshoppers or caterpillars causes defoliation of leaves but comparatively less when compared to other legumes.

Conventional breeding for pest- and pathogen-resistant varieties is a difficult and time taking task due to genetic barriers. The genetic engineering methods appear to offer better solutions for mungbean improvement. However, an efficient, simple and reproducible regeneration system is a pre-requisite for developing transgenic plants against various pathogens and pests. Recalcitrant nature of the plant makes it difficult to regenerate shoots *in vitro*. Reports on regeneration of shoots through organogenesis from the callus (Mendoza et al. 1992; Gulati and Jaiwal 1990; 1992; 1994) and somatic embryogenesis (Girija et al. 2000; Devi et al. 2004) indicated that cotyledons, primordial leaves, hypocotyls, shoot tips of mungbean are not ideal explants for *in vitro* regeneration. Organogenic callus was derived from explants like cotyledons (Gulati and Jaiwal 1990; Mendoza et al. 1992; Chandra and Pal 1995; Amutha et al. 2003), hypocotyls, leaves (Gulati and Jaiwal 1990) and shoot tips (Gulati and Jaiwal 1992) but was not usable for raising plants. Direct shoot regeneration protocols are preferred for developing transgenic plants to avoid somaclonal variation. Hence, cotyledonary node explants are being, of late, preferred in recalcitrant grain legumes for simple and effective shoot regeneration. Though direct shoot organogenesis without callus phase was reported from the cotyledonary node explants (Avenido and Hautea 1990; Gulati and Jaiwal 1994, Chandra and Pal 1995; Avenido et al. 2001), immature cotyledonary node was shown to produce more shoots (a maximum of 8 to 9 shoots per explant) in mungbean (Avenido and Hautea 1990; Gulati and Jaiwal 1994) and other related grain legumes such as pea (Jackson and Hobbs 1990), pigeon pea (George and Eapen 1994) and chickpea (Ignacimuthu and Franklin 1999). In the present study, we have attempted to optimize further the number of shoots in a rapid way using seedling cotyledonary node explants, which was used further in *Agrobacterium*-mediated transformation studies.

Legume transformation:

The productivity of the legume can be improved by value addition, the desirable traits which have the ability to withstand the biotic and abiotic stresses. Through conventional plant breeding methods, we achieved the improvement in the pulse crops, but transgenic technology has promising output. Hence, it is gaining popularity in the recent past. Despite the development of transgenic technology in 1980's and the first report on development of a transgenic pulse crop, *Vigna aconitifolia* in the same decade (Eapen et al. 1987), the progress achieved was not significant compared to their counterpart crops-namely cereals. Cereals were considered to be recalcitrant for regeneration in the 1970's and for transformation in the 1980's. However, concentrated efforts and free flow of funding support for cereal transformation have pushed transgenic cereals to the forefront of transgenic success stories (Shrawat and Lorz 2006), while transgenic pulse crop research, although has moved forward, is still confined to the four walls of the laboratory. Since these tropical grain legumes are of prime importance more to the developing countries, less effort have been put forward on these crops compared to cereals. Besides, different factors like recalcitrance of pulses for regeneration, low competence of regenerating cells for transformation and lack of a reproducible *in planta* transformation system have been pointed out as reasons for non-development of transgenic pulse crops with high efficiency (Somers et al. 2003; Popelka et al. 2004; Dita et al. 2006). However—soybean, a leguminous oil crop and a source of protein is success story in the commercialization of transgenic plants, as largest acreage under transgenic crop cultivation belongs to this crop.

The major pulse crops of the world are bean (*Phaseolus vulgaris* L.), pea (*Pisum sativum* L), broadbean (*Vicia faba* L), chickpea (*Cicer arietinum* L), pigeonpea (*Cajanus cajan* L Millsp), blackgram (*Vigna mungo* L), green gram (*Vigna radiata* L Wilczek), grasspea (*Lathyrus sativus* L), lupin (*Lupinus* spp), lentil (*Lens culinaris* Medik L Walsp), cowpea (*Vigna unguiculata*) and winged bean (*Psophocarpus tetragonolobus* L) and majority of them are grown in tropical and subtropical regions of the world.

In general, the frequency of transformation in legume is very less because of their recalcitrant nature. It was about 0.1 – 1.2% in soybean (Hinchee et al. 1988; Di et al. 1996) but with the improvement in the *Agrobacterium* strain and the use of sonication-assisted *Agrobacterium* transformation, it became 2% i.e., cotyledonary node explants gave transformed shoots with the new *Agrobacterium* strain KYRT118. Glufosinate selection reported to enhance the recovery of transgenics in soybean (Zhang et al. 1999) after transformation. A novel method of genotype independent *Agrobacterium*-mediated transformation of cotyledonary nodes and subsequent regeneration of shoots has been patented (Thomas and Townsend 1994). Here, the induction of virulence genes and the use of sequential inoculations have been shown to enhance the frequency of transformation and achieve infections in non-susceptible cultivars. In the case of pigeonpea, LBA 4404 strain increased the frequency of transformation in cotyledonary node to 62% and it was 45% from the shoot apex (Geetha et al. 1999). However, this is a preliminary report and no confirmed transgenics are available. Recently, the strain GV2260 has been used to recover transgenic plants carrying the cowpea protease inhibitor (Lawrence and Koundal 2001). Transgenic plants were characterized by Northern blotting to confirm the presence of mRNA and the frequency of transformation in this study was ~ 1%. In early attempts at recovering transgenics of chickpea with *Agrobacterium* mediated transformation of cotyledonary nodes, frequencies of 2–4% were reported (Fontana et al. 1993; Kar et al. 1996), but in later report (Krishnamurthy et al. 2000), transformation efficiency of 0.4% only was reported. A low frequency of transformation has been reported in pea also. Recently, it was about 3.5% when using a new binary vector, pGreen (Hellens et al. 2000) using the cotyledonary node explants. Enhanced recovery of transgenic plants by including 5-azacytidine in the selective medium has also been reported (Orczyk and Orczyk 2000). In their study, abnormal transgenic shoots were recovered on media with kanamycin as selection agent, while selection on phosphinothricin containing medium proved more useful for regenerating normal plants. In groundnut, a non-tissue culture approach has been employed for recovering transgenic plants (Rohini and Sankar Rao 2000). Embryo axes lacking one cotyledon were wounded by pricking with needles and infected with *Agrobacterium* strain LBA4404 (pKIWI105). Interestingly, the infectivity of *Agrobacterium* could be

enhanced by using of tobacco leaf extract instead of acetosyringone. Transgenic groundnut plants have also been recovered (Cheng et al. 1996; Rohini and Sankar Rao 2000) from leaf explants infected with EHA101 (pBI121), but frequency was very low (0.02–0.03%). Transmission of the transgene (*Gus*) to the progeny was also shown in groundnut (Rohini and Sankar Rao 2000). For transformation of *Vigna sesquipedalis*, cotyledonary node explants were infected with the strain EHA105 and a transformation frequency of up to 2% was reported (Ignacimuthu 2000). Transformation frequencies of 23% with LBA4404 and 10% with EHA105 using leaf discs of *V. mungo* have been reported (Karthikeyan et al. 1996). However, transformed calli did not give rise to any shoots. In *V. unguiculata*, transformed callus (Garcia et al. 1986) was recovered by leaf disc transformation with strains C58C1 (pGV3850) and C58C1 (pGV3850 :: 1103neo). Transformation of mature embryos with the strain C58 (pGV2260/ p35S*gus*int) (Penza et al. 1991) gave rise to chimeric plants. Cotyledon explants infected with the strain LBA4301 (pUCD2340) yielded six transformants (Muthukumar et al. 1996). However, using shoot apices from mature dry seed, transgenic plants (Sahoo et al. 2000) were recovered. An enhanced transformation frequency of cotyledon and hypocotyls explants has been observed with *A. rhizogenes* strain LBA9402, but the transformed tissue gave rise to roots only (Jaiwal et al. 1992). Transgenic calli and shoots were recovered from primary leaves and cotyledonary nodes respectively (Jaiwal et al. 2001). Transformation frequency is up to 50% was reported using the strain EHA105 (pBin*gus*int) and hypocotyls explants; however the callus did not regenerate shoots. Cotyledonary nodes infected with the strain LBA4404 (pTOK233) gave rise to transgenic plants at an overall frequency is 0.9%. Integration of foreign genes was confirmed by Southern analysis.

The particle bombardment method has been used for genetic transformation of many grain legume species. Shoot apical meristems have been shown to form multiple shoots in soybean (Yang et al. 1990), groundnut (Ponsamuel et al. 1998) and *V. radiata* (Gulati and Jaiwal 1992). In soybean, bombarded apices were allowed to grow into plants that flowered and set seed. The seeds were tested for GUS expression to confirm the transgenic nature of the parent plant. No *in vitro* selections were done and thus a larger number of plants

had to be screened. Although transformations were at low frequencies (Christou et al. 1989; Sato et al. 1993) ~ 0.4–4%, transgenic plants could be recovered. However, a large proportion of these were chimeras. Southern analysis of T_0 plants and their progeny revealed that mostly two to hundred copies of the gene were integrated in the genome. However, these copies were linked and co-segregated as a single locus in the progeny. At present, all the transgenic plants of soybean under commercial cultivation have been generated via particle bombardment.

In *V. radiata*, the transformation was first successfully attempted by Jaiwal et al. (2001) and achieved the stable transformants using the primary leaves as well as cotyledonary node explants using the gene for *GUS* reporter and *npt*II marker. Similarly, it was obtained using the primary leaf explants with *GUS* reporter and *npt*II gene from pCAMBIA2301 (Sitamahalakshmi et al. 2006). Report of transformation in mungbean with α-amylase inhibitor was achieved by Sonia et al. (2007). But in all the cases, the frequency of transformation was very low.

In the present study, we are reporting first time in *V. radiata* transformation with disease resistance signaling pathway gene such as *NPR1* from *Brassica juncea* to ameliorate the biotic stress tolerance. In SAR pathway, SA gets accumulated after pathogen challenge, particularly biotrophs. This process activates the NPR1 from oligomer into monomer in the cytoplasm and the monomer gets translocated into the nucleus, where they activate the expression of PR genes.

***NPR1* (Nonexpressor of pathogenesis related genes 1):**

NPR1 (**N**onexpressor of **P**athogenesis **R**elated genes **1**), also known as *NIM1* and *SAI1* is shown to be involved in SA (salicylic acid) mediated signal transduction leading to SAR (Cao et al. 1994; Delaney et al. 1995; Glazebrook et al. 1996; Shah et al. 1997) in plants and the gene was first cloned by different groups from *Arabidopsis thaliana* (Cao et al. 1997; Ryals et al. 1997). *Arabidopsis npr1* mutants are non-responsive to SA, do not mount an effective SAR and are compromised in their ability to express Pathogenesis-related (PR) genes (Cao et al. 1994; Delaney et al. 1995). Moreover, these plants are unable to

restrict the spread of infection by virulent pathogens at the primary site of invasion. The Overexpression of *NPR1* in rice, tomato, *Arabidopsis*, tobacco, wheat and carrot led to enhanced resistance against various pathogens (Cao et al. 1998, Chern et al. 2001; Friedrich et al. 2001; Lin et al 2004; Makandar et al. 2006; Meur et al. 2008; Wally et al. 2009). The *Arabidopsis npr1* mutants also showed reduced tolerance to SA toxicity and accumulated high endogenous levels of SA, suggesting a role for NPR1 in both detoxification of SA and feedback regulation of SA biosynthesis (Cao et al. 1997; Kinkema et al. 2000).

NPR1 protein has two protein–protein interaction motifs (Cao et al. 1997) such as BTB/POZ (Broad-Complex, Tramtrack, and Bric-a-brac/Pox virus and Zinc finger) (Aravind and Koonin 1999) and Ankyrin repeats (Bork 1993). The protein is present, constitutively, in the cytoplasm and upon SAR induction, localizes to the nucleus (Després et al. 2000; Kinkema et al. 2000; Fan and Dong 2002). Absence of any known DNA binding domains in NPR1 and presence of protein-protein interaction domains suggested that it might play an indirect role in regulating *PR-1* gene expression involving interaction with other transcription regulator(s). By means of yeast two-hybrid analysis, NPR1 was shown to interact with the members of the TGA family of transcription factors containing basic domain leucine zipper motif (bZIP) with cognate DNA binding elements containing the core sequence TGACG (Zhang et al. 1999; Després et al. 2000; Niggeweg et al. 2000b; Zhou et al. 2000; Chern et al. 2001). These transcription activators interact with activated NPR1 and then binds to SA responsive elements (also called as-1 element) present abundantly in the promoters of many *PR* genes and regulate their expression. Localization studies with *Arabidopsis* NPR1 revealed that it is present in both cytoplasm and the nucleus (Kinkema et al. 2000; Després et al. 2000). However, only at the onset of SAR, cytosolic NPR1 translocates to the nucleus, interacts with TGA factors (Kinkema et al. 2000; Fan and Dong 2002), and enhances their DNA binding activity. Nuclear localization is controlled by a bipartite nuclear localization sequence located at the C-terminal end and is important for the induction of PR genes (Kinkema et al. 2000). The various transcription factors and regulatory proteins that are identified and shown to interact with NPR1 are elaborated in the following section (Durrant and Dong 2004).

NPR1 binding transcription factors:

The absence of any obvious DNA-binding domain and the presence of protein-protein interaction domains in NPR1 leads to the identification of NPR1-interacting proteins and it was identified as NIMIN (non inducible immunity) 1, NIMIN2, and NIMIN3 (NIM interactor). NIMIN1 and NIMIN2 interact with the C-terminus of NPR1, while NIMIN3 interacts with the N-terminus (Weigel et al. 2001). NIMINs contain stretches of acidic amino acids and are hypothesized to be transcription factors. However, more elaborate experiments are required to demonstrate their biological activity. Recently, biochemical evidence for NIMIN-1 interaction with NPR1 and NIMIN-mediated negative regulation of distinct functions of NPR1 was demonstrated (Weigel et al. 2005).

NPR1 interactors are from the members of the TGA family of b-ZIP transcription factors. NPR1 interacts with the *Arabidopsis* TGA factors, TGA2, TGA3, TGA5, TGA6, and TGA7 but only weakly or not at all with TGA1 and TGA4 (Zhang et al. 1999; Despres et al 2000; Zhou et al. 2000; Kim and Delaney 2002; Zhang et al. 2003a). *Arabidopsis* NPR1 also interacts with TGA factors from tobacco and rice (Niggeweg et al. 2000b; Chern et al. 2001). Using truncated or mutant forms of NPR1, the Ankyrin-repeat domain in the middle of the protein was shown to be essential for binding TGA factors, while the N-terminal region appears to enhance the binding (Zhang et al. 1999; Despres et al. 2000; Zhou et al. 2000). TGA factors bind to activator sequence-1 (as-1) or as-1-like promoter elements (Katagiri et al. 1989), which have been found in several plant promoters, activated during defense, including Arabidopsis *PR-1* (Lebel et al. 1998). It was identified that LS5 and LS7 as-1 like elements in the promoter help in the binding of TGA transcription factors. Among the two, LS5 is a weak, negative regulatory element (Lebel et al. 1998) and LS7 is a positive regulatory element. Despres et al. (2000) showed that both TGA2 and TGA4 could bind to LS7, whereas only TGA2 could bind to LS5. Furthermore, binding of TGA2 but not TGA4 was enhanced by the addition of NPR1. Although NPR1 was clearly a positive regulator of *PR* genes, it might execute its function by either enhancing a transcriptional activator or by inhibiting a transcriptional repressor. The presence of multiple as-1-like elements in the *PR-1* promoter and the differential binding affinities of each TGA factor to these elements, as well as, to NPR1 highlight the complexity of the regulatory mechanism. Indeed, in an EMSA performed by

Despres and coworkers, binding to the as-1 element from the CaMV 35S promoter was significantly enhanced in protein extracts from SA-treated plants (Despres et al. 2000). However, extracts from untreated *NPR1* plants also contained strong as-1 binding activity and this was not changed upon SA treatment, suggesting that different TGA factors were responsible for the observed as-1-binding in wild type and *NPR1* transgenic plants.

It is also possible that the different subgroups of TGA factors regulate different sets of defense genes and that loss of induction of one set would be sufficient to impede SAR. As an alternative to mutant analysis, dominant-negative versions of TGA factors that can no longer bind to DNA were expressed in tobacco and *Arabidopsis*. In tobacco, overexpression of a dominant-negative TGA2.2 decreased as-1-binding activity and *PR* gene induction (Niggeweg et al. 2000b). These studies do support the idea that TGA factors can play both positive and negative roles in PR gene regulation.

Besides TGAs, WRKY transcription factors have been suggested to play a role in controlling *PR* gene expression. The cis-element that is recognized by WRKY factors, the W-box (with core element of TTGAC in tandem repeats), was over-represented in the PR1 regulon (Maleck et al. 2000), and mutations in a W-box led to repression of the PR-1 promoter (Lebel et al. 1998). It was hypothesized that WRKY factors are negative regulators of *PR* genes. The WRKY family consists of many more genes than the TGA family (Eulgem et al. 2000). Therefore, it would be difficult to identify a specific WRKY factor that is involved in *PR* gene expression. Overexpression of WRKY70 leads to constitutive *PR* gene expression, indicating that this transcription factor is a positive regulator of PR genes (Li et al. 2004). Surprisingly, W-box sequences were also identified in the promoter region of the *NPR1* gene in *Arabidopsis* and these W-boxes are recognized, specifically, by SA-induced WRKY DNA–binding proteins (Yu et al. 2001). This study provides strong evidence that certain WRKY genes act upstream of NPR1 and positively regulate its expression during the activation of plant defense responses.

Another interesting study suggests that the transcription factor Whirly1 could be a likely candidate for regulators of NPR1-independent *PR*-gene expression and resistance (Desveaux et al. 2002). A knockout mutation in the

Arabidopsis thaliana Whirly1 (*AtWhy1*) gene was lethal. However, two lines that carry point mutations in this gene, *atwhy1.1* and *atwhy1.2*, were viable and made it possible to identify this gene. Studies had shown that these mutants were compromised in SA-induced PR gene expression and resistance to *Peronospora parasitica*. The unusual feature of the Whirly1 transcription factor was its single-stranded DNA-binding activity. It had also been observed that binding elements GTCAAAA/T were enriched in some of the *PR* gene promoters. However, the SA-induced AtWhy1 DNA-binding activity was NPR1-independent. The NPR1-independent activation of AtWhy1 and the decrease in *PR-1* expression, observed in the *atwhy1* mutants, suggest that AtWhy1 is important for NPR1-independent *PR* gene expression.

Redox activation of NPR1:

NPR1 protein was found constitutively, at a low level, in the cytosol, but was localized to the nucleus in response to chemical and biological activators of SAR. Moreover, nuclear-localization was essential for activation of *PR* gene expression (Kinkema et al. 2000). Previous studies had suggested that changes in SA concentration after pathogen infection could affect the redox state of the cell (Chen et al. 1993; Noctor et al. 2002). This, together with the observation that NPR1 and NPR1-like proteins from four plant species contained ten conserved cysteines, led to hypothesis that NPR1 protein conformation might be sensitive to cellular redox changes (Mou et al. 2003). In the absence of SA, NPR1 existed as an oligomer formed through intermolecular disulfide bonds and was sequestered from transport into the nucleus. After SAR induction, following an initial oxidative burst, plant cells attained a more reducing atmoshpere due to the accumulation of antioxidants. Under these conditions, NPR1 was reduced from an oligomeric to a monomeric state. The monomeric NPR1 protein then moved into the nucleus to control SAR-related gene expression (**Fig. 2.1**).

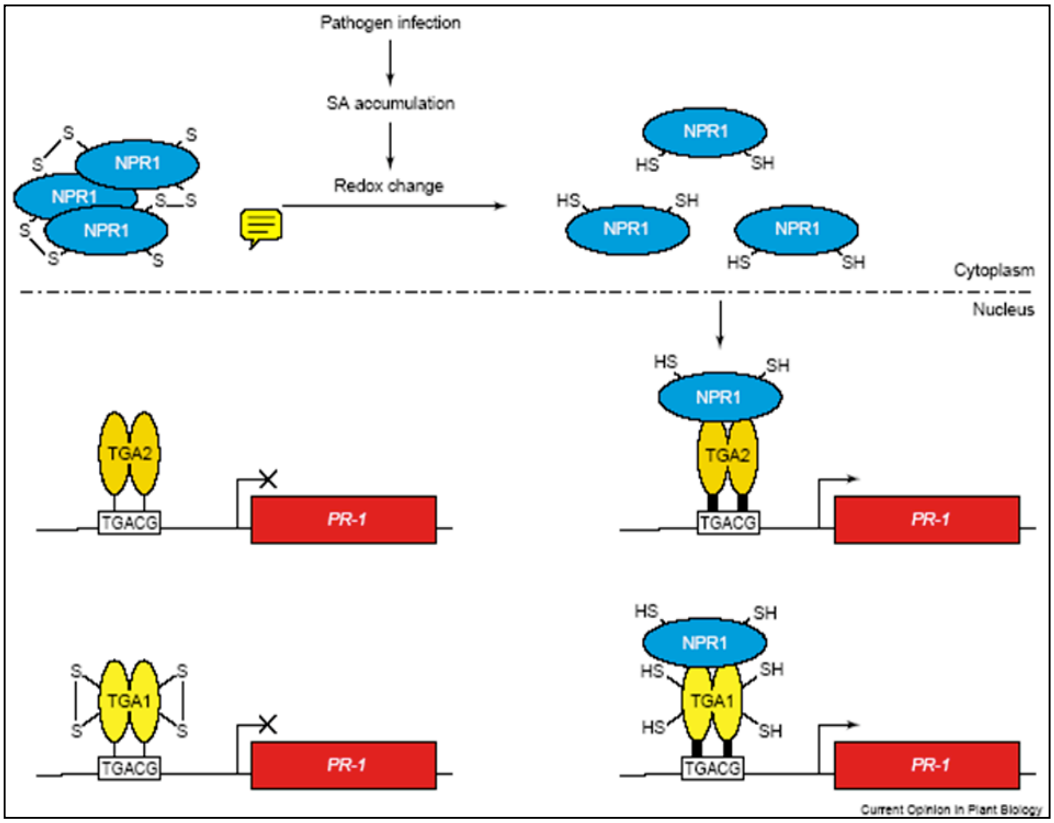


Figure 2.1. Model illustrating the role of SA-mediated redox changes, NPR1 and TGA transcription factors in SAR-related gene expression (Pieterse and Van Loon 2004)

The appearance of this NPR1 monomer was followed by activation of *PR* gene expression. Any mutations in the cysteine residues that cause monomer accumulation resulted in constitutive nuclear localization of the mutant proteins and constitutive *PR* gene expression. These results demonstrated that the NPR1 monomer is the biologically active form, and that the oligomer to-monomer switch controls NPR1 nuclear transport.

Heterologus expression of NPR1:

Overexpression of some PR genes in transgenic plants conferred modest protection against pathogens (Broglie et al. 1991; Alexander et al. 1993; Liu et al. 1994; Zhu et al. 1994; Jach et al. 1995). However, the protection provided by a single specific PR gene is usually very limited in its spectrum, degree and duration, compared to that of a native SAR response (Jach et al. 1995). Therefore, more durable resistance to a broader spectrum of pathogens may be

expected from engineering defense reactions that are more closely related to the natural SAR defense mechanisms employed in most incompatible plant-pathogen interactions.

The overexpression of *Arabidopsis NPR1* showed enhanced resistance against the pathogens such as *Pseudomonas syringae* and *Peronospora parasitica* (Cao et al. 1998). Further evidence from *Arabidopsis* indicated a crucial role of *NPR1* in the local and systemic plant response to the broad host range for the necrotrophic pathogens such as *Botrytis cinerea* and *Sclerotinia sclerotiorum* (Ferrari et al. 2003; Guo and Stotz 2007), which do not initiate SAR responses (Govrin and Levine 2002). The function of *AtNPR1* is expected to remain the same in different plant species and has led to pathogen and SA-induced increases in *PR* gene expression in transgenic rice (Fitzgerald et al. 2004; Quilis et al. 2008), wheat (Makandar et al. 2006), tobacco (Meur et al. 2008) and tomato (Lin et al. 2004). This indicates that *AtNPR1* when expressed in a heterologous system reacts in a similar fashion to the endogenous NPR1-like proteins. The plants constitutively expressing *AtNPR1* have shown resistance against fungal diseases such as Fusarium wilt caused by *Fusarium oxysporum f. sp*. *lycopersici*, gray leaf spot caused by *Stemphylium solani* and late blight caused by *Phytophthora infestans*, the bacterial diseases such as bacterial spot caused by *Xanthomonas campestris p.v. vesicatoria* and bacterial wilt caused by *Ralstonia solanacearum* and viral diseases caused by *Cucumber Mosaic Virus* (CMV), *Tomato mottle Virus* (ToMV) and *Tomato Yellow Leaf Curl Virus* (TYLCV) in tomato (Lin et al. 2004). When it is overexpressed in wheat, it showed resistance against the fungus *F. graminearum* (Makandar et al. 2006). Hence, heterologus expression of *AtNPR1* has enhanced resistance against both necrotrophic (Lin et al. 2004; Makandar et al. 2006) and biotrophic fungal, viral and bacterial pathogens (Lin et al. 2004) in addition to insect pests such as *Spodoptera litura* in tobacco (Meur et al 2008). Overexpression of rice *NPR1* showed enhanced resistance against the bacteria *X. oryzae p.v. oryzae* and the fungus *Magnoporthe grisea* but susceptible to insect herbivory (Yuan et al. 2007). Hence in the present study, *Brassica juncea NPR1* (*BjNPR1*) were selected for mungbean transformation.

Objectives for the chapter 4:

Hence, with this background information about mungbean, legume transformation and *NPR1* gene, we framed the objectives for the chapter 4 as follows:

I. To develop simple, effective and reliable protocol for direct shoot regeneration from cotyledonary node explants using different combination of plant growth regulators.

II. To develop standard protocol for mungbean transformation using the gene *BjNPR1*(*Brassica juncea* Nonexpressor of pathogenesis related genes 1)

1. Optimization of some variables before transformation.
2. Transformation of mungbean with binary vector pCAMBIA2300 harboring *BjNPR1* gene driven by 35S promoter and polyA signal.
3. Molecular analysis of the putative transgenic plants through PCR, RT-PCR and Southern hybridization.
4. Antifungal bio-assay for the transgenic plants.

Defensin

During pathogen attack, ET and JA gets accumulated during necrotroph interaction and it directly induces the expression of PR-12 protein called PDF 1.2(defensin) along with other PR proteins such as PR-6 (**P**roteinase **IN**hibitor).

Various mechanisms to fend off microbial invaders have been devised by all living organisms, including microorganisms themselves. The most sophisticated of these mechanisms rely on the synthesis of immunoglobulins directed against specific microbial targets. However, immunoglobulin-based immunity operates only in a relatively minor subset of living species, namely the higher vertebrates. A much more ancient and widespread defense strategy involves the production of small peptides that exert antimicrobial properties. The suite of defense-related proteins can either be expressed constitutively and/or be induced as a result of wounding by herbivores or by microbial invasion. As such, these proteins form pre- and post-infection defensive barriers, respectively. Examples of these proteins include enzyme inhibitors such as α-amylase and proteinase inhibitors, hydrolytic enzymes such as 1, 3-β-glucanases and

chitinases and other low molecular weight, cysteine-rich antimicrobial proteins. The accumulation of antimicrobial compounds such as oxidized phenolics, tannins and other low molecular weight secondary metabolites (such as phytoalexins) also play an important role in the chemical defense strategy of plants.

As products of single genes, antimicrobial peptides can be synthesized in a swift and flexible way, and because of their small size, they can be produced by the host with a minimal input of energy and biomass. Of the plant antimicrobial proteins that have been characterized to date, a large proportion share common characteristics. They are generally small (<10 kDa), highly basic proteins and often contain an even number of cysteine residues (typically 4, 6 or 8). These cysteines all participate in intramolecular disulfide bonds and provide the protein with structural and thermodynamic stability (Broekaert et al. 1997). Based on amino acid sequence identities, primarily with reference to the number and spacing of the cysteine residues, a number of distinct families have been defined. Antimicrobial peptides include lipid transfer proteins (Kader 1997), puroindolines, thionins (Bohlmann 1994), plant defensins (Broekaert et al. 1995; Broekaert et al. 1997; Lay et al. 2003), hevein-like peptides (Broekaert et al. 1992), knottin-like peptides (Cammue et al. 1992), glycine-rich peptides, snakins as well as antimicrobial proteins from *Macadamia integrifolia* (Marcus et al. 1997; MacManus et al. 1999) and *Impatiens balsamia* (Tailor et al. 1997), which play an important roles in protecting the hosts from the invading pathogens. All these antimicrobial proteins appear to exert their activities at the level of the outer plasma membrane of the target microorganisms, although it is likely that the different antimicrobial protein families act via different mechanisms (Broekaert et al. 1997). The cyclotides are a new family of small, cysteine-rich plant peptides that are common in members of the Rubiaceae and Violaceae families (Craik et al. 1999; Craik 2001; Craik et al. 2004) and it has various biological activities including antibacterial (Tam et al. 1999), anti-HIV (Gustafson et al. 1994), and insecticidal (Jennings et al. 2001) properties.

Although, the *in vitro* antifungal properties of these cationic peptides are well established, this property by itself is not sufficient to prove that they play a role in plant defense. Another approach to establish their contribution to plant

defense has been the analysis of transgenic plants by overexpressing the antifungal peptides. Demonstration of enhanced resistance to microbial pathogens has been accomplished for transgenic plants expressing a defensin, a thionin and a lipid transfer protein.

Plant defensin:

Plant defensins are small (~ 5 kDa,) basic, cysteine-rich, antifungal peptides ranging from 45 to 54 amino acids. They are positively charged, very stable to temperature and pH variations and are characterized by a broad spectrum of biological activities including inhibition effects on pathogens [bacteria (Chen et al. 2005) and fungi (Balandin et al. 2005)], digestive enzymes [trypsin (Wijaya et al. 2000; Melo et al. 2002) and insect α-amylases (Liu et al. 2006)], HIV-1 reverse transcriptase (Wong and Ng 2003; Wong and Ng 2006; Wong et al. 2006). The defensinVrD1 (VrCRP) displays insecticidal activity (Liu et al. 2006). Some plant defensins induce mitogenic response in mouse splenocytes, show cytotoxic activity, and inhibit proliferation of tumor cells (Kushmerick et al. 1998; Wong and Ng 2003; Wong and Ng 2006; Wong et al. 2006). Recently, a lethal effect of a sunflower defensin Ha-DEF1 on *Orobanche cumana* parasitic plant was demonstrated (Zelicourt et al. 2008)

Plant defensins are widely distributed in the plant kingdom and are likely to be present in most, if not all, plants. Most plant defensins have been isolated from seeds where they are abundant and have been characterized at the molecular, biochemical and structural levels such as Pa-AMP1 (Liu et al. 2000), Mj-AMP1and Mj-AMP2 (De Bolle et al. 1995), etc. Some of the defensins like Rs-AFP3 and Rs-AFP4 together with Rs-AFP1 and Rs-AFP2, and Lm-def are expressed in leaves of *R. sativus* (Terras et al. 1995) and *Lepidium meyenii* (Solis et al. 2007) respectively. *Arabidopsis sp* plants express a leaf-specific defensin, PDF1.2 upon pathogen challenge (Thomma et al. 2000).

Plant defensins exhibit clear, although relatively limited, sequence conservation. Strictly conserved are the eight cysteine residues and a glycine at position 34 (numbering relative to Rs-AFP2). In most cases, a second glycine (position 13), a serine (position 8), an aromatic residue (position 11) and a glutamic acid (position 29) are also conserved (Lay and Anderson 2005). Although their primary sequences are not well conserved between different

species, they present a very similar three-dimensional structure, composed by α-helix, followed by three β-sheets and stabilized by four disulfide bonds. This conserved structure is also known as the cysteine-stabilized α/β motif, which was also are found in insect defensins and scorpion neurotoxins (Lay and Anderson 2005).

Classification of plant defensin:

Based on the structure:

Plant defensins can be divided into two major classes according to the structure of the precursor proteins predicted from cDNA clones (Lay et al. 2003) (**Fig. 2.2**). In the first and largest class, the precursor protein is composed of an endoplasmic reticulum (ER) signal sequence and a mature defensin domain. These proteins enter the secretory pathway and have no obvious signals for post-translational modification or sub cellular targeting. The second class of defensins is produced as larger precursors with C-terminal prodomains of about 33 amino acids. To date, these defensins have only been found in Solanaceous species where they are expressed constitutively in floral tissues and fruit (Gu et al. 1992; Milligan and Gasser 1995; Aluru et al. 1999; Brandstader et al. 1996; Lay et al. 2003).

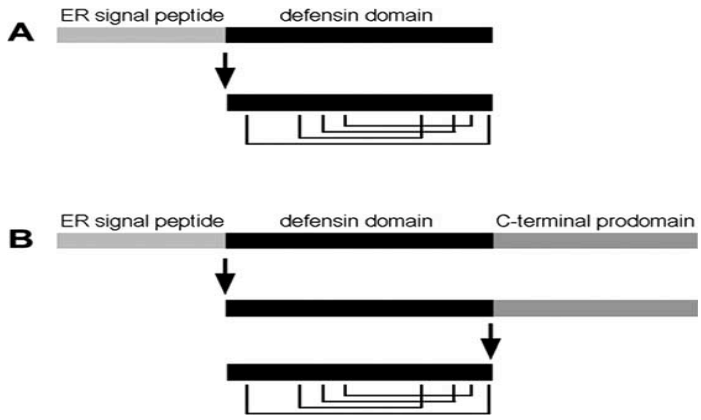


Figure 2.2. Classification of Plant defensins A) All plant defensins are produced with an ER signal sequence in addition to the mature defensin domain. (B) In some Solanaceous plants, cDNA clones have been isolated that encode plant defensins with an additional C-terminal prodomain (Lay and Anderson 2005).

Moreover, defensin expression can also be induced by salt stress in the leaves of some *Nicotiana* sp (Yamada et al. 1997; Komori et al. 1997). The prodomain of the Solanaceous defensins from *Nicotiana alata* (NaD1) and

Petunia hybrida (PhD1 and PhD2) is removed proteolytically during maturation (Broekaert et al. 1997).

The prodomains on these Solanaceous defensins have an unusually high content of acidic and hydrophobic amino acids. Interestingly, at neutral pH, the negative charge of the prodomain counter-balances the positive charge of the defensin domain. This feature is reminiscent of the prodomains (also referred to as propieces or prosegments) present on the mammalian (Michaelson et al. 1992; Yount et al. 1995) and insect defensins (Lowenberger et al. 1998), as well as the plant thionins (Bohlmann 1994; Romero et al. 1997). One difference, however, is that the prodomains in the mammalian and insect defensins are located on the N-terminal side of the defensin domain. It is interesting to speculate on the possible role(s) that the C-terminal prodomain serves in the context of the defensin domain. One hypothesis is that it may function as a targeting sequence for sub cellular sorting. Such a function has been proposed for the prodomain of human neutrophil a-defensin 1 (HNP-1) where it may be important for normal subcellular trafficking and post-translational proteolytic processing (Liu and Ganz 1995). Similarly, the prodomain on the plant thionins appears to have a role in vacuolar targeting and processing (Romero et al. 1997). On the other hand, the disparity in the electrostatic charges associated with the defensin and prodomain suggests that the prodomain could assist in the maturation of the defensin by acting as an intramolecular steric chaperone and/or by preventing deleterious interactions between the defensin and other cellular proteins or lipid membranes during translocation through the secretory pathway. These hypotheses have been proposed for the mammalian α– defensins, insect defensins and the thionins (Michaelson et al. 1992; Bohlmann 1994; Florack and Steikema 1994; Florack et al. 1994; Liu and Ganz 1995).

Based on the function:

According to their functions, plant defensins can be divided into two main groups' antimicrobial peptides and enzyme inhibitor peptides. The first group comprises antifungal and antibacterial defensins and includes most of the peptides isolated so far (Janssen et al. 2003; Lay et al. 2003; Franco et al. 2006). They are able to inhibit one specific microorganism, from Gram-negative and

Gram-positive bacteria to yeast and filamentous fungi (Terras et al. 1993; Thevissen et al. 1996) and can also inhibit the growth of about 2-5 different pathogens such as *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas syringae* (Franco et al. 2006). The second group corresponds to defensins that have the capability to inhibit proteinases and/or α-amylases from different sources. Mainly, these defensin inhibitors have demonstrated specificity to enzymes from insect midguts, such as from *Callosobruchus maculatus* and *Tenebrio molitor* (Melo et al. 2002; Farias et al. 2007).

Plant defensins, like many other plant defense-related proteins, are encoded by small multigene families (Gu et al. 1992; Terras et al. 1995; Epple et al. 1999; Thomma et al. 2002). The plant defensin family may be divided into two main groups (A and B), sharing only 25% similarity (Harrison et al. 1997). Group A can be further divided into four subfamilies (A1, A2, A3, and A4) with at least 50% similarity within each subfamily. Members of subfamily A2, formerly termed nonmorphogenic plant defensins (Broekaert et al. 1995) including Dm-AMP1 from *Dahlia merckii* reduce hyphal elongation without affecting fungal morphology. In contrast, members of subfamilies A3 and A4, including Rs-AFP2 from *Raphanus sativus* and Hs-AFP1 from *Heuchera sanguinea*, respectively, induce tip ballooning and branch formation in susceptible fungi (Osborn et al. 1995). These plant defensins have therefore been termed morphogenic plant defensins (Broekaert et al. 1995).

Structure of plant defensins:

The first plant defensin was isolated from wheat and barley grains (Colilla et al. 1990; Mendez et al. 1990). At that time these proteins were called γ-thionins, since their size and cysteine content were found to be similar to formerly described thionins (Carrasco et al. 1981). Subsequent structure analysis has demonstrated, however, that γ-thionins are not related to thionins (Bruix et al. 1993). Because of their structural similarity to mammalian and insect defensins, γ-thionins were renamed as plant defensins (Terras et al. 1995). All plant defensins identified so far have eight cysteines that form four structure-stabilizing disulfide bridges. Study of the three dimensional structure of some

plant defensins have shown that the structure comprises a triple-stranded β-sheet with an α-helix in parallel.

In insect defensins, the α-helix is combined with a double-stranded β-sheet, stabilized by three disulfide bridges between six cysteine residues (e.g. insect defensin A, **Fig. 2.3**; Bonmatin et al. 1992; Cornet et al. 1995). Interestingly, the two pathogen-inducible peptides, drosomycin and heliomicin isolated from the fruit fly *Drosophila melanogaster* and from the lepidopteran *Heliothis virescens,* respectively, combine a triple-stranded β-sheet with the α-helix (Landon et al. 1997, 2000; Lamberty et al. 2001). Three disulfide bridges stabilize heliomicin, like insect defensins, while the structure of drosomycin, like plant defensins, is stabilized by four disulfide bridges (Landon et al. 1997, 2000; Lamberty et al. 2001). Defensins have also been identified in a different phylogenetic group of invertebrates, namely mollusks (Mitta et al. 2000). From the blue mussel *Mytilus edulis*, defensins containing six cysteines have been characterized, while from the Mediterranean mussel, *M. galloprovincialis*, a defensin (MGD-1) containing eight cysteines was isolated (Charlet et al. 1996; Hubert et al. 1996). The structure of MGD-1 consists of an α-helix and two antiparallel β-strands stabilized by four disulfide bridges (**Figure 2.3;** Yang et al. 2000). The core of the global fold of plant defensins as well as invertebrate defensins includes a cysteine-stabilized α-helix β-sheet (CSαβ) motif (Cornet et al. 1995). In this motif, two cysteine residues, located one turn apart in the α - helix, form two disulfide bridges with two cysteine residues separated by a single amino acid in the last strand of the β-sheet. Before they were identified in invertebrates and plants, defensins were first identified as a family of peptides in rabbits (Selsted et al. 1984) and subsequently in other vertebrate species including humans. Until now, three types of defensins have been identified in mammals. Apart from the 18-amino-acid cyclic peptide only discovered in macaques and termed θ-defensin (Tang et al. 1999; Trabi et al. 2001), α- and β-defensins occur generally. These latter two subgroups differ in size and the arrangement of cysteines within their sequences (Lehrer et al. 1993; Selsted et al. 1993). Mammalian defensins of the a - class do not comprise α-helix (Hill et al. 1991; Pardi et al. 1992) and thus do not share the CSαβ motif found in invertebrate defensins and plant defensins.

Structure analysis of two mammalian defensins of the b-class, namely human β-defensin (HBD)-1 and HBD-2, revealed that these defensins combine the α-helix with a triple-stranded anti-parallel β-sheet (Hoover et al. 2000, 2001; Sawai et al. 2001). Nevertheless, the global fold of these defensins does not comprise a CSαβ motif, because the α-helix is located at the N-terminus of the peptide (αβββ-fold in mammalian defensins versus βαββ-fold in plant defensins). However, the size and spatial orientation of the triple-stranded β-sheet in mammalian defensins is comparable to that found in plant defensins (Broekaert et al. 1995). Moreover, the α-helix appears to be approximately in the same position relative to the β-sheet (Hwang and Vogel 1998). Because three genes encoding the human β-defensins HBD1, HBD2 and HBD3 have been localized close to a region carrying α-defensins, it was suggested that at least these types of defensins descend from a shared ancestral gene (Harder et al. 1997; Liu et al. 1997; Jia et al. 2001). But, based on the overall three-dimensional structure, there is a closer relationship between plant defensins, insect defensins and mammalian β-defensins than between mammalian α- and β-defensins. This suggests that defensins are ancient peptides conserved across the eukaryotic kingdom, originating before the evolutionary divergence of plants and animals.

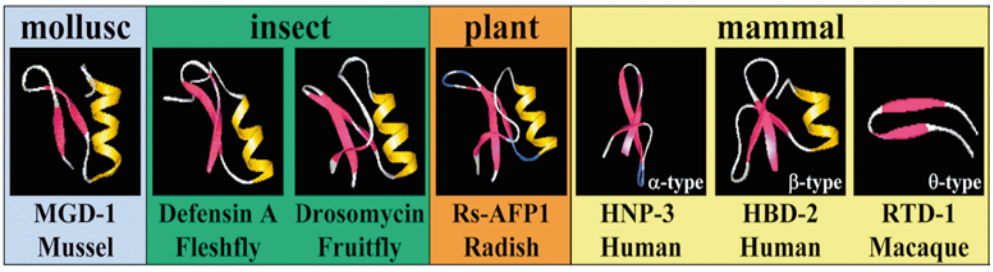


Figure 2.3. A three-dimensional structure of defensins of plant, invertebrate (insect and mollusc) and vertebrate (mammalian) origin. (Thomma et al. 2002)

Stucture-activity relationship:

The plant defensins Rs-AFP1 and Rs-AFP2 are constitutively expressed in radish (*Raphanus sativus*) seeds. These defensins only differ in two amino acids, resulting in an increase in positive charge for Rs-AFP2. At two positions (glycine-9 and valine-39), the substitution of neutral residues by arginines increased the activity of Rs-AFP2 against the filamentous fungus *Fusarium culmorum*. Analogously, replacement of a basic lysine residue at position 44 by a

neutral glutamine residue decreased antifungal activity (De Samblanx et al. 1997). The difference in antifungal activity of these Rs-AFP2 variants, compared to the antifungal activity of native Rs-AFP2, was found to be dependent on the test fungus, which suggests a very specific recognition mechanism between the plant defensin and its fungal target site. Based on a mutational analysis, it was shown that Rs-AFP2 possesses two adjacent sites important for antifungal activity against *Fusarium culmorum*. The first one is the loop connecting β-strands 2 and 3 forming a highly hydrophobic patch (De Samblanx et al. 1997; Schaaper et al. 2001). The second comprises the loop connecting β-strand 1 and the α-helix and residues on the α-helix and β-strand 3, together forming a path of contiguous residues despite their scattered positions along the Rs-AFP2 sequence (De Samblanx et al. 1997). These two regions important for antifungal activity might constitute two sites contacting a single putative receptor. Alternatively, the presence of two sites could be indicative of two distinct features necessary for the antifungal activity of Rs-AFP2, such as binding of Rs-AFP2 to its receptor on one hand, and subsequent permeabilization of the membrane on the other hand (De Samblanx et al. 1997).

Mode of action:

The precise mechanism of action that is employed by plant defensins to inhibit the growth of fungi is not completely understood, although it is generally accepted that they act at the level of the fungal plasma membrane. Thevissen et al. (2003) have demonstrated that the radish (Rs-AFP2) and dahlia (Dm-AMP1) defensins induce rapid Ca^{2+} influx and K^{+} efflux when added to the hyphae of the fungus *Neurospora crassa* at concentrations that are inhibitory for growth (**Fig. 2.4**).

Given that the fungal species grow at the tip and require the maintenance of an intracellular Ca^{2+} concentration gradient to drive polarized growth, it has been suggested that the growth inhibition may be due to dissipation of this gradient.

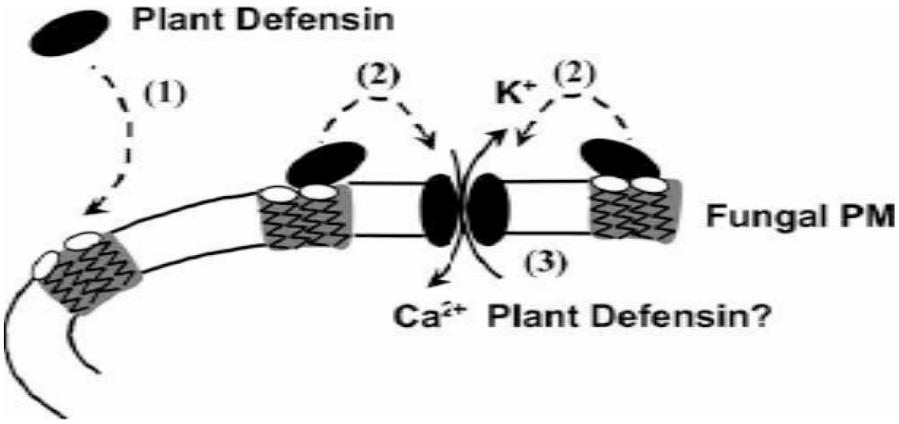


Figure 2.4. Proposed model for the mode of action of Plant Defensins (1) plant defensins, represented by dark ovals; (2) bind to rafts composed of sphingolipids (represented by grey squares in the fungal plasma membrane (PM)), where after permeability of the membrane is altered; (3) resulting in increased Ca^{2+} uptake and K^{+} efflux. Whether plant defensins are internalized and interact with intracellular targets is not known (Thevissen et al. 2003)

In support of a link between ion fluxes and antifungal activity, De Samblanx et al. (1997) demonstrated that a variant (V39R substitution) of Rs-AFP2 that displayed enhanced antifungal activity induced even more Ca^{2+} uptake (up to 2.5-fold), while a variant (Y38G substitution) that displayed almost no antifungal activity caused no Ca^{2+} uptake. The molecular basis for the antifungal inhibitory activity of other plant defensins has not been elucidated except in the cases of Dm-AMP1 and Rs-AFP2 where their targets are known in the yeast species, *Saccharomyces cerevisiae* and *Pichia pastoris* respectively.

As amylase inhibitors:

Plant defensins constitute one of six structural classes of proteinaceous amylase inhibitors. The first examples of α-amylase inhibitory plant defensins were three isoforms from *Sorghum bicolor* (Blotch and Richadson 1991). These proteins were strongly inhibitory to the digestive amylases from the gut of locusts and cockroaches. Later, two defensins have been reported with trypsin inhibitory activity, namely a defensin from *Cassia fistula* seeds and a defensin from cowpea. Wijaya et al. (2000) isolated two defensins from the seeds of *C. fistula* with molecular masses of 5144 and 5459 Da. Melo et al. (2002) noted that while these proteins share 30% amino acid sequence identity, they had different

disulfide bond connectivity and structures and thus may not share trypsin inhibitory activity.

Furthermore, it has been demonstrated that a defensin from *Vigna radiata*, named VrD1, was able to inhibit *T. molitor* α-amylases (TMA) (Liu et al. 2006). It seems that the mechanism of action between plant defensins differs depending on the inhibitor structure, although most of the interactions with the inhibitors occur at the active site of the enzyme. Therefore, VrD1 was able to form hydrogen bonds with three amino acid residues from the *T. molitor* active site (Glu222, Asp287, and Asp332) and also showed that the third loop was important for interaction and enzyme inhibition (Lin et al. 2007). The same mechanism was observed by a γ-hordothionin (γ-H) from barley (Mendes et al. 1990; Liu et al. 2006). It could be observed that loop 3 of γ-H inserts into the active site of TMA, impeding the entrance of the substrate. Moreover, it was found that positively charged residues such as Lys12 and Arg38 in VrD1, Arg39 and Arg40 in γ-H) are important for interaction with α- amylases (Mendes et al. 1990; Liu et al. 2006). The mechanism of action for insecticidal plant defensins is not completely understood, but another hypothesis for the mechanism of action has been proposed, being related to the essentiality of the Ca^{2+} ion for some insect α-amylase activity (Pelegrini et al. 2006). In this sense, some authors have suggested that the defensins are able to chelate calcium, destabilizing the enzymes and further causing their inhibition (Castro and Vernon 2003; Pelegrini and Franco 2005).

As proteinase inhibitors:

Plant defensins also have activity towards insect proteinases. The first proteinase inhibitor from the defensin family was described in *Cassia fistula*, which was able to decrease the activity of insect serine proteases, trypsin or chymotrypsin (Wijaya et al. 2000). Furthermore, there are other reports describing the inhibitory activity of defensins. These data were verified in a defensin from *N. alata*, NaD1, which showed activity towards trypsin and chymotrypsins from *Helicoverpa armigera* and *H. punctigera*, in addition to its antifungal activity (Lay et al. 2003). The conservative CSαβ motif present in plant thionins may be the key to explain the diversity of NaD1's function, as there is no hypothesis to explain clearly how this peptide can work both as an

antifungal agent and as a proteinase inhibitor (Lay et al. 2003). Moreover, the most studied proteinase inhibitor pertaining to the defensin family was Cp-thionin I, isolated from *V. unguiculata* seeds. It showed activity towards bovine pancreatic trypsin (BPT), but was unable to inhibit chymotrypsin (Melo et al. 2002). The mechanism of action of Cp-thionin I is not well understood, but the main hypothesis lies in the fact that this defensin interacts specifically with the enzyme in a water-mediated environment, where the Lys11 residue of the inhibitor was identified as being extremely important for its inhibitory activity. This amino acid acts in a canonical style fashion, by occupying a specific enzyme cavity, blocking its catalytic site and impeding the entrance of the substrate (Melo et al. 2002). In summary, although there are only a few reports describing proteinase and α-amylase inhibitors related to the plant defensin family, they are extremely important for understanding the novel mechanisms of action proposed for this multi-family. Cloning and expression of these peptides in transgenic plants could lead to an enhanced resistance to pest and pathogen predation, by reducing the protein and starch adsorption during digestion processes.

As ion channel inhibitors:

In 1998, Kushmerick and colleagues isolated two defensins from *Zea mays* called γ1- and γ 2-zeathionin (γ 1-Z and γ 2-Z, respectively) and demonstrated rapid and reversible inhibition of the sodium ion channel in a rat tumor cell line using the whole-cell patch clamp technique. However, these defensins have not been tested, for the antifungal or insecticidal activities.

Plant defensin with antibacterial activity:

Phytopathogenic bacteria are not very common enemies as fungi and insect pests (Thomma et al. 2002). Probably, for this reason, only few plant defensins demonstrate anti-bacterial activity, in comparison to defensins from others organisms. Among these exceptions, some anti-bacterial defensins have been studied such as pseudo-thionin (Pth-St1) of *Solanum tuberosum* (Moreno et al. 1994), So-D1-7 of *Spinacia oleracea* (Segura et al. 1998), Pa-AMP-1 of *Phytolacca americana* (Liu et al. 2000), Fa-AMP1 and Fa-AMP2 of *Fagopyrum esculentum* (Fujimura et al. 2003), VaD1 of *Vigna angularis* (Chen et al. 2005) and Cp-thionin II of *Vigna unguiculata* (Franco et al. 2006). Fa-AMPs, which

belong to the defensin family also, were included in the glycine-rich family due their primary structural features, which exhibit both 8 cysteine resides and continuous sequences of cysteines (–CC–), characteristic of defensins, and 10 glycine residues and continuous sequences of glycines (–GGG– and –GG–), characteristic of glycine-rich proteins. Thus, Fa-AMPs were the first to be classified into two families. Pa-AMP-1, which was active against the harmless bacterium *Bacillus megaterium*, with an IC_{50} of 8 μg ml^{-1} concentration and phytopathogenic fungi *Alternaria panax*, *Fusarium sp.,* and *Rhizoctonia solani*, with an IC_{50} of 20 μg ml^{-1} (Liu et al. 2000). In the same way, some plant defensins appear to be active towards both Gram-negative and Gram-positive bacteria as well as fungi. Furthermore, it was also observed that plant defensin So-D1-7 at 20 μg ml^{-1} concentration, was able to reduce 50% of the development of the bacteria *Clavibacter michiganensis* and *Ralstonia solanacearum*, which causes black rot and work against some phytopathogenic fungi (*Fusarium culmorum*, *F. solani*, *Bipolaris maydis*, and *Colletotrichum lagenarium*) at 25 μg ml^{-1} concentration (Segura et al. 1998). Moreover, similar data was obtained by VaD1, which inhibits the growth of *Staphylococcus epidermidis* – a cause of common infections in immune deficient patients – and *Salmonella typhimurium* – the root cause of intragastric infections – as well as the fungus *F. oxysporum* (Chen et al 2005). Recently, diverse plant defensins with bactericidal activity have been isolated from bean seeds and characterized. Ground beans (*Vigna sesquipedalis*) synthesizes sesquin, which exerts antibacterial activity toward *E. coli*, *Proteus vulgaris*, *Mycobacterium phlei* and *Bacillus megaterium* also antifungal activity against *Botrytis cinerea*, *Fusarium oxysporum* and *Mycosphaerella arachidicola* (Wong and Ng 2005).

Plant defensins with antifungal activity:

Antimicrobial defensins are also able to inhibit fungal growth. Most plant defensins inhibit yeasts or filamentous fungi (Thomma et al. 2002). Antifungal peptides usually are capable of inhibiting more than one fungal species, such as the defensin isolated from *P. vulgaris*, which showed activity against *F. oxysporum* and *M. arachidicola* (Wang and Ng 2007). Furthermore, a peptide from *Trigonella foenum-graenum* also showed activity against *R. solani* and *Phaeoisariopsis personata* (Olli and Kirti 2006). However, plant defensins

generally present a specific activity towards a unique pathogen, as observed for a peptide from *P. sativum*, which was able to inhibit the activity of *Neurospora crassa* (Lobo et al. 2007). Another example is NaD1, a defensin from *N. alata* that was able to inhibit in 56% the growth of *F. oxysporum* at a 2 μg ml^{-1} concentration (Lay et al. 2003).

Biological role of plant defensin *in planta*:

The best-characterized defensins have been isolated from seeds. Defensins account for about 30% of the proteins released upon mechanical wounding of radish seeds, even though it only represents 0.5% of the total seed proteins (Terras et al. 1995). The extent of defensin released from a single seed was estimated to be at least 1 μg, which is sufficient to form an inhibitory zone around the seed to protect the germinating seedling against soil-borne fungal pathogens (Broekaert et al. 1995). Plant defensins are also expressed in vegetative tissues where they accumulate in the peripheral cell layers of cotyledons, hypocotyls, endosperms, tubers and floral structure. These locations are consistent with a role in a first line of defense against potential pathogens (Gu et al. 1992; Moreno et al. 1994; Terras et al. 1995; Penninckx et al. 1996; Lay et al. 2003). Kragh et al. (1995) also described the accumulation of defensins in the leaves, stomatal cells and in the cell walls that line the substomatal cavities in sugar beet. This is interesting as stomata are potential entry points for fungal pathogens. The floral defensin from *N. alata* (NaD1) is expressed in the epidermal cells of the sepals and petals and in the cortical cells of the style and the connective cells of the anther (Lay et al. 2003). Its expression in these outermost layers that surround, but do not come into direct contact with, the pollen or pollen tubes as they grow through the style, is consistent with a role in protecting the valuable germ cells against damage by potential pathogens (Lay et al. 2003). This is also analogous to the expression of mammalian defensins in reproductive tissues (Rodriguez-Jimenez et al. 2003).

Table 2.1. Various biological activities displayed by plant defensins (Lay and Anderson 2005)

Biological activity	Examples	Plant source
Antifungal	Rs-AFP1-4 Ah-AMP1 AlfAFP	*Raphanus sativus* *Aesculus hippocatanum* *Medicago sativa*
Antibacterial	Pth-St1 Fabatin-1 and -2 SoD1-7	*Solanum tuberosum* *Vicia faba* *Spinacia oleracea*
Insecticidal	VrCRP	*Vigna radiata*
Protein synthesis inhibitor	γ1-H γ1-P ω-H HvAMP1	*Hordeum vulgare* *Triticum turgidium* *Hordeum vulgare* *Hardenbergia violacea*
α-amylase inhibitor	SIα1-3	*Sorghum bicolor*
Proteinase inhibitor	CfD2 Cp-thionin	*Cassia fistula* *Vigna unguiculata*
Sodium channel inhibitor	γ1-Z and γ2-Z	*Zea mays*

Expression of plant defensin in plants:

Pathogen induction:

In general, the expression of plant defensin is induced during pathogen infection in the vegetative tissues. The two well-characterized seed defensins, Rs-AFP1 and Rs- AFP2are strongly induced in radish leaves following the pathogen infection with *Alternaria brassicola* (Terras et al. 1992; Terras et al. 1995). These defensins share about 90% sequence identity with their seed counterparts and

exert comparable *in vitro* antifungal activity (Terras et al. 1995). In immature pea pods, two defensin transcripts (pI39 and pI230) are induced upon fungal infection (Chiang et al. 1991). These defensins were later renamed defense response related genes, DRR230-a and DRR230-b (Wang et al. 1998; Lai et al. 2002), respectively. Lai and colleagues also isolated a third defensin cDNA (DRR230-c) from pea tissue that was homologous to DRR230-b (Lai et al. 2002). In the uninfected pea tissue, relatively high levels of DRR230-a and DRR230-c transcripts are present in mature leaves and stems, with intermediate levels in young leaves, tendrils and flowers, and low levels in roots and pods. Infiltration of bacterial or fungal pathogens into pea leaves results in elevated levels of defensin expression (Lai et al. 2002). Interestingly, infiltration of leaves with buffer only also resulted in accumulation of defensin RNA, suggesting these genes are also induced by wounding (Lai et al. 2002). The pathogen induced expression of defensins has also been observed in the sepals of tobacco (Gu et al. 1992) as well as in *Arabidopsis* (Penninckx et al. 1996). Furthermore, systemic activation of defensin genes in distal, non-infected leaves has been observed in *Arabidopsis* and *Radish* leaves (Moreno et al. 1994; Terras et al 1995). Several researchers have described the signaling pathways involved in the pathogen-induced systemic activation of the *Arabidopsis* defensin PDF1.2 and the involvement of the plant hormones salicylic acid, jasmonic acid (and its analogue methyl jasmonate) and ethylene (Penninckx et al. 1996; Thomma et al. 1998; Tierens et al. 2002). These hormones have variously been implicated in signal transduction pathways leading to systemic acquired resistance and cross-talk in plants (Ryals et al. 1994; Heil and Bostock 2002). Expression of PDF1.2 is induced following treatment of leaves with methyl jasmonate or ethylene, but not with salicylic acid or 2,6-dichloroisonicotinic acid (a synthetic compound that mimics the action of salicylic acid) (Penninckx et al. 1996).

Metal induction:

Another improvement that defensins could give to genetically modified plants is the resistance to metals (Mirouze et al. 2006). A recent work investigated and confirmed the potential of defensins to confer zinc (Zn) resistance. In *A. thaliana* Columbia 0 ecotype plants overexpressing the *A. halleri* AhPDF1.1 cDNA, which clearly displayed Zn tolerance, when compared to

control *A. thaliana* plants. In addition, defensins not only induced Zn tolerance but the mRNA and protein accumulation of some of these defensins was increased by Zn treatments. Surprisingly, AhPDF1.1 seems to be extremely specific since it confers tolerance to Zn but not to cadmium (Cd), iron (Fe), cobalt (Co) or copper (Cu) and nor to salt treatments (Mirouze et al. 2006). It leads to the development of a hypothesis that defensins could interfere with divalent metal cation trafficking and thus conferring Zn tolerance, probably binding to transport membrane proteins. Further studies, with certainty, will shed some light over the mechanism of action.

Environmental stress induction:

In addition to pathogen-induction, defensins can also be induced by environmental stress. Maitra and Cushman (1998) identified a soybean defensin gene (Dhn8) that was induced by artificial drought stress. The level of the Dhn8 transcript was 10-fold higher in the leaves and roots of a drought resistant cultivar than in a drought-sensitive cultivar (Maitra and Cusman 1999). In response to salt stress the defensin genes from *N. excelsior* (NeThio1 and NeThio2) and *N. paniculata* (NpThio1) leaves are expressed at 250 mM NaCl (Yamada et al. 1997; Komori et al. 1997).

Later, Koike et al. (2002) reported that the up-regulation of several defensin transcripts in winter wheat in response to cold induction and acclimation. The induction of these defensins was suggested to enhance pathogen resistance during winter hardening and potentially, to have a role in freezing resistance. The inducibilty of defensins by pathogens, salinity, drought and cold stress provides examples of cross-talk between signal transduction pathways and gene expression programs that are regulated by various stress stimuli in plants. This pattern of gene expression, together with their cellular location supports a role for defensins as important defense molecules in plants. Their potential role in plant defense could be investigated further by the creation of transgenic plants.

Defensin in transgenic approaches:

To date, several plants have been transformed with plant defensins genes. A list of these genes, their recipient plants and target pathogens is presented (**Table 2.2**). Constitutive expression of the radish defensin (Rs-AFP2) enhanced resistance in tobacco plants to the fungal leaf pathogen *Alternaria longipes* and similarly in tomato against *A. solani*. Canola (*Brassica napus*) constitutively expressing a pea defensin had slightly enhanced resistance against blackleg (*Leptosphaeria maculans*) disease. However, perhaps the most extensively studied and best example of the potential of defensins in transgenic crops the alfalfa defensin (alfAFP) in potato (Gao et al. 2000). It was demonstrated that the constitutive expression of alfAFP in potatoes provided robust resistance against the agronomically important fungus *Verticillium dahliae*. Levels of fungus in the transformed plants were reduced by approximately six-fold compared to the non transformed plants.

From the above information, it seems obvious that the potential of defensins as a biotechnological tool for the discovery of new, natural drugs or in the construction of transgenic plants with enhanced resistance toward pests and pathogens exists (Pelegrini and Franco 2005). Due to wide variation of defensin functions, several efforts in different fields have been made in order to obtain products derived from high technology that could bring a better life as well high production and lower cost to crop farmers.

Table 2.2 Biotic and Abiotic Stress Induction of Plant Defensin Expression (Lay and Anderson 2005)

Stress	Plant defensin	Plant source	Tissue
Pathogen infection	DRR230-a, DRR230-b	*Pisum sativum*	Immature pea pod
		Pisum sativum	
	Rs-AFP3,	*Raphanus sativus*	Leaves
	Rs-AFP4	*Raphanus sativus*	Leaves
	DRR230-c	*Pisum sativum*	Leaves
	PDF1.2	*Arabidopsis thaliana*	Leaves
	PDF2.3	*Arabidopsis thaliana*	Leaves
Wounding	DRR230-c	*Pisum sativum*	Leaves
Drought	Dhn8	*Glycine max*	Leaves and roots
Salt	NeThio1, NeThio2	*Nicotiana excelsior*	Leaves
	NpThio1	*Nicotiana paniculata*	Leaves
Cold	Tad1	*Triticum aestivum*	Crown tissue, young seedlings

In fact, not only have plant defensins have been cloned and expressed in transgenic plants, other peptides have also been utilized as a particular resistance source such as lipid transferproteins (Carvalho et al. 2006), digestive enzyme inhibitors (Franco et al. 2002) and also animal defensins (Langen et al. 2006). A human β-defensin was expressed in genetically engineered *A. thaliana* Columbia 0 (Aerts et al. 2005) and gallerimycin, an antifungal peptide from the greater wax moth *Galleria mellonella* was used to produce transgenic tobacco against the

fungal pathogens *Erysiphe cichoracearum* and *Sclerotinia minor* (Langen et al. 2006). Thus, plant defensins have occupied the first position in a world race to produce high productive agricultural crops with resistance against biotic and non-biotic stresses. Some of the heterologus expression of plant defensin was listed in the **Table 2.3**. This excellent position could be strictly related to a multi-functionality of defensins that, as described before, could act against bacteria, fungi, insects and non-biotic stresses (Pelegrini and Franco 2005).

Mutational analysis:

We have cloned and characterized a defensin TvD1 from a weedy legume, *Tephrosia villosa* and it was found to be a very effective among the legumes and showed detrimental to several plant pathogenic filamentous fungi (Vijayan et al 2008). The mode of action of plant defensins is still not clear. From the earlier studies, it was indicated that it may be due to glycosyl ceramide fungal membrane damage followed by pore formation that leads to ionic leakage such as efflux of K^+ and influx of Ca^+ and cell death effected due to ionic imbalance within (Thevissen et al 2003; Vander ween et al. 2008).

The homology modeling showed that TvD1 has 91% similarity with that of *Vigna radiata* defensin (VrD2). Previously, it was reported that changing of non-polar or uncharged aminoacid in the loop3 greatly affects the activity of defensin. Through site directed mutagenesis, increasing the positive charge potential in this loop3 by a single amino acid substitution arginine into glutamine at position 38 (R38Q) in *Medicago sativa* defensin (MsDef1) by Spelbrink et al. (2004) or valine into arginine at position 39 (V39R) *Raphanus sativus* (RsAFP2) by De Samblanx et al. (1997) dramatically affected

Table 2.3. Heterologus expression of plant defensins in transgenic plants (modified after Lay and Anderson 2005)

Transgene	**Source plant**	**Recipient plant(s)**	**Promoter**	**Increased resistance against test organism(s)**
Rs-AFP2	Radish	Tobacco	CaMV 35S	*Alternaria longipes*
Rs-AFP2	Radish	Tomato, oil rape	CaMV 35S	*A. solani, Fusarium oxysporum, Phytophthora infestans, Rhizoctonia solani, Verticillium dahliae*
AlfAFP	Alfalfa	Potato	Figwort mosaic virus 35S	*V. dahliae*
Spi1	Norway spruce	Tobacco, Norway spruse embryonic culures	CaMV 35S	*Erwinia carotovora, Heterobasidion annosum*
DRR230-a	Pea	Canola	CaMV 35S	*Leptosphaeria maculans*
DRR230-a *DRR230-c*	Pea	Tobacco	Duplicated CaMV 35S	*F. oxysporum, Asochyta pinodes, Trichoderma reesei, Ascochyta lentis, F. solani, L. maculans, Ascochyta pisi, Alternaria alternata*
BSD1	Chinese cabbage	Tobacco	CaMV 35S	*P. parasitica*
WT1	Wasabi	Rice	Maize Ubiquitin-1	*Magnaporthe grisea*
BjD1	mustard	Peanut, tobacco	CaMV 35S	*Phaesariopsis personata, Fusarium moniliforme* (Swathi Anuratha et al. 2008)

the cationic antagonism with respect to their activity against some phytopathogenic fungi. In VrD2, a defensin from *Vigna radiata*, introduction of 5 amino acids by replacing the existing four amino acids in the loop3 has showed the enhanced α-amylase inhibitory activity against the insect *Tenebrio molitor*

and it is more than that of with VrD1 (Lin et al. 2007). Hence, replacing the uncharged to charged aminoacids or hydrophobic amino acid or insertion of amino acids by replacing the existing amino acids at the loop3, through site directed mutagenesis is predicted to be results in more effective TvD1, when compared to native defensin.

In the present study, we made mutation in the loop 3 of TvD1, a defensin from *Tephrosia villosa* through site directed mutagenesis to study its effectiveness against plant fungal and bacterial pathogens by *in vitro*. Additionally we elucidated the α-amylase inhibitory activity of the defensin against the insect *Tenbrio molitor*.

Objectives for the chapter 5:

Tephrosia villosa is a wild, weedy, herbaceous legume plant without having any external disease symptoms. Hence, we are interested in this legume defensin, which could be a potent defensin. With the above said background information about defensins, we framed our objectives as follows:

I. Isolation, cloning and functional characterization of the gene *TvD1*.
   1. Isolation and cloning of the ORF cDNA for a defensin, *TvD1* from the leaves of *Tephrosia villosa*.
   2. Prokaryotic expression and purification of the recombinant peptide rTvD1.
   3. To assess the antifungal property of the purified recombinant peptide.

II. Sub-cellular localization of rTvD1 within the plant.
   1. Cloning of *TvD1* gene in the GFP fusion vector pEGAD vector driven by 35S promoter and polyA signal with *basta* resistance gene as a selection marker.
   2. Generation of putative transgenic plants through *Agrobacterium* mediated transformation and to analyze the leaves through confocal microscopy.

III. Cloning and transformation of *TvD1* in tobacco for *in vivo* characterization.
   1. Cloning of *TvD1* gene in the pCAMBIA2300 vector driven by 35S promoter and polyA signal with *npt*II gene as selection marker.
   2. Generation of putative transgenic plants through *Agrobacterium* mediated transformation.

3. Molecular analysis such as PCR, RT-PCR and Southern to confirm the transgenic plants.
4. To carry out antifungal and anti-insect bioassay for the high as well as low expression plants.

IV. Mutational analysis of TvD1 for enhanced inhibitory activity against the microbial pathogens.

1. To develop mutants such as S32R, D37R, Alpha TvD1 and native TvD1 through PCR.
2. Prokaryotic expression and purification of the recombinant peptides.
3. To carry out antifungal, antibacterial and insect α-amylase inhibitory activity with the purified recombinant peptides.

Chapter 3

Materials and methods

The methods used in this study are briefed as follows:

Preparation of competent cells of *Escherichia coli*:

A loop full of *E. coli* (DH5-α, Bangalore GENEi, India) culture was inoculated in 25 ml of LB (Luria Broth, Himedia, India) and was incubated overnight at 37ºC on a rotary shaker at 200 rpm. From the overnight culture, 1.0 ml was taken and inoculated in a fresh batch of 25 ml of LB and incubated again until the OD reached 0.2 at 600 nm. From here onwards all the steps were performed at 4ºC on ice. Sterile micro-tubes were cooled on ice and 1.5 ml of the chilled bacterial suspension was transferred to each one of them. The cells were centrifuged in a cooling centrifuge (Eppendorf®, Germany) at 4ºC and 5000 rpm for 10 minutes. The supernatant was removed and the pellet was thoroughly suspended in 0.5 ml of ice cold 0.1 M $CaCl_2$ solution and was stored on ice for 5 to 10 minutes. The cells were centrifuged at 4ºC and 5000 rpm for 10 minutes, and the pellet was resuspended in ice cold 0.5 ml of 0.1 M $CaCl_2$. These competent cells were stored at -70ºC after adding 50% sterile glycerol in batches of 100 µl, or used immediately for transformation after keeping the cells on ice for 30 minutes.

Transformation of *E. coli* competent cells:

To 100 µl of *E. coli* competent cells, were used immediately or taken from -70ºC freezer of competent cells 50 to 100 ng of the plasmid containing the desired gene was added and the cells were incubated on ice for 10 to 30 minutes. A heat shock was given at 42ºC in a water bath for 90 to 120 seconds and the culture was immediately chilled on ice. The volume was made up 0.5 or 1.0 ml by adding 400 or 900 µl of sterile LB medium. The tubes were then incubated on a rotary shaker at 37 ºC for 1 to 2 hrs. After recovery and growth of the transformed cells, they were plated on LA (Luria Agar, Himedia, India) medium containing appropriate selection antibiotics using different volumes (50, 100, 150, 200 µl). The plates were incubated in an oven for 12 to 16 hrs at 37ºC for the appearance of colonies. Once the colonies were visible, the plates were stored

at 4ºC in a refrigerator. The cells stayed viable for one month at this temperature. Plasmid DNA was isolated from the colonies to confirm transformation. For long-term storage of the transformed cells, liquid cultures of the cells were stored at -70ºC after adding sterile 50% glycerol or 0.075% of DMSO.

Plasmid DNA isolation from *E. coli* (mini preparation) (Sambrook et al. 1989):

A single colony of *E. coli* cells after transformation was incubated in 5.0 ml of LB medium with appropriate antibiotics on a rotary shaker at 37ºC and 200 rpm for 12-16 hrs. This overnight grown culture was taken in 1.5 ml micro tube and was centrifuged at 5000 rpm for 30 to 60 seconds at 4ºC. Supernatant was removed and the pellet was suspended thoroughly in 100 µl sterile ice cold Solution I [25 mM Tris Cl (pH 8.0), 10 mM EDTA [Ethylene Diamine Tetra Acetic Acid (pH 8.0)], 50 mM Glucose, stored at 4 ºC] using a vortex mixer. To the suspension, 150 µl of Solution II [0.2 N NaOH, 1% SDS, freshly prepared and stored at RT] was added. The contents were mixed thoroughly by inversion till the solution became clear. The tubes were incubated on ice for 10 minutes. After the lysate got chilled, 200 µl of Solution III [3.0 M potassium acetate (pH 4.8), autoclaved and stored at RT] was added. The solution was mixed thoroughly by gentle inversion. The tubes were incubated on ice for a further 5 minutes. The contents of the tubes were centrifuged at 4ºC and 12,000 rpm for 10 minutes in a cooling centrifuge. The supernatant was transferred to a fresh 1.5 ml micro tube. From a stock of 10 mg ml^{-1} of RNase, 2-3 µl was added to the lysate and was incubated at 37ºC in a water bath for 1 hr. The lysate was treated with phenol: chloroform: isoamyl alcohol (25:24:1) and chloroform: isoamyl alcohol (24:1) successively and was centrifuged after each treatment at RT and 12,000 rpm for 15 minutes to separate the aqueous phase from the organic layer. The upper aqueous layer was separated to a new tube without disturbing the middle protein layer. The purified dsDNA was precipitated with two volumes of 100% ethanol or an equal volume of isopropyl alcohol. The mixture was allowed to stand at -20ºC for 30 minutes and the DNA was collected at the bottom of the tube by centrifuging at 4ºC and 12,000 rpm for 10 minutes. The supernatant was decanted completely and the pellet was rinsed with 1.0 ml of 70% ethanol. The pellet was air dried and dissolved in 30 to 50 µl TE buffer [10 mM Tris HCl and

1.0 mM EDTA (pH 8.0)]. The isolated plasmid DNA was stored at -20ºC. Plasmid isolations were also carried out using kit (Eppendorf®, Germany) following the manufacturer's instructions

Preparation of competent cells of *Agrobacterium*:

A loop full of parental *Agrobacterium* culture was inoculated in LB medium with appropriate antibiotics and was incubated on a rotary shaker at 28ºC and 200 rpm for 24 hrs. When the OD reached 0.5-0.8 at 260 nm, the culture was placed on ice. The bacterial suspension was dispensed into pre-chilled (to 4ºC) micro tubes under sterile conditions and the cells were collected at the bottom of the tubes by centrifuging at 4ºC and 5000 rpm for 3 minutes. The supernatant was discarded and 0.5 ml of 20 mM $CaCl_2$ was added under sterile conditions. These competent cells were stored at -70ºC after adding 50% sterile glycerol/ 0.075% DMSO, or used immediately after keeping the cells on ice for 30 minutes.

Transformation of *Agrobacterium* cells (Holsters et al. 1978):

To 100 µl of competent cells, 50 to 100 ng of the binary vector with the desired gene was added. Cold and heat shocks were given by dipping the tubes in liquid nitrogen for 1-2 minutes and immediately transferring them to 37ºC in water bath for 5 minutes. After heat shock, 0.5 ml of LB medium was added and the tubes were incubated in a rotary shaker at 28ºC and 200 rpm for 4-5 hrs. The cells were then spread evenly on LA plates containing appropriate antibiotics and were incubated in a BOD incubator at 28ºC for 48-72 hrs for the appearance of transformed colonies. The plates were stored at 4ºC in a refrigerator, where the cells stayed viable for two months. Plasmid DNA was isolated from the colonies to confirm the transformation. For long-term storage of the transformed cells, liquid cultures of the cells were stored at -70ºC after adding sterile 50% glycerol as in the case of *E. coli*.

Plasmid isolation from *Agrobacterium*:

After transformation, a single colony of *Agrobacterium* cells was inoculated in 10 ml of LB medium containing appropriate antibiotics and incubated overnight on a rotary shaker at 28ºC and 200 rpm. The overnight

grown culture was transferred to micro tubes and was centrifuged at 1000 rpm for 1 minute. The supernatant was discarded and the cells were resuspended in 0.1 ml of ice-cold Solution I [4.0 mg l-1 lysosyme, 50 mM Glucose, 10 mM EDTA, 25 mM Tris HCl (pH 8.0)] and the tubes were incubated at RT for 10 minutes. To this suspension, 0.2 ml of freshly prepared Solution II [1% SDS, 0.2 N NaOH] was added. After mixing the content thoroughly, the tubes was incubated at RT and 30 µl of phenol equilibrated with 2 volumes of Solution II was added. The contents were vortex mixed for a few seconds or till the suspension became viscous. To this viscous lysate, 150 µl of 3.0 M sodium acetate (pH 4.8) was added and was mixed well. The tubes were incubated at -70ºC for 15 minutes. The tubes were centrifuged at 5000 rpm for 3 minutes. The supernatant was taken in a fresh tube and ice cold 100% ethanol was added to fill the tube. The content was mixed thoroughly by inversion and was stored at -70ºC for 15 minutes. After incubation, the tubes were centrifuged at 5000 rpm for 3 minutes and the supernatant was discarded. The pellet was suspended in 0.5 ml of 0.3 M sodium acetate (pH 7.0) and the DNA was precipitated by adding ice-cold 95% ethanol. The DNA in sodium acetate and ethanol were mixed well by inversion and stored at -70ºC for 15 minutes. After incubation, the tubes were centrifuged at 5000 rpm for 3 minutes. The supernatant was decanted and the tubes were allowed to stand in inverted position until the supernatant drained off completely. The pellet was rinsed with 1.0 ml of 70% ice-cold ethanol; vortexed briefly and was centrifuged at 5000 rpm for 1 minute. The supernatant was discarded and the pellet was air-dried. The dried DNA pellet was suspended in 50 µl TE buffer and was stored at -20 ºC.

Agarose gel preparation and electrophoresis:

For resolving the DNA fragments, 0.8% agarose gel was prepared by melting 0.8 g agarose (A-9539, Sigma-Aldrich, St. Louis, USA) in 100 ml of 1x TAE buffer [50x TAE: 2.0 M Tris Cl, 1.0 M Acetate, and 100 mM EDTA (pH 8.0)]. The melted agarose was cooled to 50ºC and 2 µl of ethidium bromide (EtBr) from a stock of 10 mg ml^{-1} was added. This was poured into a gel-casting tray with a comb placed properly in the grooves provided. After the gel got polymerized, it was kept inside the electrophoresis tank containing 1x TAE buffer so as to cover the gel. The DNA mixed with 6x loading dye [0.15%

bromophenol blue, 0.15% xylene cyanol, 5.0 mM EDTA, 40% sucrose] to a concentration of 1x and was loaded in the wells created by the comb. The gel was electrophoresed at 60 V for 1 h or till the dye front covered almost 3 to 4 cm length of the gel. A molecular weight marker [λ DNA digested with E*co*RI/ *Hin*dIII or *Hin*dIII alone (MBI Fermentas)] was loaded along with the samples for reference.

Gel elution:

For gel elution, the DNA fragments were electrophoresed on 0.8% agarose gel stained with ethidium bromide(EtBr). The fragment of interest cut out along with the gel slice was weighed and taken in a micro tube. Perfectprep Gel Cleanup kit (Eppendorf®, Germany) was used for extracting DNA from agarose gel following manufacturer's instructions. The DNA was eluted in 20 µl of warm sterile water (65ºC). A small amount (1.0 µl) of the eluted DNA was electrophoresed on agarose gel to check the concentration and quality before proceeding further. The gel eluted DNA was stored at -20ºC.

DNA extraction from leaf tissues (miniprep):

CTAB method (Murray and Thompson 1980; Doyle and Doyle 1990): DNA isolation was done from the second leaf from the shoot tip of young plants. The leaves were freshly collected or frozen in liquid nitrogen and stored at -70ºC. The leaf tissue (100-500 mg) was ground using mortor and pestle in liquid nitrogen to a fine powder along with a pinch of PVPP (Polyvinyl Polypyrrolidone). Warm (65ºC) CTAB (Cetyl/ Hexadecyltrimethyl Ammonium Bromide) extraction buffer (1.0 ml) [2% CTAB, 100 mM Tris HCl (pH 8.0), 20 mM EDTA (pH 8.0), 1.4 M NaCl and 2% β- mercapto ethanol (β-merc)] was taken in 2.0 ml micro tubes and 200 mg of the powder was transferred to the tube, and mixed well to suspend the powder uniformly by repeated inversion of the tubes. The tubes were incubated at 65ºC for 1 hr with intermittent mixing. After incubation, 0.5 ml of Chloroform: Isoamyl alcohol (24:1) mixture was added and mixed thoroughly by inversion. The two phases were separated by centrifugation at 12,000 rpm for 15 minutes. The upper aqueous layer was taken in a fresh 2.0 ml tube without disturbing the middle and lower layers. The nucleic acid content was precipitated from the aqueous phase by mixing well by

inversion after adding an equal volume of isopropyl alcohol and incubating the tubes at -20ºC for a minimum of 30 minutes. After the cold treatment, the tubes were centrifuged at 4ºC and 12,000 rpm for 15 minutes to sediment the nucleic acids. The solution was decanted completely and 1.0 ml of 75% ethanol was added, and incubated for 5 minutes at RT. The tubes were centrifuged at 12,000 rpm 3-5 minutes and ethanol was decanted. The pellet was air-dried and a required volume of TE [10 mM Tris HCl and 1.0 mM EDTA (pH 8.0)] solution was added. For further purification, the pellet was dissolved in 500µl of TE buffer.

Purification of genomic DNA:

The genomic DNA isolated from the tissue was purified from contaminating RNA and protein in the purification procedure. This was necessary if the DNA was to be used for molecular analysis. To the nucleic acids, dissolved in TE buffer, 2-3 µl of RNase A from a stock of 10 mg ml^{-1} solution was added and the tubes were incubated at 37ºC in a water bath for 1-2 hrs. An equal volume (here 500 µl) of phenol: chloroform: isoamyl alcohol (25:24:1) mixture was added and mixed carefully by inversion. When the two phases got mixed properly, the tubes were centrifuged at 12,000 rpm for 15 minutes. The upper aqueous layer was removed to a fresh tube without disturbing the middle protein and lower phenol mixture layers. An equal volume of chloroform: isoamyl alcohol (24:1) mixture was added, mixed thoroughly and centrifugation was repeated. Two volumes of 100% ethanol were added to the upper aqueous layer. The content was mixed well by inverting the tubes for 5 to 6 times and was incubated at -20ºC for a minimum of 30 minutes. After incubation, the tubes were centrifuged at 12,000 rpm for 10 minutes. The solution was decanted and the pellet was rinsed with 75% ethanol as explained earlier. The final pellet was air dried and suspended in a minimum volume (20 to 50 µl according to the pellet size) of deionized water or TE buffer. The isolated DNA was stored either at 4ºC (short term) or -20ºC (long term).

DNA Precipitation:

To the aqueous phase, 0.225 ml of 100% ethanol was added and mixed by inversion 6-8 times and was stored at RT for 5 minutes. Precipitated DNA was sedimented by centrifuging at 5,000 rpm for 4 minutes, and resulting supernatant was removed.

Quantification of DNA:

An aliquot (5.0 µl) of the solubilized DNA was mixed with 1.0 ml of TE buffer (pH 8.0) and measured A_{260} and A_{280} of the resulting solution. The DNA content was calculated assuming that one A_{260} unit equals 50 µg of double stranded DNA/ml.

RNA isolation:

Total RNA was isolated from the plant organs using the TRI reagent(Sigma-Aldrich, USA) or TRIZOL(Invitrogen, Germany) as per the manufacturer's instruction.

PCR (Polymerase Chain Reaction):

Primers used for the PCR analysis:

*NPT*II 'Forward': 5'-GAG GCT ATT CGG CTA TGA CTG-3'

*NPT*II 'Reverse': 5'- ATC GGG AGC GGC GAT ACC GTA-3'.

These primers amplified the full length (700 bp) gene.

TvD1 Forward: 5'GGGTACCATGGAGAAGAAATCACTAGC 3'

TvD1 Reverse: 5' GGGATCCTTTAACATCTTTTAGTACACCA 3'.

These above primers used for the amplification of 228 bp gene

Each reaction was carried out in a 50 µl (total volume) reaction mixture consisting of 1x PCR buffer, 0.2 mM each dNTPs, 1.5 mM $MgCl_2$, 20 pmol of each primer, 0.5 units of Taq DNA polymerase and 10 to 20 ng plant DNA from putative transgenic and control non-transgenic plants or plasmids. Amplification was carried out in a thermal cycler (Eppendorf® Master Cycler) under following conditions:

94ºC for 3 minutes initial denaturation,

94ºC for 1 minute denaturation,

58ºC for 55 seconds annealing,

72ºC for 1 minute elongation,
10 minutes at 72ºC final extension
35 cycles of amplification (steps 2-4).

Lid temperature was set at 105ºC before the reaction was started. Amplified DNA fragments were electrophoresed on 0.8% agarose gel stained with EtBr. Gels were photographed under UV-light in a Gel-Documentation System (LTF Labortechnik, Germany).

Digestion of the genomic DNA:

For Southern analysis, 10-20 µg of genomic DNA was taken for digestion using restriction enzymes. *Hin*dIII and *Eco*RI enzyme was the most frequently used enzyme in this study for Southern analyses. 20-30 units of the enzyme were used along with 1x reaction buffer. Usually 40 µl reactions were set.

Southern Blotting:

Gel Treatments:

Digested DNA samples were electrophoresed on 0.8% neutral agarose gel at 30 V as explained earlier. The gel was allowed to run till the dye front reached 12 to 13 cm. EtBr (0.1 µl/ ml) was included in the gel for visualization and gel documentation. Before starting the gel treatments for blotting, the EtBr was removed by gently agitating the gel in sterile double distilled water for 30 minutes.

Depurination:

The gel was immersed in freshly prepared 0.125 M HCl and agitated by gentle rocking for 7 to 10 minutes on a gel rocker. During this time the bromophenol blue dye present in the samples change its color from blue to yellowish green. Depurination was not done for DNA fragments less than 10 kb in size (when Southern analysis was done on PCR fragments).

Denaturation:

The gel was submerged in sufficient denaturation buffer [1.5 M NaCl, 0.5 M NaOH] and incubated for 30 minutes with gentle agitation. During this time the bromophenol blue dye returns to its original color.

Neutralization:

After 30 minutes in denaturation buffer, the buffer was replaced by sufficient volume of neutralization buffer [1.0 M Tris-Cl, 1.5 M NaCl (pH 7.5)] to submerge the gel. The gel was incubated for 30 minutes with gentle agitation and the capillary blot was set up as described below:

Capillary blotting:

Capillary blotting technique, first described by Southern (1975), is still the most widely used technique for transferring separated nucleic acid fragments from an agarose gel to a solid support. Wet neutral blotting using SSC (Sodium Chloride Sodium Citrate) or SSPE (Sodium Chloride Sodium Phosphate EDTA) and semi dry alkaline blotting using 0.5 M NaOH + 1.5 M NaCl were used in blotting.

Neutral wet blotting using 20x SSC/SSPE:

A sheet of nitrocellulose membrane was cut to an appropriate size. A tray or a suitable dish was half filled with transfer buffer [20x SSC/SSPE (pH 7.2)]. A platform was made that was covered with a wick made from 3MM Whatman sheet saturated in transfer buffer. The treated gel was placed on the wick platform. Any air bubble getting trapped between the gel and wick were avoided. The gel was surrounded with a cling film or clean polythene sheet to prevent the transfer buffer being absorbed directly by the paper towels. The membrane was positioned on the top of the gel taking care not to trap any air bubbles in between the membrane and gel. Three sheets of 3MM paper cut to size and saturated in transfer buffer was placed on top of the membrane taking care not to trap air bubbles. A stack of absorbent crude filter papers cut to size (at least 5 cm high) was placed on the top of 3MM paper. Finally, a glass plate and a weight (500 g) were kept on top of the paper stack. The transfer was allowed to proceed overnight. After blotting, the transfer set up was carefully dismantled. Before

separating the gel and the membrane, the wells were marked to track the identification using a pencil. Membrane was not rinsed following the transfer before it was fixed properly, as it removes the nucleic acid from the membrane. The nucleic acid fragments were fixed to the membrane using a UV - cross linker by exposing the membrane to 120 milli joules of ultra violet radiation. Blots were used immediately or thoroughly dried and stored between the folds of Whatman filter paper.

Random Priming in Radioactive DNA labeling:

Random priming radioactive DNA labeling was done using HexaLabelTM DNA Labelling Kit (Prime-a-gene labelling system (Promega). PCR amplified DNA fragment of the gene of interest was used for preparing the probe for Southern analyses. The probe was prepared according to the manufacturer's instructions. The procedure is as follows:

In a fresh 1.5 ml micro tube, 100 ng of the DNA fragment was taken and to this 10 µl of hexanucleotide in 5x reaction buffer was added and the volume was made up to 40 µl using deionized water. The contents were mixed well, incubated in a boiling water bath for 5-10 minutes and the tube was quick chilled on ice. A quick spin was given to accumulate the content at the bottom of the tube and 3 µl of dNTP (0.25 mM each) mix devoid of dATP was added. α-^{32}P labeled dATP (50 µCi) and Klenow fragment exo- (5u) were added to the tube. The contents were mixed well by tapping and a quick spin was given in a micro centrifuge for 3-5 seconds. This mixture was incubated at 37 ºC for 10 to 30 minutes. After 30 minutes of incubation, 4.0 µl of dNTP mix was added and incubated further for 5 minutes more at 37 ºC. The reaction was stopped by adding 1.0 µl of 0.5 M EDTA (pH 8.0). The labeled DNA was used directly for hybridization after denaturation at boiling temperature in a water bath for 5 minutes, or stored at -20ºC.

Southern Hybridization:

Hybridization of Southern blots was performed in glass tubes in a hybridization oven/shaker (Amersham Biosciences, USA) at 65ºC. Modified Church and Gilbert (1984) buffer [0.5 M phosphate buffer (pH 7.2), 7% SDS, 10 mM EDTA, and 1% BSA] was used for hybridization. Approximately not less

than 50 µl of hybridization buffer was used per cm^2 of the membrane. The blot was pre-wet in sterile double distilled water in a tray followed by 1x SSC. The membrane was rolled (nucleic acid side facing towards middle of the tube) along its length to avoid overlapping and was placed inside the hybridization tube already containing the required volume of warm (65ºC) pre-hybridization buffer (hybridization buffer devoid of probe). Addition of BSA to the prehybridization solution reduced background and nonspecific hybridization. The blot was allowed to be in the pre-hybridization solution for 4 hrs inside the hybridization oven at 65ºC and 3 rpm. Radioactive probe was prepared fresh as explained above. The probe was added to the pre-hybridization buffer, taking care not to add it directly onto the membrane. Prior to adding, the probe it was denatured in a boiling water bath for 5 minutes and quickly chilled on ice. Hybridization was continued for 16 hrs at same temperature and rpm.

Stringency wash:

For washing of the blots after hybridization, the wash solutions were prepared in excess and preheated to 65ºC. The hybridization buffer was discarded in to a brown bottle kept for the purpose. Blots were washed in excess (1-5 ml/ cm^2) wash buffers. Three washes were given each for 5 minutes in Solution I [2x SSC, 0.1% (w/v) SDS], Solution II [1x SSC, 0.1% (w/v) SDS], and Solution III [0.1x SSC, 0.1% (w/v) SDS] at 65ºC. Second wash onwards, washes were performed in trays and count was monitored using a GM counter, and the wash was continued till the background reduced to 3 or below. After the washes, the blots were removed and the solution was completely drained off the membrane by holding the corners with forceps and touching the other corner to a filter paper. The membrane was then wrapped in Saran Wrap or neatly placed in between clean thin polythene sheets. Blots were exposed to Konica/ Amersham X-ray film inside the X-ray film cassette with intensifying screens and stored at -70ºC as required. Cassettes were taken out of -70ºC and allowed to come to RT before they were developed and fixed using X-ray film developer and fixer (Indu, India). Autoradiographs were washed thoroughly under running tap water and air-dried.

Stripping/ De-probing the blots:

If the blots were to be used again after hybridization for hybridizing it with the same or a different probe, the previous probe hybridized to the DNA should be removed. For the successful removal of probes, the membranes were never allowed to dry after hybridization and washing since this process of drying has the effect of fixing the hybrid. Two procedures were used for this.

Hot SDS procedure:

The moist membrane is placed in an appropriate sized tray. Boiling solution of SDS [0.1% (w/v)] is prepared and poured onto the membrane and was allowed to cool to RT. This step is repeated until the GM counter shows a near 0 value. After removing the probe, the blots are rinsed in 2x SSC, air dried and stored at RT in between the folds of Whatman papers.

Alkali procedure:

In this procedure instead of hot SDS, warm 0.2 M (42ºC) NaOH solution is used. After adding the alkali solution, the membranes are incubated at 42ºC for 10 minutes with gentle agitation. This step is repeated after adding fresh alkali solution till the GM counter showed 0 value. After removing the probe, the membranes were stored as explained above.

Total Protein Extraction:

For testing the presence of protein in the transgenic plants, total protein was extracted. Leaf tissue was homogenized in liquid nitrogen using mortar and pestle adding a pinch of PVPP and 1.0 g of the fine powder was collected in pre-chilled micro tubes. Into the tubes kept on ice, 1.0 ml of chilled extraction buffer [50 mM Tris-Cl and 1.0 mM PMSF (Phenyl Methane Sulphonyl Fluoride)] was added, mixed thoroughly and were made to stand at 4ºC for half an hour with intermittent shaking. The tubes were centrifuged at 4ºC and 15,000 rpm for 20 minutes and the supernatant containing the protein was collected in a fresh tube. All the steps were performed at 4ºC. Isolated protein samples were immediately used or stored at 4ºC. It stayed without degradation for one week.

Quantification of the leaf-extracted total protein:

Bradford (1976) method for protein quantification:

Bradford dye concentrate was prepared by dissolving 100 mg Coomassie Brilliant blue G250 in 50 ml of 95% ethanol. To this, 100 ml concentrated phosphoric acid was added and the volume was made up to 200 ml with H_2O. The Bradford dye concentrate was stable for 6 months at 4ºC. Samples of BSA were prepared with 10, 20, 30, 40, 50, 75 and 100 µg/ 100 µl of BSA (fraction V) in the same buffer solution in which the protein samples were extracted. The Bradford dye concentrate was diluted to 5x with double distilled water and 1.0 ml was added to each sample. The red dye turned blue after binding to the protein. The samples were allowed to develop color for at least 5 minutes (not longer than 30 minutes). Absorbance at 595 nm was read, and a linear standard curve was prepared to calculate the concentration of protein. The results were cross confirmed using Lowry method.

Lowry (1951) method:

Reagent A: Sodium carbonate (10 g) was dissolved in 500 ml H_2O and 0.5 g $CuSO_4$ 5 H2O and 1.0 g sodium tartrate in 500 ml of H_2O separately. The sodium carbonate solution was slowly added to the copper/ tartrate solution on a magnetic stirrer (this solution stays for one year at 4 ºC). Before use, this solution is activated by adding one volume of reagent A with 2 volumes of 5% SDS (w/v) and 1 volume of 0.8 M NaOH, which was stable for a couple of weeks at RT.

Reagent B: One volume of 2 N Folin-Ciocalteau Phenol reagents is combined with 5 volumes of water (This solution is stable for several months at RT when kept in a amber color bottle). Samples of BSA are prepared in water as explained above. One ml of activated reagent A is added to the samples mixed thoroughly and was incubated at RT for 30 minutes. Absorbance was taken at 750 nm and a linear standard curve was prepared to calculate the concentration.

Polyacrylamide Gel Electrophoresis (PAGE):

SDS–Polyacrylamide Gel Electrophoresis (SDS-PAGE) with acrylamide: N,N'-bis acrylamide (30:1) was carried out according to the standard protocol (Laemmli, 1970). A 15% (standardized for defensin protein) 7.5 cm gel was prepared with 1 cm stacking gel. Electrode buffer was prepared by dissolving 10

g SDS, 30.3 g Tris and 144.1 g glycine in 800 ml H_2O [25 mM Tris and 192 mM glycine with 1% SDS]. The volume is adjusted to 1 liter with H_2O and stored at RT. Protein samples are prepared by boiling in a water bath for 3-5 minutes after mixing 50 µg of each sample with one-tenth volume of the sample buffer [0.125 M Tris-HCI (pH 6.8), 4% SDS, 20% glycerol, 10% 2-mercaptoethanol and 0.002% bromophenol blue]. Electrophoresis is carried out at 90 V in an electrophoresis and electro-transfer unit (Hoefer mini VE, Amersham Pharmacia Biotech, USA) till the dye came out of the gel into the electrode buffer.

Coomassie Blue staining of the gel:

Coomassie Brilliant Blue R250 staining detection limit is 300 to 1000 ng protein and is used in the experiments. The gel with electrophoretically separated proteins is incubated for staining in Coomassie solution (0.025% Coomassie Brilliant blue- R250 in 45% methanol and 10% acetic acid) for 30 minutes. Destaining was done with a destaining solution [45% methanol and 10% acetic acid] to remove background staining. The destaining solution is replaced every 10-15 minutes with a fresh solution, until the protein bands are visible.

Western Blotting and Immuno staining:

After electrophoresis, the protein samples are electro-blotted on to Polyvinylidine fluoride (PVDF)/ nitrocellulose membrane (PALL®, India) using Trans-Blot apparatus (electrophoresis and electro-transfer unit, Hoefer mini VE, Amersham Pharmacia Biotech, USA) according to the manufacturer's instruction using Towbin buffer (Towbin et al. 1979). The gel is first equilibrated in Towbin buffer (25 mM Tris, 192 mM glycine and 20% methanol) and the membrane is kept above the gel towards the positive end of apparatus and is stacked in between Whatman filter papers taking care not to trap any air bubbles. Transfer is conducted for 4 hrs at 25V. The transfer of protein to the membrane was checked by reversible Ponceau S staining (100 mg Ponceau S in 5% acetic acid). The stain was removed by 3-4 washes with TBST [10 mM Tris-HCl (pH 7.4), 150 mM NaCl and 0.1% Tween-20 (v/v)]. Blocking of the membrane is carrying out using 3% BSA (w/v) in TBS for 1 hr at RT followed by 5 washes with TBS for 10 minutes each. The blot was then incubated overnight with the primary antibody diluted in TBS containing 3% BSA at 4ºC. This is again followed by 5 thorough

washes, each of 10 minutes in TBS. Then the blot is incubated in secondary antibody - Goat anti-rabbit IgG (Bangalore GENEi, India) ALP conjugate. The bands are visualized after staining with BCIP/NBT (Bangalore GENEi, India), which is a substrate for ALP conjugate. Proteases are ubiquitous and difficult to separate from proteins. Hence, protease inhibitor, PMSF is used throughout the procedure for successful isolation of total protein from the plants. Protein solutions are stored in aliquots at 2-8ºC or at -70ºC after addition of 20% glycerol and shock freezing in liquid nitrogen. During experiments, proteins are always maintained on ice. Vigorous pipetting and vortexing are avoided. β-merc is avoided in the extraction buffer as it can form disulfide bridges with thiol groups of proteins leading to aggregation and/ or inactivation. Molecular weight of amino acids ranges between 75 and 204 Da. For calculations, the average molecular weight of amino acid (110 Daltons) is considered. Ponceau stain is used only in the initial experiments; later this step is avoided in the western blot for checking the transfer, as the proteins are visible even without using the stain while drying the blot.

RT-PCR:

Total RNA is also isolated from the leaves without any stress or pathogen treatment using TRI-reagent (Sigma-Aldrich, USA). For RT-PCR, the first strand of the cDNA is synthesized by reverse transcription with an oligo-(dT) primer and M-MLV reverse transcriptase enzyme (Sigma-Aldrich, USA). The program for the RT-PCR is followed as per the instruction previously.

Biological Software used for detection of deducing the amino acid sequence:

Nucleotide and deduced amino acid sequence comparisons are made using the BLAST (Basic Local Alignment Search Tool) programs such as BLASTN (Zhang et al. 2000) and BLASTX (Atschul et al. 1997) respectively, on the non-redundant database of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/). Expasy tool is used to deduce the amino acid sequence coded by the gene *TvD1*.

Expression and purification of *Tephrosia* (TvD1) defensin protein:

The coding region of TvD1 gene is cloned between *Kpn*I and *Bam*HI sites in pET32a (+) vector (Novagen, USA). The cloned vector is transformed into *E. coli* BL21 (DE3) pLys S and the transformed cells are grown at 37^{o}C in LB Broth (Himedia, India) overnight at 200 rpm in a shaker. A 10 ml aliquot from the overnight culture is added into one litre of fresh LB medium for 1 hr till the OD_{600} reached 0.6 – 0.8. These cells are induced using 0.4 mM IPTG (Fermentas, Germany). After 6 hrs incubation, the cells are harvested by centrifugation at 5000 rpm for 5 min at 4^{o}C. The pellet is washed with fresh 1x PBS buffer. Subsequently, the first lysis (prolysis) buffer (300 mM NaCl, 50 mM $NaHPO_4$, 10 mM imidazole, 15% glycerol and 0.1% SDS) was added and mixed homogeneously. The cells are lysed by sonication at 6X pulse for 5 min at 4^{o}C (every 20 second pulse, a 10 second interval was given). The lysate is centrifuged at 12,000 rpm for 20 min to remove the debris. The supernatant is mixed with the second lysis buffer (300 mM NaCl, 50 mM $NaHPO_4$ and10 mM imidazole) along with 2 ml Ni-NTA (Qiagen, Germany), and the volume is made up to 50 ml per liter culture of cells and mixed for 1 hr at 4^{o}C at 60 rpm in a rocker. The supernatant mixture is loaded on to the column (Sigma-Aldrich, USA) and the flow-through is collected at the rate of 0.5 ml per min, which was maintained till the end. Finally, the column was washed with 10 volumes of wash buffer (300 mM NaCl, 50 mM $NaHPO_4$, 20 mM imidazole and 15% glycerol). The recombinant peptide rTvD1 is eluted with elution buffer (300 mM NaCl, 50 mM $NaHPO_4$ and 250 mM imidazole), according to the manufacturer's instructions (Qiagen, Germany).

Antifungal activity assay:

Antifungal activity of rTvD1 was tested by micro-spectrophotometry as well as an *in vitro* plate assay with the following fungal pathogens: *F. oxysporum f. sp. vasinfectum*, *F. moniliforme*, *R. solani*, *A. helianthi*, *P. parasitica f. sp. nicotiana, B. cinerea* and *Curvularia sp*.

Briefly, 10 µl of protein diluted to different concentrations is pipetted into the well of a 96-well microtitre plate containing 90 µl of the test fungal spore suspension (~2.5×10^4 spores/ml) in potato dextrose broth (PDB), which is placed

in an incubator at 28°C. Each protein concentration is tested for its antifungal activity in triplicate. Fungal spore germination and growth are observed microscopically and the optical density (OD) measured with a microplate reader at a wavelength of 595 nm after inoculation for 30 min and 48 hr. Controls are tested identically except that the protein is omitted. Value of the growth inhibition lower than 10% is not considered as significant (growth inhibition is defined as the ratio of the corrected absorbance at 595 nm of the control minus the corrected absorbance of the test sample, divided by the corrected absorbance of the control). The corrected absorbance is defined as the absorbance at 48 hrs minus that at 30 min. IC_{50} is defined as the protein concentration at which 50% inhibitions is reached). Percent inhibition of fungal growth on the plate has been estimated as: area of mycelial growth in the absence of antifungal protein minus area of mycelial growth in the presence of antifungal protein / area of mycelial colony in absence of antifungal protein) x 100.

A graph plotting percent inhibition of fungal growth against the concentration of protein is used to determine the IC_{50} of antifungal activity i.e., the concentration producing 50% inhibition.

For the *in vitro* plate assay, agarblocks with the fungus of uniform size are inoculated at the center of the Potato Dextrose Agar (Himedia, India) carrying 25 ml of the medium and incubated at 28°C. When the mycelia reached 6 cm diameter, four sterile Whatman no.1 filter paper discs (1 cm diameter) are placed on the plate at equal distance from the center. Purified rTvD1 is added at various concentrations, (ranging from 25-100 μg/ml) at the center of the disc on the plate. The elution buffer served as the control. The plates were incubated at 28°C and the plates were observed periodically till the mycelial mat covered the control discs.

For the sclerotium germination assay, the sclerotium is placed at four places at equal distance from the centre of the PDA plate. The protein at respective concentration is added directly over the sclerotia and its effect on their germination and growth is observed.

Arabidopsis germination assay:

The purified protein is diluted to desired concentration in 10 mM Tris-HCl pH 7.6. This dilution is then mixed with an equal volume of Murashige and Skoog basal salts (Murashige and Skoog 1962) with B_5 vitamins (Gamborg's vitamins) in a 96-well assay plate. A single sterilized Columbia seed was placed in each well and grown at 24^{o}C under a 16 h photoperiod for 7 days (Allen et al. 2007). The experiment is carried out in triplicates.

Transformation of Tobacco leaf discs (Horsch et al. 1985):

Tobacco leaves were surface sterilized by rinsing in 400 ml of water containing 5% of commercial bleaching powder and 20 drops of Tween 20. The leaves were rinsed for 5 minutes in distilled water to remove the bleach and the surfactant. The mid ribs of the leaves were cut out and the lamina was cut into uniform squares using sterile blade. The leaf pieces were rinsed for 5 minutes in sterile distilled water 5 times followed by the treatment with $HgCl_2$ (0.01%) for 5 minutes. Then they were rewashed with sterile water 5 times for 5 minutes each. The leaf pieces were carefully placed on sterile tissue paper and the wound the leaf edges and were cut out carefully. They were carefully kept in plates containing pre-solidified MS medium with 2 mg l^{-1} BAP and 0.1 mg l^{-1} NAA. The leaf discs were co-cultivated with *Agrobacterium* for a period of 3 days and then they were transferred to regeneration medium containing the antibiotic cefatoxime to kill the bacteria.

In one to two weeks the leaf disks starts expanding and callusing. At that stage, the leaf pieces were sub cultured to the bottles containing regeneration medium i.e. MS medium with 2 mg l^{-1} BAP and 0.1 mg l^{-1} NAA. Each piece was cut into four and the pieces were pressed into the medium in order to ensure a good contact between the plant tissue and the medium. Shoots started appearing about 3 weeks after inoculation and they generally grew from the edge of the disks or from internal area wounded by the forceps. When the shoots were at least 5-mm long, they were excised at the avoiding any callus tissue and the shoots were carefully placed in the rooting medium i.e. MS medium. Cefotaxime was kept in the medium to avoid *Agrobacterium* growth. The rooted plantlets were then taken out from the medium and the agar was washed from the root system under running tap and the plantlets were transferred to a mixture of sterile

soil and vermiculite (1: 3). To harden the *in vitro* plantlets, which need to develop a cuticle to control water losses, each plantlet was covered with a polythene bag.

Screening of the Transgenic plants using 0.2% Basta solution:

Basta® is a unique broad spectrum, non-selective herbicide that provides excellent crop safety with a relatively low hazard to the operator. Basta is registered for use in a wide range of crops including avocado, banana, citrus, mango, lychee, pawpaw, rambutan, tree nuts and stonefruit (http://www.nrrbs.com.au/bayerbasta.html). The selectable bar gene encodes the enzyme phosphinothricin acetyl transferase (PAT) that inactivates Phosphinothricin (PPT), the active ingredient of the herbicide by acetylation (Chowdhury and Vasil 1992).

As the vector pEGAD contained the selectable marker for Basta resistance, the obtained plantlets were screened for tolerance to Basta treatment. One leaf from each plantlet was cut into small pieces about 1cm^2 and the pieces were incubated in a solution of 0.2% Basta for 3 days along with the leaf pieces of the control plants.

Construction of Expression Plasmids:

To produce the different recombinant protein with or without mutation, an expression vector was constructed. Firstly, a PCR based – two step DNA synthesis was used to produce the different TvD1 DNA sequence (Ke and Madison 1997). DNA sequence was obtained from the gene bank accession number: AY907349 from National Centre for Biotechnology Information for the construction of different fragments and gene synthesis without signal peptide. In the first step, the **native TvD1** DNA was synthesized by using the following primers: TvD1A (TvD1-A- F-5'-AAG ACA TGC GAG AAC CTG GCA GAT ACG TAT AGG GGT CCA TGC TTC ACC ACT GGA AGC TGT GAC GAT CAT TGC AAG-3') and TvD1B (TvD1-B-R-5'-ACA TCT TTT AGT ACA CCA GCA GCG AAA ATC GTC CCT GCA CCT TCC ACT CAG TAA GTG CTC CTT ATT CTT GCA ATG ATC G-3') in the presence of *Pfu* DNA polymerase. Similarly, second **Alpha TvD1** (mutant) was synthesized using the following the primers:TvD1 A (TvD1-A- F-5'-AAG ACA TGC GAG AAC CTG

GCA GAT ACG TAT AGG GGT CCA TGC TTC ACC ACT GGA AGC TGT GAC GAT CAT TGC AAG-3') and TvD1C(TvD1-C-R-5'-ACA TCT TTT AGT ACA CCA GCA ***AGT ACG AGT CAT ACC*** CCT GCA CCT TCC ACT CAG TAA GTG CTC CTT ATT CTT GCA ATG ATC G- 3'). Finally to produce the complete fragment, the second step DNA synthesis was performed using the following primers: forward (*Bam*HI-DP-TvD1-F-5'-C GGA TCC **GAC CCG** AAG ACA TGC GAG AAC CTG GCA-3') and reverse (*Xho*I - Stop-TvD1-R-5'- GTG CTC GAG *TTA* ACA TCT TTT AGT ACA CCA-3'). The two restriction sites for *Bam*HI and *Xho*I are underlined; the acid-sensitive dipeptide (Asp-Pro) that is immediately upstream of the TvD1 coding sequence is in bold, and the stop codon is in italics. The PCR-amplified DNA fragment containing modified TvD1 was cloned between the *Bam*HI and *Xho*I sites of the multiple-cloning site of expression vector pET32a (+) (Novagen, USA). For the second PCR, all the primers used were same as in the case of **native TvD1** and **Alpha TvD1**. Likewise, the other mutants were generated by using the native fragment as template. The other mutants including **S32R** and **D37R** were generated by using the same strategy as mentioned above. For the first PCR, the following primers were used: for **S32R**, forward primer used was TvD1 (**S32R**)-F-5'-CAC TTA CTG **AGA** GGA AGG TGC A-3' for D37R, forward primer used was TvD1 (**D37R**)-F-5'-TGC AGG GAC **GGT** TTT CGC TGC T-3'and reverse primer (*Xho I* - Stop-TvD1-R-5'- GTG CTC GAG TTA ACA TCT TTT AGT ACA CCA-3') it was same for both. The PCR program as follows for the generation of native TvD1 and Alpha TvD1: 94^{o}C for 20 sec; 60^{o}C for 20 sec; 72^{o}C for 90 sec; rep 30 cycles. It was the same for the second PCR also. For S32R and D37R, the PCR program as follows: first PCR 5 cycle; 94^{o}C 1 min; 55^{o}C for 1 min; 72^{o}C for 1 min and second PCR program as same as TvD1 and Alpha TvD1. After PCR, all the fragments were double digested with the respective restriction enzymes and cloned at the same site in the vector pET32a(+). The cloned fragment was confirmed by sequencing.

Overexpression and Purification of Recombinant Proteins:

The expression and purification of recombinant proteins were similar to the previous report. *E. coli* BL21 (DE3) cells carrying the plasmid pET32a (+)-

TvD1, pET32a(+)-**Alpha TvD1**, pET32a(+)-**S32R** or pET32a(+)-**D37R** were grown in LB medium containing ampicillin (100 μg/ml). The fusion proteins possessing an N-terminal tag that consists of a thioredoxin (Trx)-tag, His-tag, S-tag and an acid-sensitive dipeptide were overexpressed after isopropyl-β-D-thiogalactopyranoside (IPTG, 1 mM) induction. After incubation at 37^{o}C for 6 hrs, cells were collected by centrifugation and then lysed by french press or sonication in the presence of binding buffer A (8 M urea, 10 mM imidazole, 0.1 M sodium phosphate monobasic, 0.01 M Tris base, pH8.0) followed by centrifugation at 12000 g for 15 min at 4^{o}C. The supernatant was saved and passed through the Nickel – NTA agarose affinity column, followed by 10 volumes of the washing buffer B (8 M urea, 50 mM Imidazole, 0.1 M sodium phosphate monobasic and 0.01 Tris base, pH 6.3) to wash the column. Finally, the fusion proteins were obtained by using the elution buffer C (8 M urea, 250 mM imidazole, 0.1 M sodium hydrogen phosphate , 0.01 Tris base, pH 4.5).The Asp-Pro bond of the acid-sensitive dipeptide has been shown to be extremely labile when incubated at pH around 1.5. Thus, the fractions containing the fusion protein were adjusted to pH 1.4 with 6N HCl and incubated at 55^{o}C for 16–18 h to release the target proteins from their fusion partners. This acidic cleavage was stopped by neutralizing the pH to 7.0 with NaOH, and the expressed target proteins, which contain an extra proline in the N-terminus, were further purified by passing the digested fraction in the affinity column in the presence of binding buffer. The final flow through from the column was saved. The fraction was then centrifuged in the special column with 3 kDa cut off (Vivaspin, Vivascience Ltd, UK) in order to concentrate and remove the salts from the fraction.

Antifungal activity assay:

Antifungal activity of rTvD1 was tested by micro-spectrophotometry with the following fungal pathogens: *Fusarium oxysporum* and *F. culmorum*.

Briefly, 10 μl of protein diluted to different concentrations was pipetted into the wells of a 96-well microtitre plate containing 90 μl each of the test fungal spore suspension (~2.5×10^{4} spores/ml) in potato dextrose broth (PDB), which was placed in an incubator at 28^{o}C. Each protein concentration was tested for its antifungal activity in triplicate (as mentioned earlier).

Assay of chitin deposition at hyphal tips:

This assay was conducted as described by Moreno et al. (2006) to observe chitin deposition at hyphal tips of *F. culmorum*. Pre-germination and incubation of fungal cultures with the native TvD1 and the mutant Alpha TvD1 defensin was performed in 96-well microplates. Following incubation with the recombinant protein, the dye Congo red was added until a final concentration of 1 mM was reached. Fluorescence was examined under the fluorescence microscopy with the wavelength between 543 and 560 nm. The tips of growing hyphae would not stain densely with Congo red while hyphal tips with inhibited growth would be stained.

Antibacterial activity:

The antibacterial activities of the recombinant protein such as native TvD1 and Alpha TvD1 were examined against the plant pathogenic bacteria *Pseudomonas syringae p.v. lycopersci* in sterile 96-well plates (microtiter plates) in a final volume of 100 μl as follows. Aliquots (50 μl) of a suspension containing bacteria at concentrations of 10^6 CFU/ml in culture medium were added to 50 μl of double-distilled water containing the defensin in serial twofold dilutions in LB. Inhibition of growth was determined by measuring the absorbance at 595 nm with an micro plate auto reader after an incubation of 48 hrs at 28°C. The antibacterial activity was expressed as the IC_{50}, the concentration at which half of the bacteria die after the incubation (Makovitzki et al. 2007).

Membrane permeabilization assay:

Fusarium culmorum spores at a concentration of ~ $4x10^4$ were incubated in 1/4-strength potato dextrose broth for 14–18 hours at 25°C. Afterwards, the germinated spores with hyphae were incubated with the recombinant proteins (both native TvD1 and Alpha TvD1) at a concentration of 2.5 and 5 μM respectively for 3 hours with gentle agitation. Fluorescence of the spores with hyphae was visualized using the fluorescence microscope with excitation and emission wavelengths of 488 and 538 nm, respectively after 10 min of adding sytox green dye (0.5 μM).

Insect bioassay:

The third instar larvae of *Tenebrio molitor* of equal size were transferred into the ice and entire gut was removed, then homogenized in 0.9% (w/v) NaCl solution. Samples were then centrifuged for 10 min at 12000 g at 4^{o}C and precipitate was removed. The supernatant was saved as enzyme from the insect and used for assay with the recombinant protein.

Enzyme inhibition assay:

The insect α-amylase inhibitory activity of the recombinant protein was determined by using the Bernfeld method (1955). Different concentration of native and Alpha TvD1 was assayed against the purified enzyme (25 μg ml^{-1}) in a buffer containing 50 mM acetate buffer, pH6.5, 5 mM $CaCl_2$ and 10 mM NaCl. Soluble starch (1% w/v) was used as the substrate. The recombinant protein and the enzymes were pre-incubated for 20 min at 37^{o}C before the addition of 300 μL of 3, 5-dinitrosalicylic acid (DNS). The reaction was stopped by heating for 10 min at 100^{o}C and the absorbance was measured at 530 nm. One α- amylase unit was defined as the amount of the enzyme that increased the absorbance at 530 nm by 0.1 OD during 20 min assay. Each assay was performed in triplicate and distilled water was used as negative control.

Chapter 4

Heterologus expression of *BjNPR1* in mungbean for amelioration of biotic stress tolerance

4. A. Standardization of Regeneration:

Results and Discussion:

Callus with occasional regenerability was obtained in all the explants tried (data not shown) except the cotyledonary node on culture medium containing various growth regulators with different combinations and concentrations. The callus was initially whitish but later turned green, friable or greenish brown hard callus. In our experiments, the calli started browning within 15 to 20 days of proliferation and very few of them developed 3-4 shoots after 40 days of culture. Similar results were reported in mungbean from hypocotyl and cotyledon explants (Patel et al. 1991). Amutha et al. (2003) also made similar observations in mungbean, when the callus was transferred to media containing BA (2.0 mg l^{-1}) and TDZ (0.55 mg l^{-1}) for shoot development. As our primary interest was direct shoot induction from different explants, further studies on callus-mediated regeneration were abandoned.

The cotyledonary node explants turned green after two days of culture and the cut axis enlarged to double the size, and shoot bud initiation was observed at the cotyledonary node region as well as from the proximal end of the cut axis in all the treatments of BAP (1.0 - 6.0 mg l^{-1}) tested. Shoots were obtained at an average of 12.1 per explant at 5.0 mg l^{-1} BAP concentration in the present investigation and this was significantly higher than that has been reported earlier (**Figure 4.A.1**).

Other concentrations of BAP resulted in fewer number of shoot buds (**Table 4.A.1**). Even though the number of shoot buds was more, all of them did not elongate to shoots. Hence, a different strategy was adopted for shoot elongation in our studies. After 7 days of culture on 5.0 mg l^{-1} BAP, the shoot buds were transferred to half the concentration of BAP (2.5 mg l^{-1}) followed by a subsequent transfer to 1.25 mg l^{-1} by the completion of two weeks of culture. After, three weeks of culture, the regenerated cultures were transferred to a medium devoid of growth regulators. Our approach is similar to that reported in groundnut for shoot elongation, where the shoot buds were sub-cultured at 15-day intervals on lower concentrations of BAP for achieving optimal shoot

elongation (Beena et al. 2005). Within four weeks, rapid elongation of the shoot buds was obtained and shoots were more than 5 cm long with 2-3 internodes. Gulati and Jaiwal (1992) obtained a maximum of 8 to 9 shoots per explant in *V. radiata,* and 10.4 shoots in *V. angularis* and *V. aconitifolia* with 1.0 mg l^{-1} BA with a regeneration frequency of only60%. Ignacimuthu and Franklin (1999) noted similar response in mungbean. To increase the frequency of shoot differentiation in mungbean, Das and Pal (2004) recommended the addition of β-arabinogalactan in B_5 medium. Gulati and Jaiwal (1992) tried to increase the shoot number by increasing the BAP concentration but at the cost of shoot length. Prem Anand (2001) used GA_3 in combination with BAP in culturing immature cotyledonary node explants in *V. unguiculata* and also it was necessary to keep the cultures in continuous darkness for a week for elongation.

Figure 4.A.1. Regeneration of mungbean from cotyledonary node explant a) explants showing direct shoot initiation b) elongated shoots c) rooted plantlets and d) regenerated and acclimatized plants produced normal flowers

Table 4.A.1. Effect of MS + B_5 with growth regulator BAP alone or with TDZ, IBA, IAA and NAA on the cotyledonary node explants

S. No.	**MS + B_5 + Growth regulators (mg L^{-1})**					Average no. of Shoots
	BAP	TDZ	IBA	IAA	NAA	
1	1.0					6.0 ± 1.25
2	2.0					8.1 ± 0.70
3	3.0					9.7 ± 1.5
4	4.0					10.3 ± 2.25
5	**5.0**					**12.1 ± 1.45**
6	6.0					11.6 ±1.30
7	5.0	0.1				3.0 ± 0.25
8	5.0	0.5				4.2 ± 0.73
9	5.0		1.0			3.0 ± 0.32
10	5.0		2.0			4.6 ± 1.20
11	5.0			1.0		7.0 ± 1.35
12	5.0			2.0		7.1 ± 1.74
13	5.0				0.1	8.0 ± 0.45
14	5.0				0.2	10.0 ± 0.75
15		1.0	0.1			3.1 ± 1.20
16		1.0	0.01			3.8 ± 0.76
17		0.5	0.1			5.2 ± 1.35
18		0.5	0.01			6.1 ± 1.60
19		1.0		0.1		2.3 ± 0.11
20		1.0		0.01		3.7 ± 0.45
21		0.5		0.1		4.1 ± 2.21
22		0.5		0.01		5.0 ± 2.65
23		1.0			0.1	2.3 ± 1.0
24		1.0			0.01	2.5 ± 0.55
25		0.5			0.1	3.4 ± 1.33
26		0.5			0.01	3.9 ± 1.60

The treatment giving highest shoot proliferation frequency has been indicated in bold face. Each experiment was done with 10 replicas and was repeated 5 times. All the treatments gave 100% response.

Elongation was difficult for shoots regenerated on BAP concentrations higher than 5.0 mg l^{-1} and they remained clumped, and sometimes fasciated (Gulati and Jaiwal1992). The higher frequency and number of multiple shoots

obtained could be due to the cotyledon attached to the nodal explant as in the case of embryo axis observed in *V. mungo* (Saini et al. 2003) and chickpea (Sharma and Amla 1998). During explant preparation, care was taken to remove the shoot tip completely. Hence, meristematic cells remaining below the shoot tip probably differentiate further to give rise to shoot buds. In all the cultures, besides the nodal region, the proximal end of the embryo axis that is in contact with the medium also regenerated shoot buds. .

As 5.0 mg l^{-1} BAP showed maximum shoot proliferation, this concentration was considered as standard and combinations with other plant growth regulators were tried to see the effect on regeneration frequency. When 5.0 mg l^{-1} BAP was used in combination with TDZ, IAA, IBA, NAA, 2 - 10 shoots were obtained; more number of shoots were obtained in combination with NAA (0.2 mg l^{-1}) (**Table 4.A.1**). The shoot apex explants produced up to 9 shoots per explant (data not shown). Axillary shoots were produced from the elongated shoots, which could be excised and rooted as individual shoots. Further increase in the concentrations of the BAP above 5.0 mg l^{-1} did not show considerable increase in the number of shoots. This result is in corroboration with the results previously reported (Gulati and Jaiwal 1990; Chandra and Pal 1995).

TDZ in combination with IAA, IBA and NAA induced callus in the cotyledonary node explant. Average number of shoots was not significant when compared to BAP and its combinations (**Table 4.A.1**). However, an average of 6.1 shoots was produced at 0.5 mg l^{-1} TDZ in combination with 0.01 mg l^{-1} IBA.

The elongated shoots rooted easily on full strength MS salts + B_5 vitamins and ½ MS salts + B_5 vitamins. Roots were more profuse on medium supplemented with 2.0 mg l^{-1} IBA and 0.2 mg l^{-1} NAA (**Fig. 4.A.1**). The plantlets were transferred to sterile vermiculite-soil mixture for hardening. Ten days incubation of these transferred plantlets at culture room conditions was enough to acclimatize them. The hardened plantlets were transferred to field for further growth and maturity. Survival rate of plant was more than 90%. All the plants flowered and set seed normally.

The *in vitro* regenerated plants looked similar to plants raised from seeds in morphology. Regeneration involving callus phase would result in somaclonal variation, hence direct shoot initiation from the cotyledonary node is useful for regeneration as well as further transformation studies.

Results

4.B. Mungbean transformation:

The most regenerative medium (5.0 mg l^{-1} BAP) and the explants (cotyledonary node) similar to that of other legumes were used in all the transformation experiments. The number of shoot buds developed and remained green after transformation was far less than that obtained in regeneration trials. If the bacterial suspension was blot-dried from the explants before culture, after the treatment, the explants stayed intact longer on the regeneration medium. If it was not blot-dried, the bacteria overgrew the explant within 2 to 3 days time. After 3 or 5 days (before the *Agrobacterium* overgrows the explants), the explants were transferred to same medium containing 250 mg l^{-1} cefotaxime for inactivating the bacteria. Cotyledonary explants sustained the infection pressure more easily than the cotyledon explants. On cefotaxime containing medium, the explants, which were green and enlarged, started developing shoot buds from the proximal end. During co-cultivation period, there was not much difference between the two explants. But once they were freed of bacterial infection, cotyledonary node explants showed better regeneration response and suffered little from injury. Within 7 days after transferring to cefotaxime containing medium, the explants started developing shoot buds. Once the shoot bud induction started, the explants were given selection pressure to discriminate between the transformed and non-transformed shoot buds. Before proceeding into the transformation, some of the pre-requiste experiments and factors were standardized to achieve the maximum number of shoots after transformation and co-cultivation. They are mentioned as follows:

Transient Gus expression:

To check the explants, whether it is suitable for transformation or not, preliminary transformation experiments were done with two days old cotyledonary node explants through *Agrobacterium* mediated using the strain GV2260 harboring the binary vector pCAMBIA2301 with *GUS* reporter gene driven by 35S promoter. Explants after 7 days of incubation in the co-cultivation media (free of antibiotics) were used for GUS assay using the standard protocol (Jeffereson et al. 1987). It was observed that the entire surface of the transformed explants showed blue patches indicating the transient expression of GUS in all the superficial cells (**Fig. 4.B.1**). The non-transformed control explants did not develop any blue color indicating that there is no problem of 'GUS'-like activity in mungbean explants.

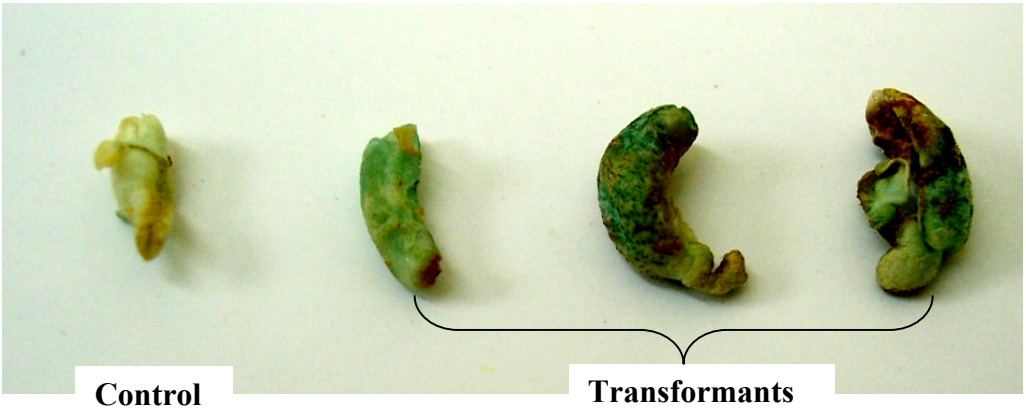
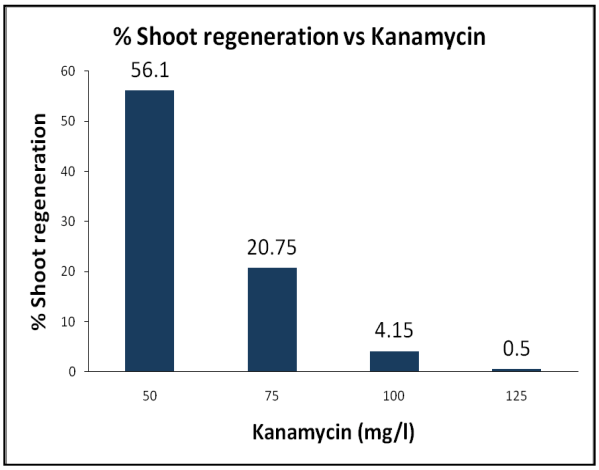


Figure 4.B.1. Gus assay for the 7 days old co-cultivated explants showing transient expression of GUS on the transformed explants.

Optimization of kanamycin:

The control explants were used for optimizing the required antibiotics kanamycin concentration for selection in the media (**Fig. 4.B.2A& 4.B.2B**). At 100 mg l^{-1} kanamycin along with regeneration media, the explants showed 4.15% of regeneration when compared to 0.5 % at 125 mg l^{-1}. And at 75 mg l^{-1} and 50 mg l^{-1}, it was observed as 20.75% and 56.1% respectively. Hence, 100 mgl^{-1} kanamycin concentration was preferred for the selection of putative transformants.



(A) **(B) Kanamycin (100 mg l^{-1})**

Figure 4.B.2. Optimization of required kanamycin for plant selection. (A) Graphical representation of percentage regeneration against the different concentration of kanamycin. (B) Explants showing necrosis at 100 mg l^{-1} kanamycin.

Selection of *Agrobacterium* strain:

In order to improve the transformation efficiency, two different strains such as GV2260 and LBA4404 were used for transformation and observed the percentage of regeneration. The strain GV2260 resulted in 24.9% regeneration after co-cultivation whereas the strain LBA4404 resulted in 40.3% regeneration (**Fig. 4.B.3**).

Factors affecting the transformation:

According to Birch (1997), there are several factors that affect the transformation. Among them, some were selected as follows and tabulated (**Table 4.B.1**):

pH:

The pH of the media during co-cultivation is largely affects the frequency of transformation. At pH 5.2, 5.5 and 5.8, the frequency of regeneration was 74%, 64% and 57% respectively.

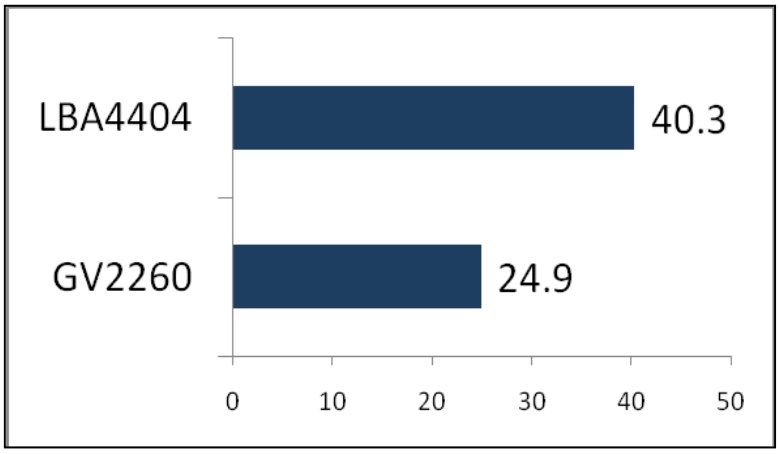


Figure 4.B.3. Graphical representation of the percentage regeneration after transformation and co-cultivation with two different strains of *Agrobacterium tumifaciens* such as GV2260 and LBA4404

Light:

The shoot regeneration response is largely affected by light during co-cultivation. It was shown that in the presence of yellow light during co-cultivation resulted in 74% shoot regeneration response, whereas there is no significant difference in the green as well as white light with 64% and 67% response respectively. In the presence of red and blue light, the response was 57% and 45% respectively.

L-cysteine:

The frequency of transformation is also largely affected by L-cystein and other thiol compounds. In the present study, we used different concentrations of L-cysteine. The shoot regeneration percentage was given in the **Table 4.B.1** at specific concentrations such as 72%, 86%, 82%, 63% and 55% at 200 mg l^{-1}, 400 mg l^{-1}, 600 mg l^{-1}, 800 mg l^{-1} and 1000 mg l^{-1} L-cysteine respectively.

Table 4.B.1. Showing the different factors affecting the transformation and subsequent regeneration of shoots immediately after co-cultivation

Factors	Variables	% Shoot regeneration
pH	5.2	74 ± 2.1
	5.5	64 ± 4.2
	5.8	57 ± 1.0
Light	Green	64 ± 2.6
	Red	57 ± 2.6
	Yellow	74 ±1.6
	White	67 ± 1.2
	Blue	45 ± 1.6
Addition of compounds	Tobacco leaf extract	84 ± 2.8
L-cyteine	200 mg/l	72 ±3.2
	400 mg/l	86 ± 2.0
	600 mg/l	82 ± 2.6
	800 mg/l	63 ± 4.2
	1000 mg/l	55 ± 4.2

Tobacco leaf extract:

The addition of tobacco leaf extract, in suspending *Agrobacterium* before co-cultivation that is when the overnight grown *Agrobacterium* culture reaches the OD~0.5 has favoured the shoot response after transformation, which was upto 84% (**Table 4.B.1**).

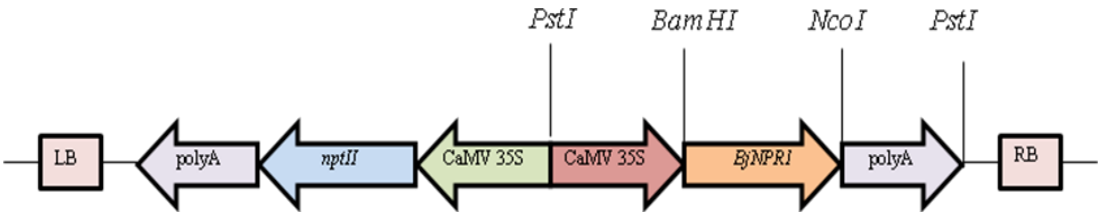


Figure 4.B.4. Construct map of the pCAMBIA2300 with *BjNPR1* and marker gene, *npt*II driven by 35S promoter and t-nos terminator separately.

Hence, with above experiments different factors were optimized for mungbean transformation. Co-cultivation were carried out using the *Agrobacterium tumefaciens* strain LBA4404 harboring the binary vector pCAMBIA2300 with *BjNPR1* under 35S promoter and polyA signal or t-nos terminator from pRT100 as shown in the **Fig. 4.B.4**. It has the *npt*II gene as selection marker.

Molecular analysis:

PCR analysis of T_0 putative transgenic plants:

T_0 plants were raised by using the standard regeneration protocol in the presence of selection antibiotics kanamycin 100 mg l^{-1}. The putative transgenic plants were confirmed through PCR using the marker (*npt*II) gene specific primers as per the protocol mentioned in the materials and methods. Genomic DNA was isolated and used for PCR analysis; the amplification is expected to give a 700 bp specific for *npt*II marker gene (**Fig. 4.B.6**). Out of 73 plants (**Table 4.B.2**) 15 plants were identified as putatively positive transgenic plants from the overall experiments, i.e., three set of experiments with 100 explants each. The PCR confirmed plants were analyzed for other molecular confirmations. At

maturity, seeds were collected from the T_0 plants to raise the T_1 generation plants for further molecular analysis.

The following **Fig. 4.B.5** shows the different stages of regeneration after transformation.

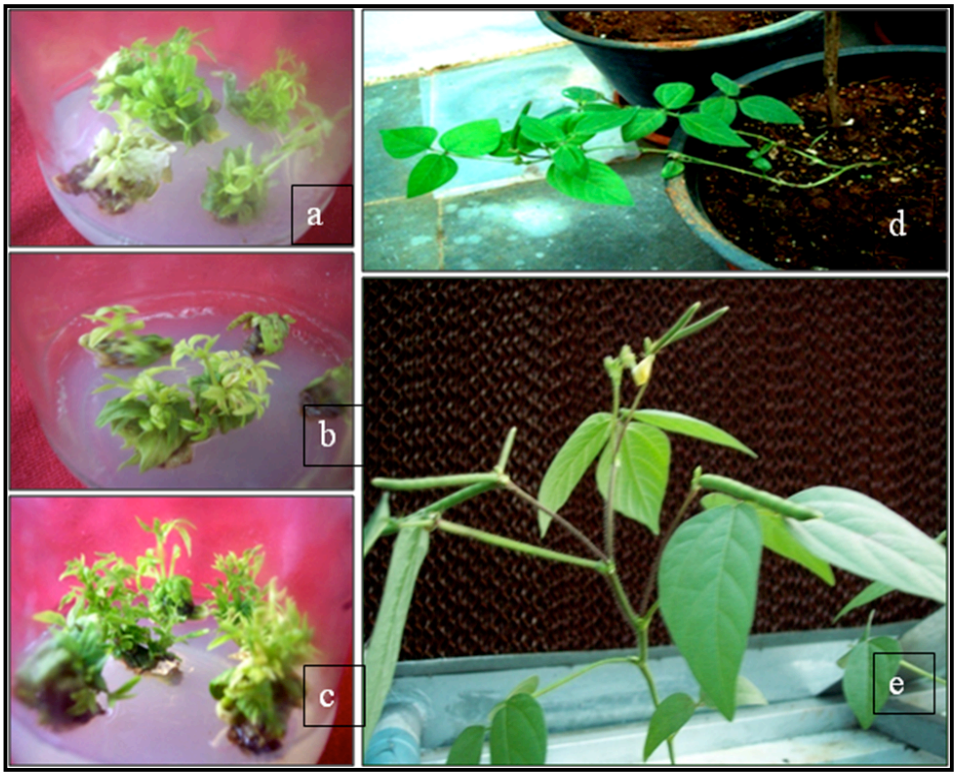


Figure 4.B.5. Different stages of regeneration after transformation with *BjNPR1* gene where (a), (b), (c) are elongating shoots on selection media (d) acclimatization of putative transgenics and (e) mature plant.

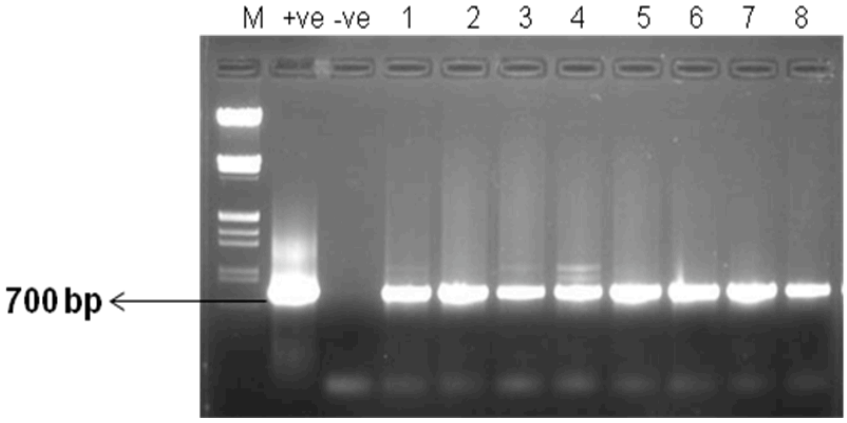


Figure 4.B.6. PCR analysis of T_0 putative transgenic mungbean plants showed amplification of 700 bp *npt*II fragment, where 1, 2, 3, 4, 5, 6, 7 and 8 are different independent plants.

Table 4.B.2. Showing the output results of the mungbean transformation

Exp. No.	No. of co-cultivated explants	No. of surviving shoots after four selection	No. of putative transformants rooted	No. of plants survived	PCR positive plants
1.	100	54	52	21	7
2.	100	61	56	29	4
3.	100	43	39	23	4

Detached leaf fungal bioassay:

Some of the T_0 PCR positive plant leaf was collected and used for fungal bioassay using the fungal pathogen *Rhizoctonia solani*. The fungus was inoculated on the leaf surface and observed the necrotic symptoms after 5 days of

post inoculation (**Fig. 4.B.7**). The plant T5 showed better tolerance and damage was only about 10% when compared to other putative transgenic plants and control. Control plants showed complete necrosis whereas the plant T2, T4, T7 and T8 showed partial necrosis and it was approximately around 35%, 20%, 40% and 27% damage of the leaves respectively and conferred tolerance towards the pathogen *R. solani*.

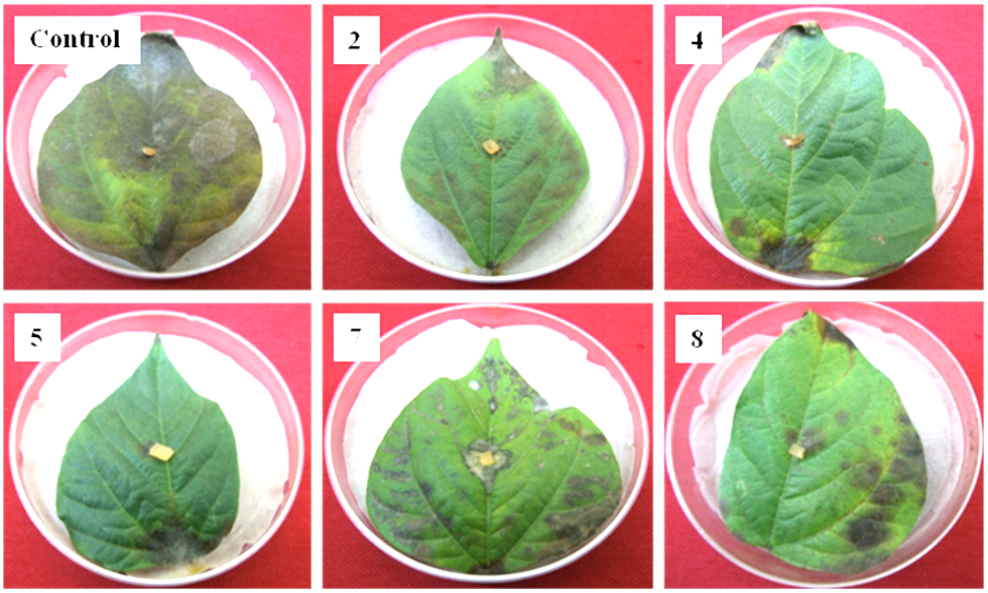


Figure 4.B.7. Detached leaf fungal bioassay using the fungal pathogen *Rhizoctonia solani* with T_0 putative transgenic plants, where 2, 4, 5, 7 and 8 are independent transgenic plants.

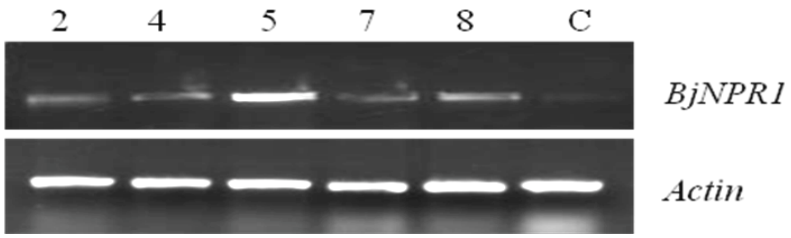


Figure 4.B.8. RT-PCR analysis using the primer for *BjNPR1* and Actin for some of T_0 putative transgenic plants, where 2, 4, 5, 7 and 8 are different plants; C is the control.

RT-PCR analysis:

The bioassay tested plant leaf was collected and used for total RNA isolation. From that cDNA was synthesized through RT and followed by PCR using the primer specific for *BjNPR1* gene and it was shown in the **Fig. 4.B.8**. The plant T5 showed high expression with other transgenic plants T2, T4, T7 and T8 showing relatively lower BjNPR1 expression, actin served as acontrol for equal loading amplification. Wild type (WT) plants did not showed any NPR1 expression, which shows that the primer used for BjNPR1 do not recognize native NPR1 of mungbean.

PCR analysis of T_1 putative transgenic plants:

T_1 plants were raised in the glass house from the T_0 seeds, genomic DNA was isolated and used for PCR analysis. Some of the selected plants gave the expected amplification at 700 bp specific for *npt*II marker (**Fig. 4.B.9**). These PCR selected T_1 plants were used for Southern analysis.

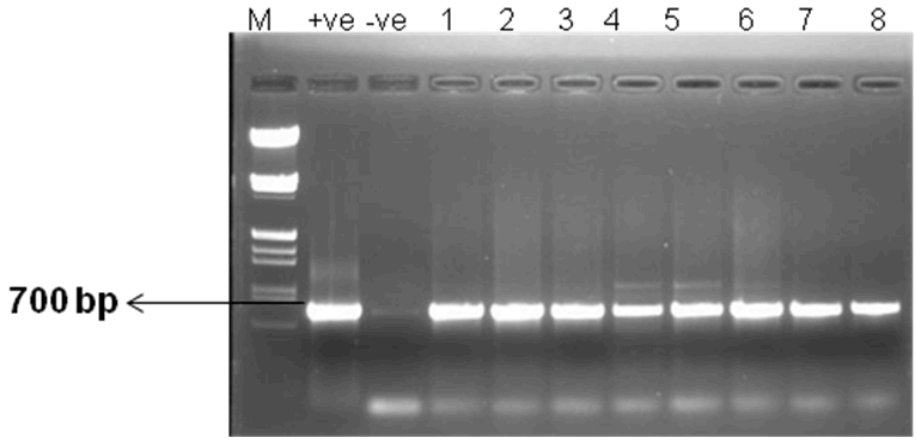


Figure 4.B.9. PCR analysis of T_1 transgenic mungbean plants showed amplification of 700 bp *npt*II fragment, where c, 1, 2, 3, 4, 5, 6, 7 and 8 are T2-1, T4-2, T5-1, T6-1, T7-1, T8-1, T21-1 and T31-1 respectively.

Southern analysis:

PCR confirmed T_1 transgenic plants were used for Southern analysis to determine the stable integration and copy number of the transgenes. Good quality of DNA was isolated from the leaf and digested with random cutter *Hind*III and then was probed with 700 bp *npt*II fragment (**Fig. 4.B.10**). In the T-DNA cassette, one internal site for *Hind*III in the MCS was present. The plant T2-1 showed single copy integration, the plant T4-2, T6-1, T7-1, T8-1 and T31-1 showed double copy whereas the plant T25-1 showed four copies of gene.

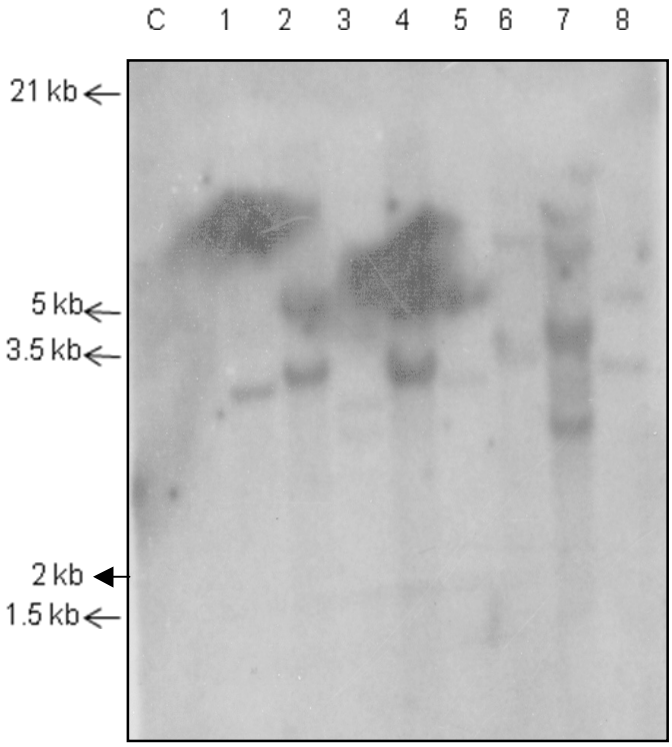


Figure 4.B.10. Southern blot analysis of T_1 transgenic mungbean plants probed with 700 bp *npt*II fragment after digestion with *Hind* III, where c, 1, 2, 3, 4, 5, 6, 7 and 8 are control, T2-1, T4-2, T5-1, T6-1, T7-1, T8-1, T21-1 and T31-1 respectively.

Discussion

Biotechnology is one of the best tools for the development of genetically modified crops in modern days when compared to conventional breeding, which requires a lot of labor work and time consuming. Among the legumes, cotyledonary node explants were best suited for regeneration as well as transformation as reported earlier by Mansur et al. (1993), Sharma and Anjaiah (2000), Venkatachalam et al. (2000) and Surekha et al. (2005) in peanut. Olhoft and Somers (2001) and Olhoft et al. (2001) have used cotyledonary nodes for efficient transformation in soybean. Similarly, mungbean transformation was also achieved using the cotyledonary node explants (Jaiwal et al. 2001; Sonia et al. 2007). Mungbean cotyledonary node explant produced more number of shoots (Vijayan et al. 2006) and was hence, selected for transformation. Since, the plant is recalcitrant in nature and the frequency of transformation reported was low, different factors were optimized before proceeding with transformation. The first step in that direction is to check whether the cotyledonary node explants are suitable for transformation or not. In that direction, we did the initial transformation experiments with cotyledonary nodes through *Agrobacterium tumifaciens* harboring pCAMBIA 2301 and observed the transient expression of GUS all along the explants and concluded that it is suitable for transformation. Similarly, in order to determine the efficiency and frequency of transformation, many investigations were carried out using the construct with *GUS* reporter genes, which were recently reviewed by Eapen (2008). Among the strains, investigations we carried out only with LBA4404 and GV2260, because the supervirulent strain EHA105 was observed to result in necrosis that is not condusive for regeneration after transformation for the used mungbean cultivar (ML-267) (data not shown). Among the two strains, LBA4404 was 15% more efficient when compared to GV2260 in regeneration after transformation, so the former strain were used for further studies. Similarly, it was observed in cowpea that higher transformation efficiency could be achieved with LBA4404 (64%) whereas GV2260 gave only 23% (Solleti et al. 2007). The transformation was done using the standard protocol in the present study such that cotyledonary nodes from two days old seedlings were immersed in the *Agrobacterium* suspension solution for 15 minutes and the treated explants were incubated for

co-cultivation. This is in accordance with the reports in mungbean (Jaiwal et al. 2001; Sonia et al. 2007) and also in *V. mungo* (Saini et al. 2003), because the younger explants were grow actively and their dividing cells are easily accessible to T-DNA.

Most of the legume transformation reports so far used kanamycin (*npt*II) as the selection marker gene in the construct (Eapen 2008). As the *BjNPR1* gene was cloned in the pCAMBIA2300, the selection marker used in the present study also is kanamycin. Hence, the required inhibitory concentration of kanamycin in the media was optimized. For *V. radiata* (ML-267), the required kanamycin was 100 mgl^{-1}, which was 75 mgl^{-1} in other varieties (Jaiwal et al. 2001). Light did not have any significant influence on regeneration efficiency of the explants, but in the presence of yellow light, percentage of regeneration was high (**Table 4.B.1**) when compared to other colors in the light spectrum during the co-cultivation period. And also at pH5.2, in the presence of tobacco leaf extract, the regeneration was higher when compared to other variables. Similarly, it was observed that the pH5.2 in the co-cultivation medium increased the frequency of transformation in mungbean (Sonia et al. 2007) and rice (Hei et al 1994). Hence, these results are in accordance with the variables reported for the optimization of conditions for *Agrobacterium* mediated transformation (Birch 1997). L-cystein is an unusual amino acid, which enhances the efficiency of *Agrobacterium* mediated transformation. In the presence of 200 mgl^{-1} L-cystein in the co-cultivation media appeared to be optimal for enhancing transformation resulting in high percentage of shoot regeneration, when compared to other concentrations. Similar results were observed in soybean transformation (Olhoft et al. 2001a, 2003; Olhoft and Somers 2001b). L-cysteine also increased the frequency of transformation in *Zea mays* (Frame et al. 2002). Hence all the optimized variables like pH5.2, L-cystein 200 mgl^{-1}, tobacco leaf extract and yellow light were combined into an optimized protocol for transformation was proceeded in order to increase the frequency.

After co-cultivation, the plants were raised from cultures that passed through three rounds of selection in the regeneration media containing 100 mg l^{-1} kanamycin. Elongated shoots were used for rooting. Over all 147 plants were rooted, among them 73 survived and were used for molecular analysis. Putative transgenic plants were confirmed through PCR analysis. It showed amplification

of 700 bp fragment corresponding to *npt*II gene indicating the presence of transgene in 15 plants out of 73 plants. This is probably due to the escapes in the selection media or incomplete insertion or unstable integration of transgene in the plant genome and was lost (Hess et al. 1990; Langridge et al. 1992). At maturity all the plants developed flowers and set seed normally, which was collected and used for raising the plants for next generation analysis.

Some of the putative transgenic plant leaves along with control were collected and used in detached leaf antifungal bio-assay using the fungal pathogen *Rhizoctonia solani* which causes stem rot diseases in mungbean. This showed varied level of resistance in all the plants except T5, which did not develops any disease symptoms. The intensity of fungal attack varies; this could be due to the level of expression of *BjNPR1* differs in each plant but the control was severely affected. This is in accordance with the transgenic papaya plants constitutively expressing *DmAMP1* showing varied level of resistance against the fungus *Phytophthora palmira* (Zhu et al. 2007) and transgenic tobacco overexpressing *BjD1* showing resistance against the fungal pathogen *P. parasitica* and *Fusarium moniliforme* (Anuradha et al. 2008). These plants were used for RT-PCR analysis in order to know the level of expression in each plant. It was observed that the plant T5 has high *BjNPR1* expression, whereas the other plants such as T2, T4, T7 and T8 showed its expression to a comparatively lesser extent. Hence, the symptoms also varied according to the level of expression of *NPR1*. Similarly it was showed in wheat transgenic plants overexpressing *AtNPR1* had high level of transcripts of PR protein genes analysed through RT-PCR and hence resistance towards the fungal pathogen *F. graminearum* (Makandar et al. 2008). NPR1 is a SAR regulator known to enhance the expression of the pathogenesis related proteins. The expression of PR1 is need as a marker for NPR1 expression. However, since there are no sequences of PR protein genes related to mungbean in the GenBank, we coud not showed the expression of PR1 related to NPR1 expression.

At maturity, the seeds were collected and used for raising plants for T_1 generation analysis. The seeds were germinated into plants and leaves were collected for genomic DNA isolation, which was used for PCR analysis. Through PCR analysis some plants were selected and Southern hybridization was carried

out using *npt*II probe to determine the copy number and stable integration of transgene within the plant genome. The plant T2-1 showed single copy integration whereas the plant T4-2, T6-1, T7-1, T8-1 and T31-1 showed double copy and the plant T25-1 showed four copies of transgene.

In conclusion, we have developed a simple and reliable regeneration and also transformation protocol in mungbean using the gene *BjNPR1*. The *Agrobacterium tumifaciens* strain LBA4404 appeared to be a better and other factors such as pH 5.2, yellow light, L-cystein 200 mgl^{-1}, tobacco leaf extract positively affected the efficiency of regeneration after transformation. After three rounds of selection on 100 mgl^{-1} kanamycin, putative transformed plants were obtained. Overall, it took three months to obtain the complete plant from the co-cultivated explants and the frequency of transformation is about 0.42%. It was confirmed through PCR and then stable integration was determined by Southern hybridization in T_1 generation. The plant T5 showed high level of tolerance towards broad spectrum fungal pathogen *R. solani*. This protocol can be used for routine genetic manipulation in mungbean for manipulating any desirable agronomic traits.

Summary

In summary, we have generated transgenic mungbean with *BjNPR1*gene using the cotyledonary node explants as in other legumes. As the legume is recalcitrant in nature, the regeneration efficiency was very less from other parts of the plant; hence cotyledonary node was opted for direct shoot regeneration. We developed a simple and reliable protocol for regeneration. Among the different growth regulator 5 mg l^{-1} BAP produced maximum number of direct shoots from the cotyledonary nodes in the mungbean variety ML-267.

Before proceeding to transformation, different factors such as pH, light, addition of compounds in the co-cultivation media such as L-cystein, tobacco leaf extract was optimized for regeneration after transformation and co-cultivation. Among the two strains of *Agrobacterium tumifaciens*, LBA4404 appear to be 15% more efficient in inducing shoot regeneration than the GV2260 after transformation. And also, the explants were pre-tested for transformation efficiency using the construct pCAMBIA2301 with *GUS* gene driven by 35S promoter and observed the transient expression of GUS was analysed in the explants. Kanamycin concentration (100 mg l^{-1}) was also optimized in the selection media.

After co-cultivation, putative transgenic mungbean plants were raised and confirmed through genomic DNA PCR. Out of 147 plants, 73 survived of which and 15 plants were confirmed as PCR positive. Among the PCR positive plants, some of them were selected for detached leaf antifungal assay using the broad spectrum fungal pathogen *R. solani*. The plant T5 showed varied levels of resistance, whereas the other transgenic plants such as T2, T4, T7 and T8 showed varied levels of tolerance. This may be due to differences in transcript levels of *BjNPR1* because of position effect or copy number of transgene (Meyer 1998). At maturity, the plants produced normal flowers and set seed and the seeds were used in T_1 generation analysis. Some of the T_1 plants were confirmed through PCR and used for Southern hybridization. By Southern hybridization, stable integration and copy number were determined in some transgenic plants

It was concluded that the gene *BjNPR1,* when expressed in any heterologus system especially in crop plants such as mungbean would confer tolerance towards the attack by phytopathogens.

Chapter 5

Characterization and heterologus expression of a legume defensin, *TvDi* in tobacco for amelioration of biotic stress tolerance

Results

I. Isolation, cloning and functional characterization of the gene *TvD1*:

Cloning of *TvD1* coding region:

The cDNA amplification from total RNA after reverse transcription resulted in a 228 bp product. No amplification was observed when the total RNA was used without reverse transcription indicating that the coding region was obtained from the amplification of the reverse transcribed cDNA. Amplification using the genomic DNA also resulted in a similar sized product indicating that the gene does not contain any intron(s).

TvD1 codes for a predicted 75 amino acid containing peptide of 8.2 kDa with the first 28 amino acid serving as signal peptide and the mature peptide of about 5.2 kDa (NCBI GenBank Accession no. AY907349 with the E-value of $3.1e^{-40}$). A BLASTX search in the GenBank protein database showed that the amino acid sequence has homology with other known defensins characterized from different plants with eight-conserved cysteine residues forming four disulfide bridges. Comparison of the deduced amino acid sequence of TvD1 (**Fig. 5.I.1**) with some of the well characterized legume defensin mature peptides using alignment tool (CLUSTALW) showed that TvD1 had significant similarity to the defensins from *Cicer arietinum* (95%), *Arachis hypogaea* (93%), *Vigna radiata* defensin-2 (91%), *Medicago sativa* (87%), *Trigonella foenum-graecum* (82%), etc.

Tephrosia villosa	-KTCENLADTYRGPCFTTGSCDDHCKNKEHLLSGRCRDDFR---CWCTKRC	100
Cicer arietinum	---CENLADTYRGPCFTTGSCDDHCKNKEHLVSGRCRDDFR---CWCTKNC	95
Arachis hypogaea	-ATCENLADTYRGPCFTTGSCDDHCKNKEHLLSGRCRDDFR---CWCTRNC	93
Vigna radiata (VrD2)	-KTCENLANTYRGPCFTTGSCDDHCKNKEHLRSGRCRDDFR---CWCTRNC	91
Medicago sativa	-KTCENLADTFRGPCFTNGACDDHCKNKEHLVSGRCRDDFR---CWCTRNC	87
Trigonella foenum-graecum	-KTCENLADKYRGPCF--SGCDTHCTTKEHAVSGRCRDDFR---CWCTKRC	82
Medicago truncatula	-KTCENLADKYRGPCF--SGCDTHCTTKENAVSGRCRDDFR---CWCTKRC	80
Arachis diogoi	-ATCENLADTFRGPCFGNSNCNFHCKTKEHLLSGRCRDDFR---CWCTKRC	80
Pisum sativum	-KTCEHLADTYRGVCFTNASCDDHCKNKAHLISGTCHN-WK---CFCTQNC	69
Vigna radiata (VrD1)	-RTCMIKKEGW-GKCLIDTTCAHSCK-NRGYIGGNCKGMTR--TCYCLVNC	19

Figure 5.I.1. Comparison of mature TvD1 with other mature peptides from legume

Constitutive expression of TvD1

Using RT-PCR, the expression of *TvD1* gene was analyzed in seeds, roots, stems, young- and mature leaves, and flowers of the plant (**Fig. 5.I.2**). Interestingly, this defensin was constitutively expressed in all the organs, however, with higher levels of expression in young and mature leaves.

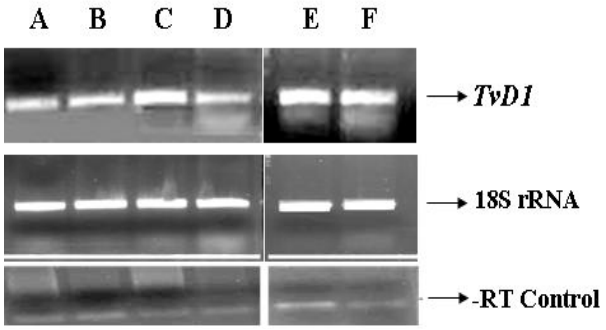
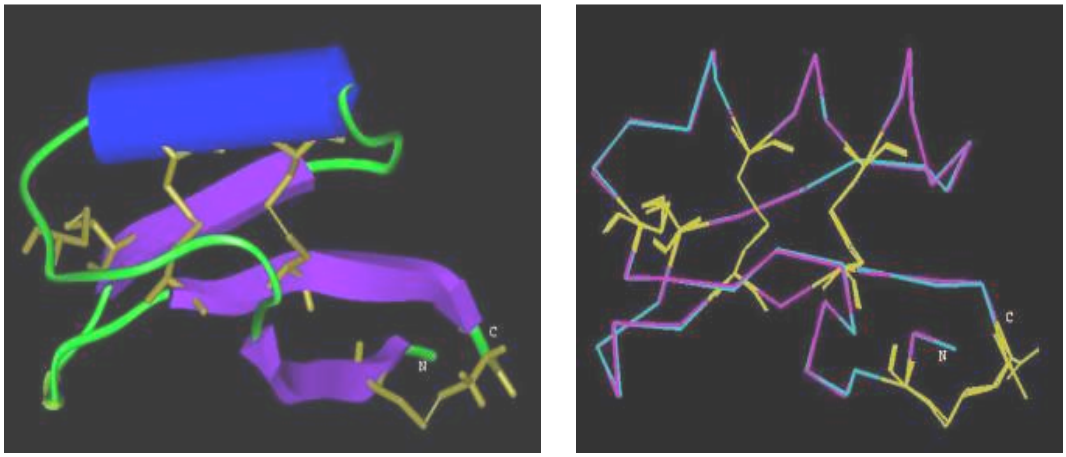


Figure 5.I.2. RT-PCR of the total RNA isolated from different tissues such as A) seed, B) root, C) stem, D) inflorescence, E) mature leaves and F) young leaves for amplifying the open reading frame (ORF) of the cDNA for studying the expression of *TvD1* in different tissues. The amplification of 18S rRNA (400 bp) was used as a control for equal loading of total RNA in the RT-PCR reactions. Minus RT control represents PCR for *TvD1* cDNA using total RNA as template without reverse transcription.

***In silico* characterization of TvD1 peptide:**

We constructed a three-dimensional structure of the mature portion of TvD1 (29-75 amino acid residue region) using standard protein modeling tools. The NMR structure (**Fig. 5.I.3**) of defensin VrD2 (PDB_ID: 2GL1) from *Vigna radiata* (Lin et al. 2007) (identified with an E value = 5e-11) shared 91% pair-wise identity with the query sequence. The pair-wise sequence alignment was used for modeling the structure of the TvD1. The overall three-dimensional fold of the model defensin comprised three β-strands and one α-helix held together with four cysteine disulphide bridges as in the case of VrD2.



(A) **(B)**

Figure 5.I.3. Homology modeling of TvD1 showed 91% similarity with that of VrD2. In the 3D model (A) the barrel-shaped blue-colored structure denotes the α-helix, pleated sheets represent β-strand and yellow-colored structures denote the cysteine bridges. In the stick model (B), the yellow colored structures represent the cysteine bridges.

Prokaryotic protein expression and purification

The rTvD1 protein from prokaryotic expression was purified and characterized further. The solubility characteristics of the recombinant protein were not largely affected either by increasing or reducing the IPTG concentration. The induction period also appeared to be crucial and maximum induction was observed after 6 h at 0.4 mM IPTG with no considerable variation afterwards. The recombinant protein had a molecular weight of approximately ~25 kDa with approximately 8.2 kDa defensin of interest and the remaining peptide is the tag region of pET32a vector (**Fig. 5.I.4**). The use of prolysis buffer has increased the protein concentration to1.8 mg/ml as against 0.7 mg/ml without this treatment. The protein concentration was observed to be similar to that of protein purified from the inclusion bodies after 10 h of IPTG induction (data not shown) using the method followed by Kirubakaran and Sakthivel (2007). This protein was used for *in vitro* antifungal assays.

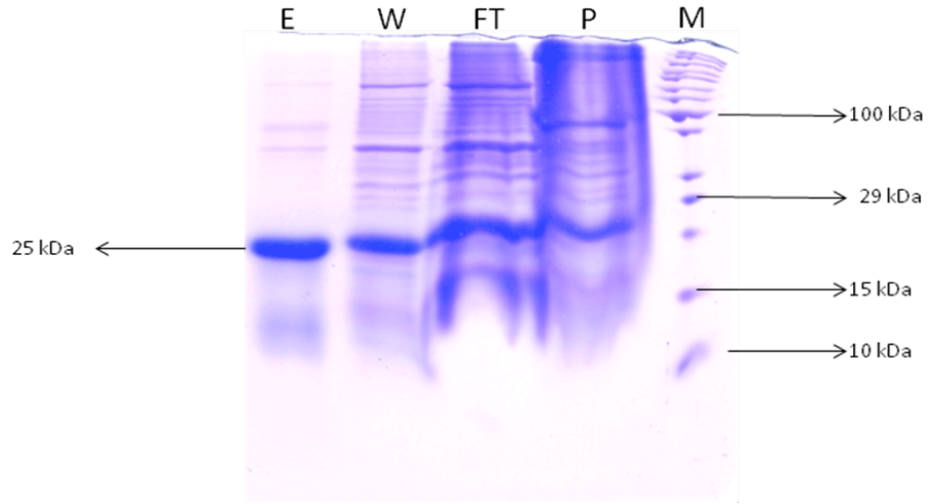
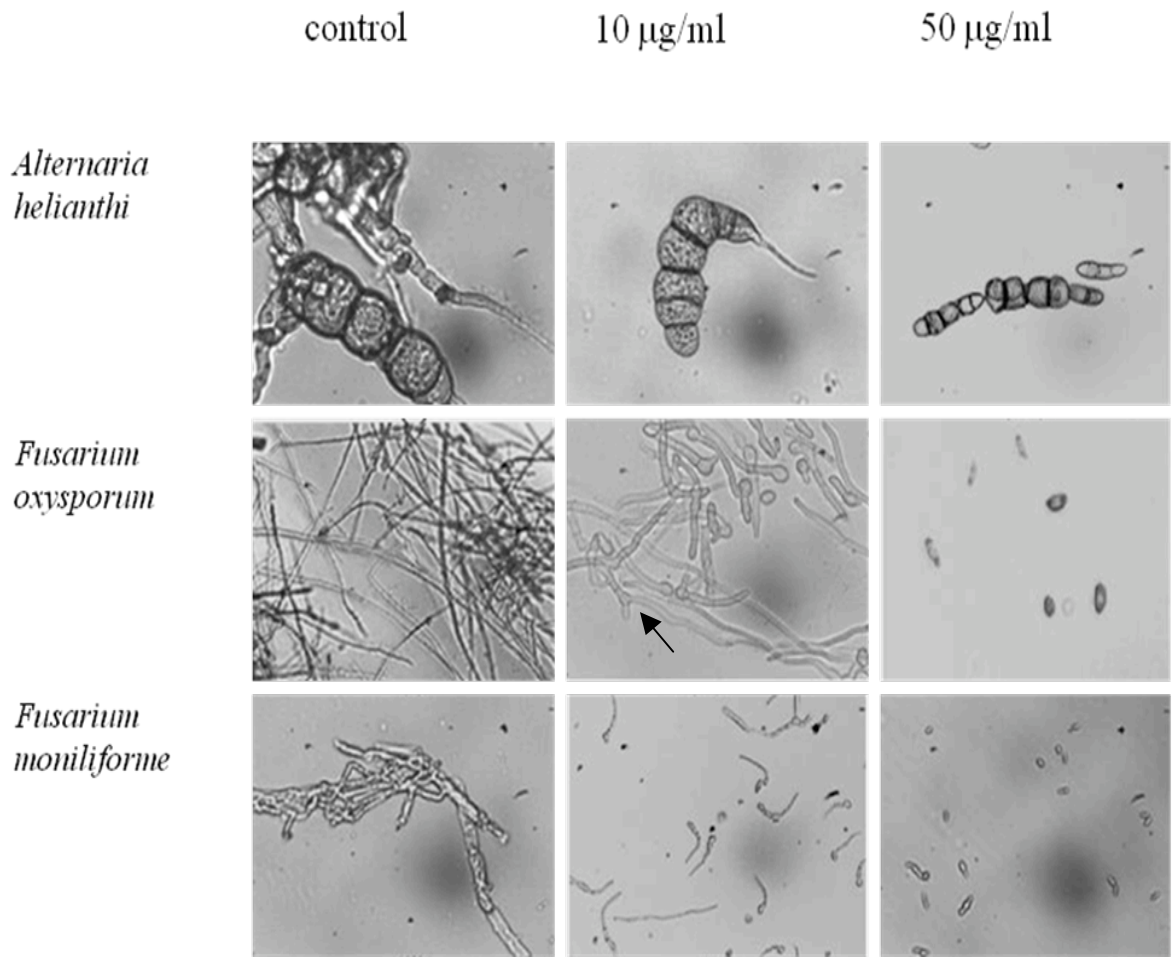


Figure 5.I.4. 15% SDS-PAGE gel showing the protein profiles after different washes and elution from the nickel NTA column. M is the marker, FT is the flow-through and E is elution of the expressed recombinant protein, respectively

In vitro antifungal assay

Antifungal activity of the rTvD1 was studied using various assays. It was observed that the antifungal activity of this protein was strongly dependent on the target fungus. Even though the peptide showed superior antifungal effect in comparison to the known defensins, the concentration at which the peptide inhibited the growth of the fungus was not uniform and varied with the fungal pathogen under consideration. In the spore germination assay (**Fig. 5.I.5**), the growth of some fungal pathogens was inhibited at very low concentrations (10-25 µg/ml) and a moderately higher concentration was needed for inhibition of other pathogens (50 µg/ml) (**Table 5.I.1**). Among the fungal species tested, the peanut late leaf spot fungus *P. personata* appeared to be the most sensitive with IC_{50} < 10 µg/ml. The antifungal activity was assessed in other fungal pathogens like *F. oxysporum*, *F. moniliforme*, *P. parasitica, B. cinerea, A. helianthi* and *Curvularia sp* with IC_{50} < 25 µg/ml. Hyperbranching of the mycelium was also observed, when the spores of *F. oxysporum* were germinated in the presence of 10 µg/ml of rTvD1 (**Fig. 5.I.5**). The growth of *R. solani* sclerotium was also tested; rTvD1 showed its detrimental effect on the growth of mycelium. Its IC_{50} value was determined as 38 µg/ml (**Fig. 5.I.6**; **Table 5.I.1**) and also it was observed that the

formation of sclerotia was greatly affected by forming a clear zone of inhibition near the disc where rTvD1 was added.



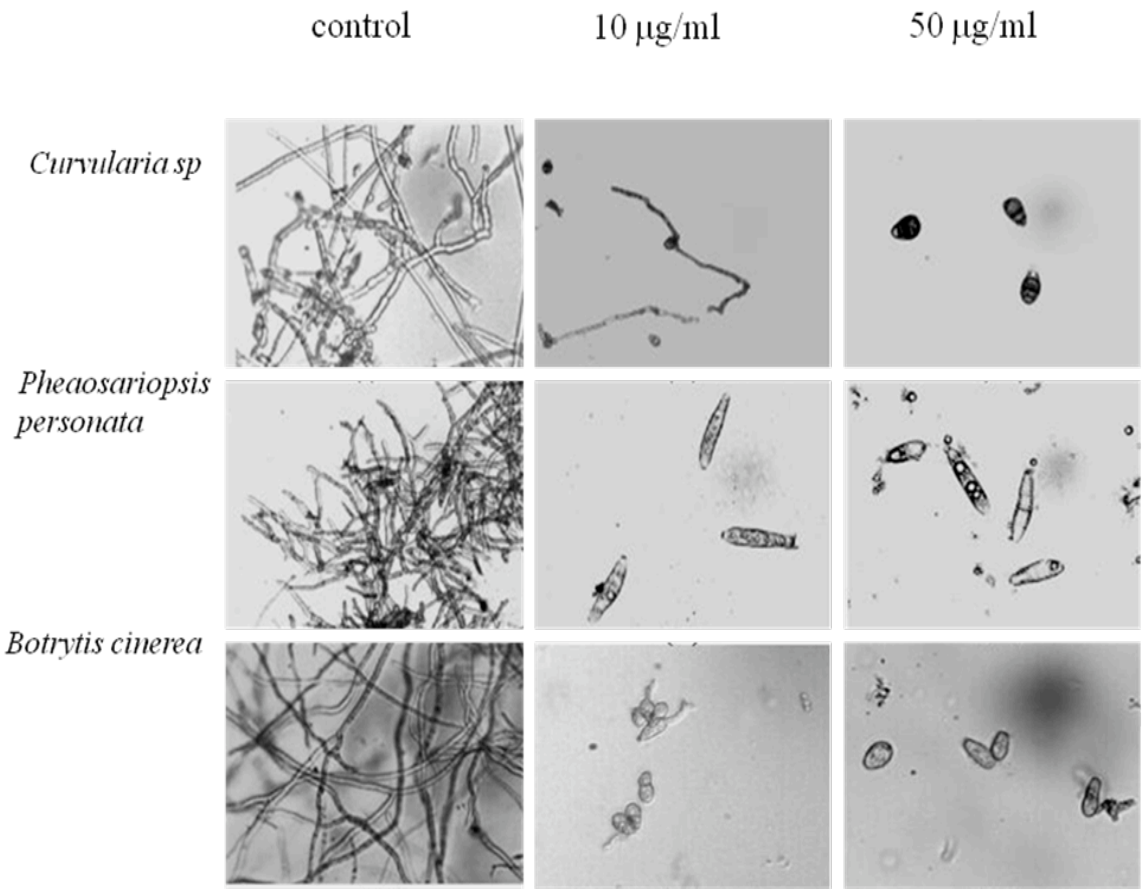


Figure 5.I.5. Spore germination assay of the fungus in the presence of different concentrations of TvD1. A) *Alternaria helianthi*, B) *Fusarium moniliforme*, C) *Fusarium oxysporum f. sp. vasinfectum* (please note hyperbranching, indicated by an arrow), D) *Curvularia sp*, E) *Pheaosariopsis personata* and F) *Botrytis cinerea*.

In the plate assays, there were varied zones of inhibition in the test fungal species depending on the peptide concentration used, but at 100 µg/ml a distinct inhibition zone was noted in all the species tested, *viz*., *R. solani*, *B. cinerea, F. moniliforme* and *P. parasitica* (**Fig. 5.I.7**).

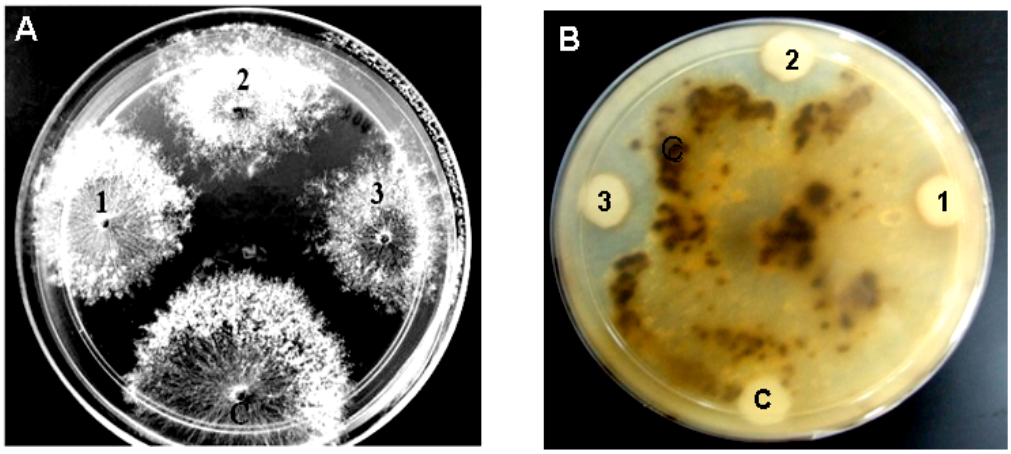


Figure 5.I.6. Effect of TvD1 on growth of *Rhizoctonia solani* sclerotia. C, 1, 2 and 3 represent control, 25 μg/ml, 50 μg/ml and 100 μg/ml, respectively. (A) Protein at respective concentration was directly added over the sclerotium and photograph was taken after 48 hours of growth and (B) Protein was added on discs at respective concentrations and formation of sclerotia was observed after three weeks.

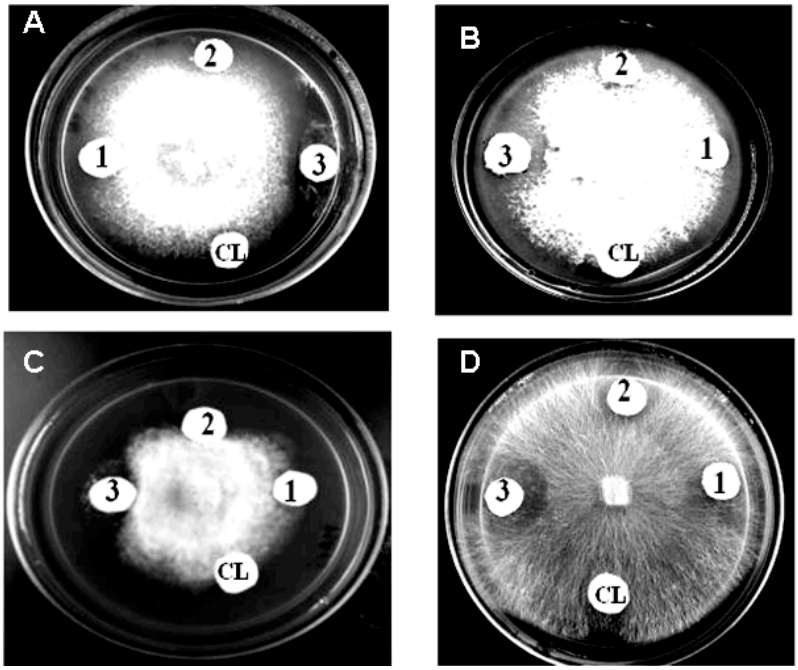


Figure 5.I.7. Effects of TvD1 defensin on growth of the fungus, where CL, 1, 2, 3 are control (Buffer), 25 μg/ml, 50 μg/ml and 100 μg/ml respectively. A) *Phytopthora parasitica f. sp. nicotianae*, B) *Fusarium moniliforme*, C) *Botrytis cinerea* and D) *Rhizoctonia solani*

Table 5.I.1. Antifungal assay with the recombinant peptide TvD1 against some fungal pathogens

Fungus	% Inhibition at different concentrations of TvD1 (µg/ml)				IC_{50}[a] µg/ml
	10	25	50	75	
Botrytis cinerea	44.16±1.3	65.08±2.1	95.45±0.4	97.70±1.7	<25
Curvularia sp	42.10±3.3	71.12±1.9	88.21±0.9	98.27±0.8	<25
Fusarium moniliforme	44.67±0.3	62.02±0.1	88.30±1.2	97.09±1.9	<25
F. oxysporum	45.50±1.0	78.60±2.2	89.50±2.4	97.83±0.1	<25
Alternaria helianthi	26.98±3.7	65.08±0.4	81.45±0.3	99.70±0.1	<25
Pheaoisariopsis personata	56.00±3.9	66.40±1.1	71.43±3.4	93.89±1.7	<10

Data represent mean ± S.D., n = 3; [a]Protein concentration required for 50% growth inhibition (IC_{50}) after 48 hour of incubation was determined from the dose-response curves (percentage growth inhibition vs. protein concentration). * represents the IC50 value from the graph plotting percentage growth inhibition vs. protein concentration for the *in vitro* plate assay.

Arabidopsis seed germination assay

We have also seen whether the rTvD1 was able to inhibit root elongation in *Arabidopsis thaliana.* Root growth in Arabidopsis was largely affected by TvD1 (**Fig. 5.I.8**), and it was inhibited in a dose-dependent manner (**Table 5.I.2**). At 10 µg/ml of recombinant TvD1, there was about 50% reduction in root length and further development was not evident. There was no root growth at concentrations of 50 µg/ml and above. It has also been noticed that the protein affected the number and extent of lateral root formation.

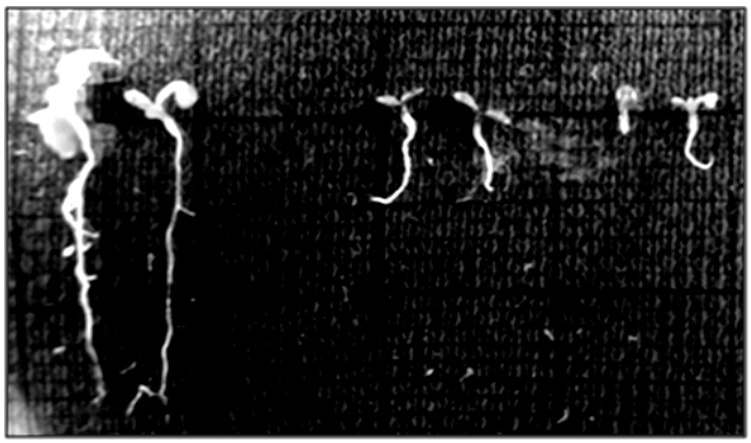


Figure 5.I.8. Seed germination assay of *Arabidopsis* showing that the growth of the roots was severely affected by rTvD1

Table 5.I.2. *Arabidopsis* seed germination assay

Sl. No.	Concentrations of the protein (µg/ml)	Length of the Root (cm)
1.	Control (buffer)	1.7 ± 0.2
2.	10	0.5 ± 0.1
3.	25	0.3 ± 0.1
4.	50	no germination

Data represent mean ± S. D., n = 3.

Discussion

Defensins are amongst the highly potent antimicrobial peptides advanced by the plant to protect itself against the invading pathogens. Hence, characterization of effective defensins from several sources is an emerging tool in plant biotechnology for enhancing disease resistance in crop plants (Osusky et al. 2000; Punja 2001).

From the pair-wise alignment it was inferred that almost all the reported legume defensins have highly conserved domains within the protein. Homology modeling of the TvD1 indicated that it has a three-dimensional structure similar to the characterized defensins like VrD2 (Lin et al. 2007) and PsD1 (Almeida et al. 2002). These secondary structural elements are held together by four disulfide bridges, thus forming a cysteine stabilized α,β-fold. This fold belongs to the Knot-1 superfamily (INTERPRO ID: IPR003614). Examination of the model structure indicated that more than 95% residues fall in the allowed regions of the phi-psi Ramachandran map indicating the confidence measure of the 3-D with a score of 17.8/21. The C-alpha traces of template and target structures were superimposed with 0.15 Å RMSD indicating high conservation in their structures. Two conserved regions distinguish plant defensins with or without antifungal activity (Almeida et al. 2002). From the alignment, it was observed that His residue at position 29 in Psd1 is also conserved in TvD1 and the conserved Phe residue at position 41 is conservatively mutated to Trp. These structural features indicate that TvD1 would also exhibit strong antifungal activity similar to Psd1.The phylogenic tree indicated that TvD1 showed a common evolutionary origin based on conserved sequence and structural characteristics such as amino acid homology and conserved motifs from the legumes.

TvD1 is expressed constitutively to high levels in all the tissues of the plant, namely seed, leaf, root, stem and flower. A similar constitutive expression of the defensin PDF2.3 was also observed in *Arabidopsis* in all organs except in roots (Manners et al. 1998). But, the latter protein was not shown to exhibit any antifungal activity.

The presence of a typical secretory signal peptide is one of the characteristic features of plant defensins (Broekaert et al. 1997). Xu and Reddy (1997) reported that bacteria failed to produce the pre-protein of a PR5-like protein of *Arabidopsis*, because its N-terminus affected *E. coli* growth. Similarly, no fusion protein could be induced under the same conditions, when the *Trichosanthes kirilowii* defensin (TDEF1) gene with its signal peptide-coding region was inserted into pET32a(+). Therefore, the partial TDEF1 cDNA, corresponding to the mature peptide, was inserted into the expression vector, and TDEF1 was produced as the fusion protein in *E. coli* without the N- terminal signal. However, the antifungal activity of the expressed protein was very low, requiring a dose up to 250 μg ml^{-1} of TDEF1 to have an effect on *F. oxysporum* (Hui et al. 2007). In the present study, the *E. coli* expressed TvD1 defensin protein with its signal peptide was purified efficiently as a recombinant protein with significant antifungal activity at comparatively lower concentrations compared to TDEF.

The assay of antifungal activity of the rTvD1 has shown that the 100 μg ml^{-1} concentration was sufficient to form an inhibitory zone for all the tested fungal pathogens. It was 100 and 150 μg ml^{-1} for Tfgd1 and Tfgd2 defensins respectively from *Trigonella foenum-graecum* against *R. solani* and *F. moniliforme* (Olli and Kirti 2006; Olli et al. 2007). The IC_{50} value of Lm-Def against *P. infestans* was observed to be 100 μg ml^{-1} (Solis et al. 2007). The other *E. coli* expressed PR proteins such as a chitinase from sorghum required higher concentration such as 300 μg ml^{-1} against several fungal species viz., *B. cinerea, R. solani, A. alternata, F. oxysporum* and wheat chitinase against *Colletotrichum falcatum, Pestalotia theae, R. solani, Sarocladium oryzae, Alternaria sp.* and *Fusarium sp.*(Kirubakaran and Sakthivel 2007; Singh et al. 2007).

In order to investigate the effect of recombinant TvD1 on fungal spore germination, we have tested some filamentous fungal pathogens. The conidia of *P. personata* failed to germinate at 10 μg ml^{-1} concentration. It was observed that 100 μg ml^{-1} concentration of the recombinant protein (Tfgd1) was an inhibitory factor for spore germination and hyphal growth even after 48h for the conidia of

P. personata (Olli and Kirti 2006).

However, similar growth with slight inhibitory effect was observed in the case of *F. oxysporum, B. cinerea, F. moniliforme,* A. *helianthi* and *Curvularia sp.* at 10 μg ml^{-1} of TvD1, but slightly higher concentrations were needed for 50% inhibition. The IC_{50} value of *R. solani* was 38 μg ml^{-1} and growth of sclerotium was distinctly arrested at higher concentration.

Generally, defensins inhibit fungal growth by inducing hyperbranching (Brassicaceae) or the growth was arrested without hyperbranching (Fabaceae, Asteraceae and Hippocastanaceae) (Osborn et al. 1995). Also it depended upon the fungal species tested (Spelbrink et al. 2004). Our studies showed that the defensin TvD1 from a member of Fabaceae, apart from inhibitory activity in spore germination also induced morphogenic changes like hyperbranching and changes in cell wall morphology in the fungal pathogens like *F. oxysporum* and *F. moniliforme.* Such an activity was similar to Rs-AFP1 and Rs-AFP2 (Terras et al. 1995) and HaDef1 (Zélicourt et al. 2007) indicating that the TvD1 possesses both types of antifungal activities.

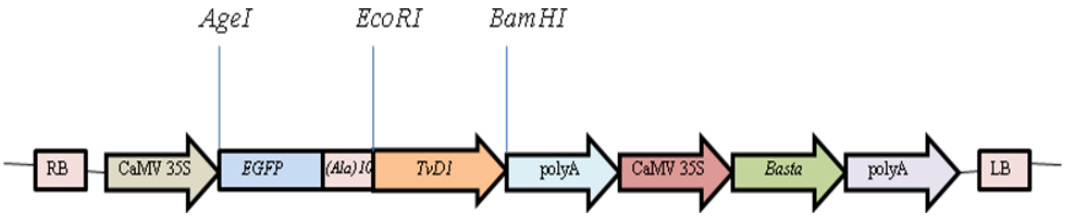
As in fungal hyphae, the root hair tip growth is also associated with an apex-high cytosolic free calcium gradient generated by a local calcium influx at the tip (Schiefelbein et al. 1992; Felle et al. 1997). TvD1 was able to inhibit root elongation and lateral root formation at 10 μg ml^{-1}. The effect was more prominent at higher concentrations. Interestingly, this effect was observed at approximately the same concentration that was necessary for antifungal activity in some species. Antifungal defensins like MsDef1, MtDef2, RsAFP2 and KP4 also blocked the whole root development causing rapid RabA4b depolarization and inhibited the extension of growing root hairs (Allen et al. 2007). Hence, the defensin appears to possess the potential to control the development and growth of the plant on external application.

Defensins with antimicrobial activity are potent candidate genes for deployment in transgenic crops for protecting them against pathogens. With the inhibitory concentration as low as 10 μg ml^{-1} against some fungal pathogens, *TvD1* appears to be a potent defensin gene for fungal disease resistance in transgenic crops.

II. Sub-cellular localization of TvD1 within the plant

Construct preparation:

Since, the peptide is secretary in nature, and to determine the localization within the cell, the ORF of the gene was cloned downstream of the *GFP* at *Eco*RI and *Bam*HI site in the binary vector pEGAD for plant transformation. The *GFP-TvD1* fusion gene was driven by 35S promoter and t-nos terminator. The plant selection marker gene in the construct is *basta* and it is also driven by 35S promoter and t-nos terminator (**Fig. 5.II.1**).



pEGAD vector

The vector was mobilized into *Agrobacterium tumifaciens* strain LBA4404 through freeze thaw method as per the protocol mentioned in the materials and methods. The transformed bacteria were characterized for the presence of the recombinant pEGAD vector and subsequently used for plant transformation.

Tobacco transformation:

The leaf discs of tobacco plants were used for transformation and putative transformants were raised along with non-transformant plants using the standard tobacco transformation protocol (Horshe et al. 1985). After transformation, the explants were kept on MS medium supplemented with 2 mg l^{-1} BAP and 0.1 mg l^{-1} NAA and 250 mg l^{-1} cefotaxime without any selection antibiotics in the media. Shoots were obtained about a month after initiation of the transformation event. Rooting occurred when the shoots transferred to the growth regulator free rooting

media, either full strength or ½ strength MS mediu m and root formation occurred in two weeks (**Fig. 5.II.2**). Rooted plantlets were hardened in a mixture of sterile vermiculite and soil (3:1).

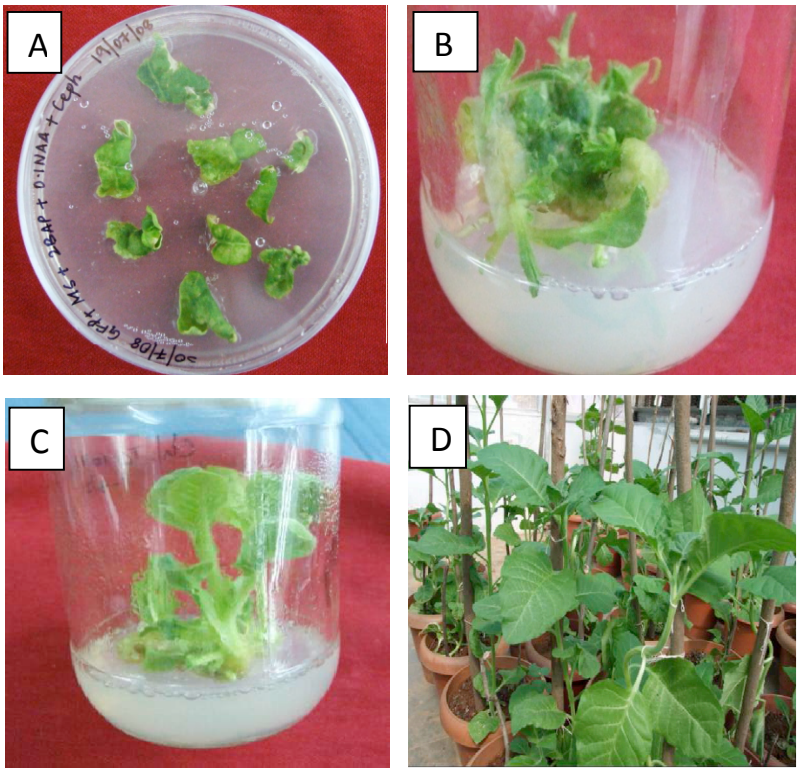


Figure 5.II.2. Different stages of tobacco regeneration after transformation. (A) Explants under co-cultivation, (B) Initiation of shoot buds, (C) Rooted plantlet and (D) Hardening of plants in the glass house.

Screening of putative transgenic plants:

Initially, about 50 plants were transferred to vermiculite: soil mixture for acclimatization. Since the plant selection marker is basta resistance, leaf discs from the acclimatized plants were used for screening the putative tobacco transgenics by incubating in 0.2% aqueous solution basta for 3 days (**Fig. 5.II.3**).

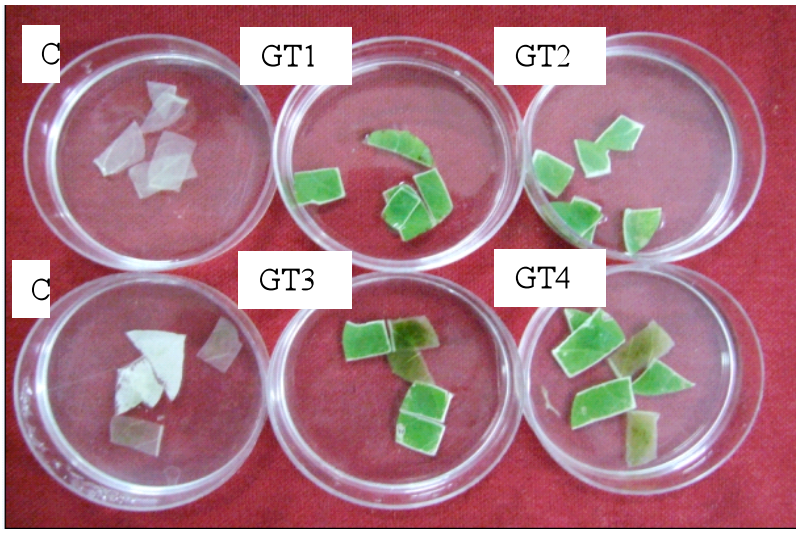


Figure 5.II.3. The screening of the putative transgenics for the presence of basta resistance. The control (C) leaf sample showed severe bleaching after 3 days of incubation whereas the putative transgenic samples such as GT1, GT2, GT3 and GT4 remained green even after 72 hours or beyond.

The untransformed plant leaf discs bleached completely and putative transformed plants such as GT1, GT2, GT3 and GT4 leaf discs were green after 3 days and beyond. These plants were analyzed for further experiments. The selected plants were transferred to soil for hardening and acclimatization. The leaves of the transgenic plants were observed under the confocal microscope for the GFP localization. From the microscope observations, it was concluded that the GFP tagged TvD1 protein was localized in the apoplastic region and it was not the case for the GFP control which showed cytosolic localisation (**Fig. 5.11.4**). Hence, it is evident that the protein is present in the apoplastic region and help in preventing the entry of fungal pathogen into the cells as well as haustoria formation into the cell.

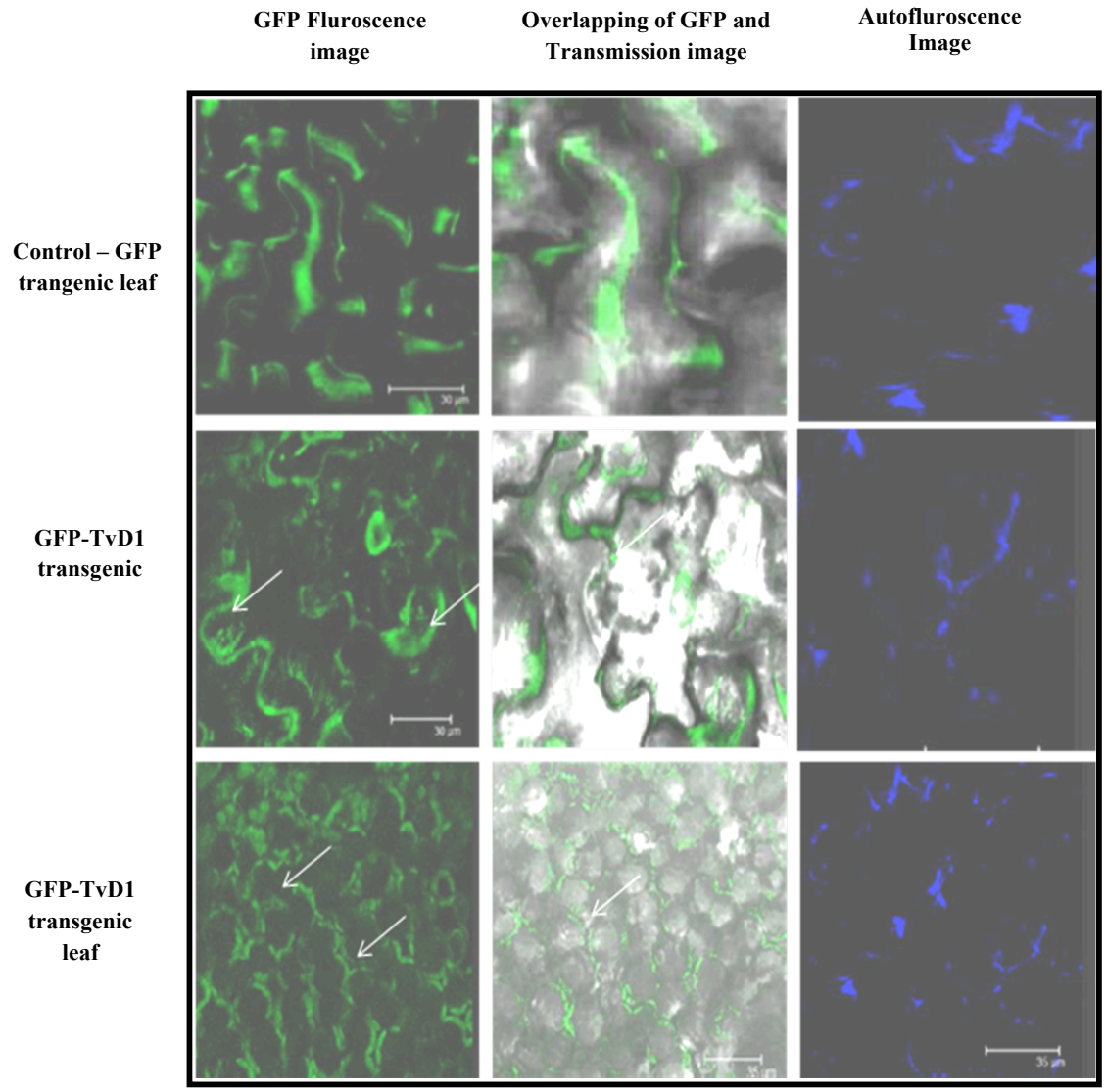


Figure 5.II.4. Confocal images showing GFP fluorescence in the cells below the epidermal layer in putative transgenic sample

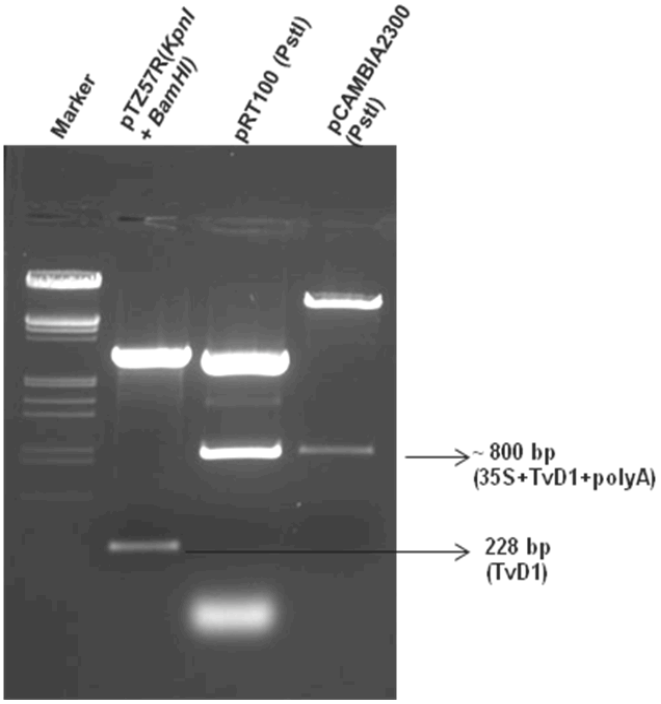
Discussion

In order to determine the sub-cellular localization of TvD1, the ORF of the cDNA of *TvD1* was cloned in the binary vector pEGAD at the *Eco*RI and *Bam*HI in a translational fusion site. The GFP at the N-terminal region and the fusion protein coding gene driven by the 35S promoter and t-nos terminator. In general, for subcellular localization studies, onion peels are used and the transient transformation is performed through biolistic methods (Scott et al. 1999). However, we attempted to develop transgenic plants using *Agrobacterium* mediated transformation of tobacco leaf discs. For achieving this, the binary vector with fusion gene insert was then mobilized into the *Agrobacterium tumifaciens* strain LBA4404. Transformation was done using the tobacco leaf explants (Horch et al. 1985). After transformation, putative transgenic plants were raised in the glass house and the leaf discs of some of the plants were screened through senescence assay in the presence of 0.2% basta. Identified putative transgenic plants were used in confocal microscopy analysis for confirming the GFP localization. Since GFP is involved in the fusion with the TvD1, its localization would confirm the actual localization of the TvD1 protein.

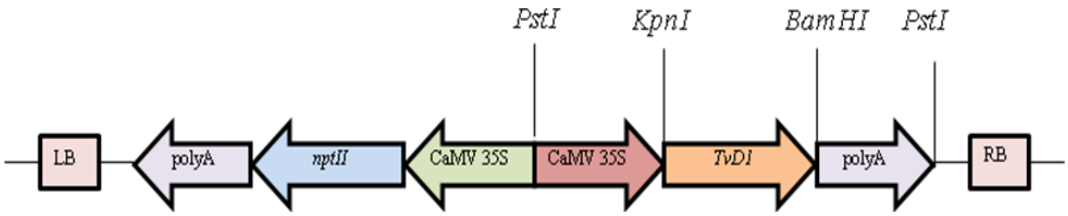
Some of the putative transgenic plants were used for confocal microscopy to determine the subcellular localization of GFP-TvD1 fusion peptide along with the control GFP putative transformants. From these observations, it could be confirmed that the peptide TvD1 appears as secretary and is targeted towards the apoplastic region. Hence, it can form the front line of host defense against the impeding pathogen challenge. It was also shown that the signal peptide of Vv-AMP1, a ripening induced peptide from *Vitis vinifera* cloned with GFP as a fusion in the binary vector pART27 and transformation was done, when the peptide was clearly localized in the apoplastic region of the tissue (Beer and Vivier 2008). Hence from the present experiment, it can be concluded that the presence of TvD1 in the apoplastic region could have a possible role in defence against the pathogens especially against the fungus as a front line defense.

III. Cloning and transformation of *TvD1* in tobacco for *in vivo* characterization

From the previous chapter it was concluded that the recombinant peptide TvD1 has potent activity against many soil borne fungal pathogens. Hence, the cDNA of *TvD1* was cloned in between the *Eco*RI and *Bam*HI sites in the plant expression cassette in pRT100 vector flanked by 35S promoter and polyA signal on either side. The cloned fragment *TvD1* was released from the vector using the enzyme *Pst*I and further cloned in the binary vector pCAMBIA2300 (**Fig. 5.III.1A& 5.III.B**) with the same site for plant transformation with the marker gene *npt*II driven by 35S promoter with t-nos terminator. The pCAMBIA2300 harboring the gene *TvD1* was mobilized into the *Agrobacterium tumifaciens* strain LBA4404 using the freeze thaw method and used for plant transformation.



(A)



(B)

Figure 5.III.1. Preparation of construct for plant transformation. **(A)** Gel picture showing the release of 228 bp *TvD1* fragment from pTZ57R, cloned in pRT100 vector and it released the ~ 800 bp TvD1 cassettes with 35S promoter and polyA signal and same from the pCAMBIA2300. **(B)** Pictorial representation of pCAMBIA2300 vector with *TvD1* gene

Tobacco transformation and regeneration:

Tobacco transformation was done using the leaf explants as per the standard protocol (Horsh et al. 1985). After transformation, the explants were kept on MS medium supplemented with 2 mg l^{-1} BAP and 0.1 mg l^{-1} NAA with 125 mg l^{-1} kanamycin and 250 mg l^{-1} cefotaxime. Shoots were obtained about a month after the transformation event. After their transfer to root inducing media i.e., hormone free media either full strength or ½ strength MS medium, rooting was observed in two weeks (**Fig. 5.III.2**). Rooted plantlets were hardened in a mixture of sterile vermiculite and soil (3:1). They were covered with polythene bags to avoid desiccation and to allow proper cuticle formation for proper acclimatization.

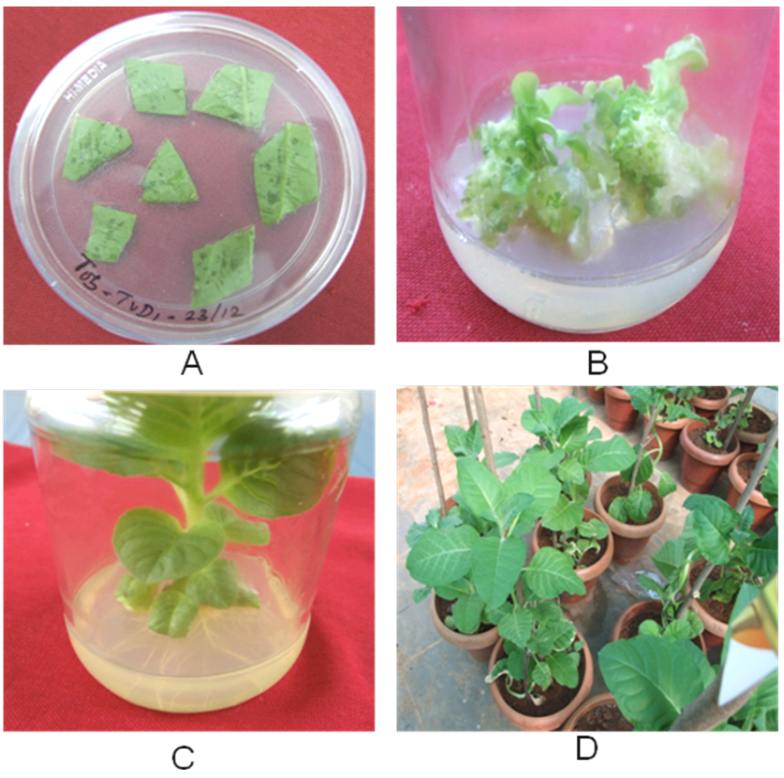


Figure 5.III.2. Different stages of tobacco regeneration and transformation. (A) Explants under co-cultivation, (B) Initiation of shoot buds, (C) Rooted plantlet in culture and (D) Hardening of plants in the glass house.

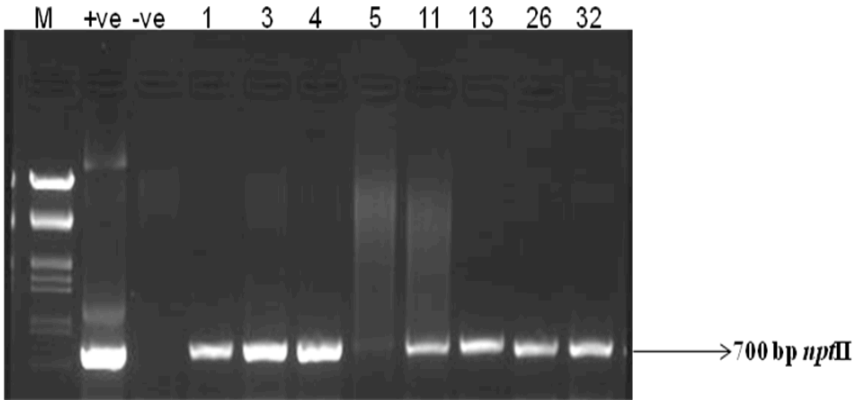


Figure 5.III.3. PCR analysis of the T_0 putative transgenic plants showing the amplification of 700 bp *npt*II marker gene, where M, +ve, -ve, 1, 3, 4, 5, 11, 13, 26, 32 are marker, positive control, wild type (negative control) and different putative transgenic plants.

About 32, T_0 putative transgenic plants were raised after different stages of tissue culture selection (**Fig. 5.III.2**) and they were confirmed through PCR. Genomic DNA was isolated from some of the plants and used for PCR using the primer for the marker gene (*npt*II). This analysis showed the expected amplification of 700 bp *npt*II marker gene (**Fig. 5.III.3)**.

Expression analysis of the T_0 putative transgenic plants:

The PCR confirmed plants were selected for expression analysis through RT-PCR. The total RNA was isolated from nine independent plants and RT was performed followed by PCR using the primers specific for *TvD1*. It was showed that out of 9 plants selected for analysis, 2 plants such as T4 and T13 showed low expression whereas the other plants such as T1, T3, T5, T6, T11, T26 and T32 have high expression of *TvD1 in vivo*. Amplification of actin was used as the control for equal loading in the RT-PCR (**Fig. 5.III.4**).

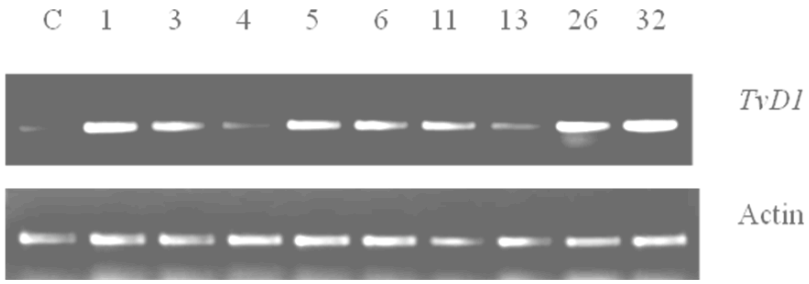


Figure 5.III.4. RT-PCR analysis of T_0 putative transgenic plants using the primers specific for *TvD1* and *actin*, where c is the control and 1, 3, 4, 5, 6, 11, 13, 26 and 32 are independent putative transgenic plants.

Detached leaf antifungal bio-assay:

The tobacco transgenic plants such as T1 and T13 were selected as high and low expression plants respectively along with wild type control and used for detached leaf antifungal bio-assay using the fungal pathogens, *Phytopthora parasitica p.v. nicotiana*, which is specific for tobacco and *Rhizotonia solani.*

The high expression plant T1 showed very limited necrosis that is 7.5% damage whereas the low expression plant (T13) as well as control plants showed more than 90% damage across the leaf within 5 days of fungal disc inoculation over the leaf (**Fig. 5.III.5A**) with *P. parasitica p.v. nicotiana*.

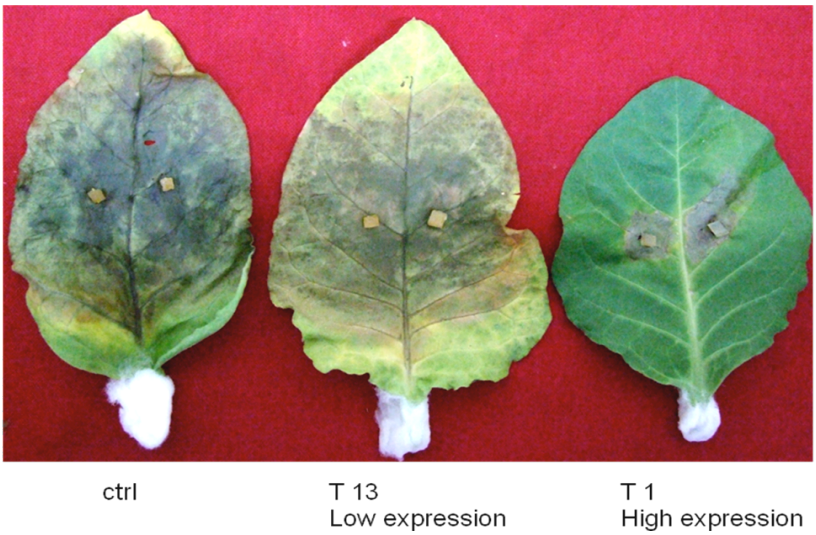


Figure 5.III.5A. Detached leaf fungal bioassay using the fungus, *Phytopthora parasitica* (Photograph was taken after 5 days post inoculation).

Similarly for the fungus *R. solani*, the necrosis was prominent in the control plant which showed 39% damage of the leaf after five days of post inoculation. It was limited in the low expression plant T13 showed less than 7% damage. But it was controlled in the high expression plant (T1) with less than 2.5% damage (**Fig. 5.III.5B**).

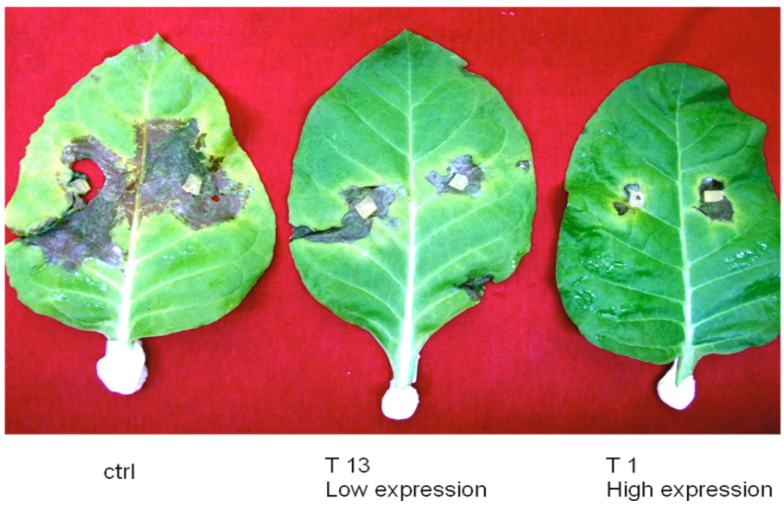


Figure 5.III.5B. Detached leaf fungal bioassay using the fungus *Rhizoctonia solani* (Photograph was taken after 5 days post inoculation).

T_1 generation molecular analysis:

PCR analysis:

The high expression plants such as T1, T26 and T32 as well low expression plant T13 seeds were collected and used for T_1 generation analysis. The transgenic plants were selected by growing the seedlings in the ½ MS media containing 125 mg^{-1} kanamycin for 15 days. Then green seedlings were selected and transferred to the pots for further growth and maturity. These T_1 generation seedlings were used for molecular analysis. Prior to Southern analysis, the transgenic plants were confirmed through PCR for the marker gene (**Fig. 5.III.6**).

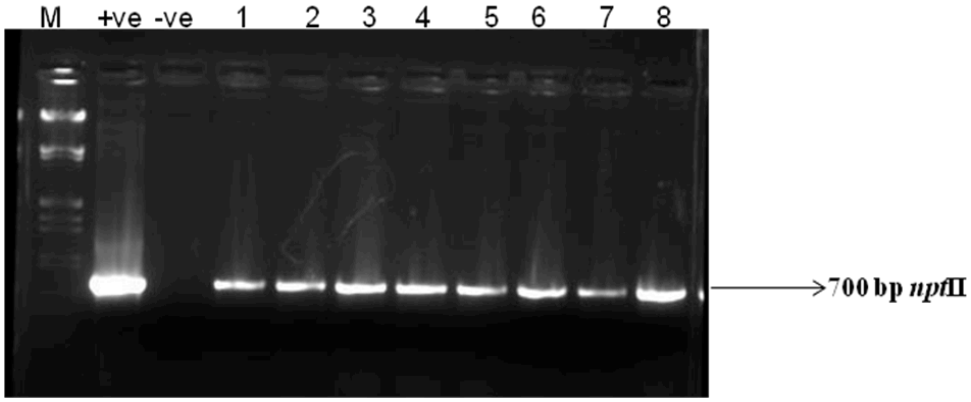


Figure 5.III.6. PCR for the T_1 transgenic plants showing the amplification of 700 bp *npt*II marker gene, where M, +ve, -ve, 1, 2, 3, 4, 5, 6, 7, 8 are marker, positive control, negative control, 1-1, 1-2, 13-1, 13-2, 26-1, 26-2, 32-1 and 32-2 respectively.

Southern analysis:

The genomic DNA was isolated from two plants of each in high expression as well as low expression plants along with wild type control plants and used for Southern analysis to check the stable integration and copy numbers of the transgene in the selected plants. The Southern analysis was done with the genomic DNA of the above mentioned plants after restriction digestion with random cutters such as *Eco*RI, which did not have any site in the T-DNA. After blotting, the membrane was probed with the PCR amplified fragment of *npt*II marker gene as a probe. Different copy numbers were observed in the transgenic plants ranging from single copy to multiple copies **(Fig 5.III.7)**. It was a single

copy integration in the plant T26 (T26-1 and T26-2), two copies in the plant T1 (T1-1 and T1-2), four copies in the plant T32 (T32-1 and T32-2) and it was multiple copy number in the plant T13 (T13-1 and T13-2).

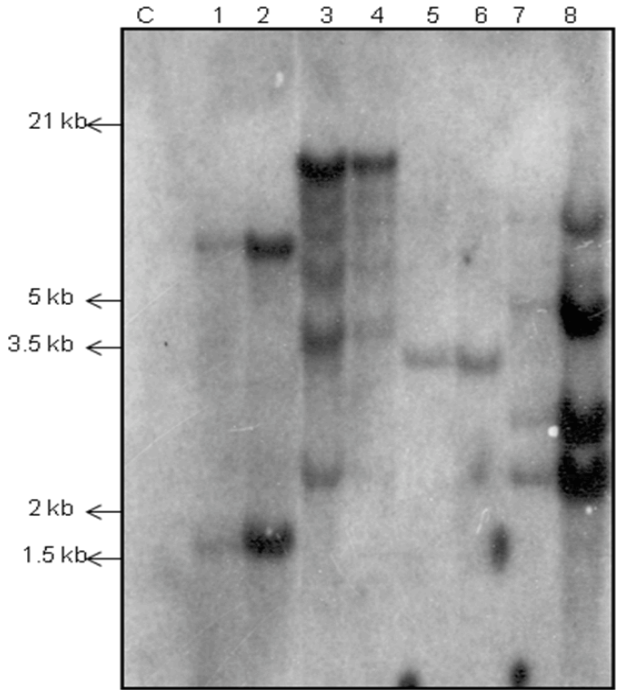


Figure 5.III.7. Southern blot analysis of the T_1 generation transgenic plants after restriction digestion with *Eco*RI and probed with *npt*II marker gene where, C 1, 2, 3, 4, 5, 6, 7, 8 are control, T1-1, T1-2, T13-1, T13-2, T26-1, T26-2, T32-1 and T32-2 respectively.PCR amplified *npt*II fragment was used as a probe.

Expression analysis of the T_1 transgenic plants:

Two progeny plants of each high expression plant such as T1, T26, T32 and low expression plant T13 were used for expression analysis through RT-PCR (**Fig. 5.III.8)**. It showed that high expression plant such T1-1, T1-2, T26-1, T26-2, T32-1, T32-2 evident high expression as in T_0 studies. Similarly, the low expression plants such as T13-1 and T13-2 showed low level of expression. The amplification of actin was used as a control for equal loading of RNA.

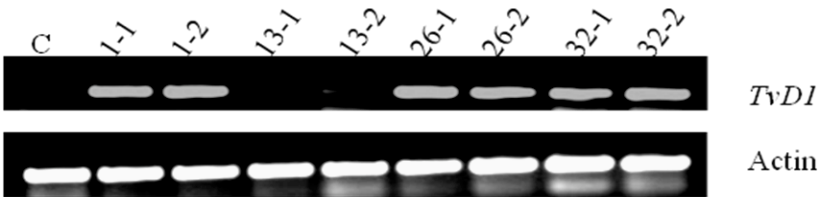


Figure 5.III.8. RT-PCR analysis of T_1 transgenic plants using the primers specific for *TvD1* and *actin*, where c is the control, 1-1, 1-2, 26-1, 26-2, 32-1 and 32-2 are progeny plants of high expression plants and 13-1 and 13-2 are from low expression plants.

Antifungal bioassay:

The high expression and low expression plants were used for whole plant antifungal bioassay along with wild type control plant using the fungal pathogen *R. solani*. The sclerotia of the fungus were inoculated on to the 30 day old plants from the high expression plant T1 and low expression plant T13 along with wild type control. The control and low expression plants T13 showed leaf wilting symptoms within 5 days of post inoculation (**Fig. 5.III.9**). The symptoms starts from base of the plant i.e., older leaves gets affected early, whereas the high expression line persists without any external symptoms.

Control **T13** **T1**

Figure 5.III.9. Whole plant fungal bioassay (*Rhizoctonia solani*) for the 30 days old plant (Photograph was taken after 5th day of post fungal inoculation)

Anti-insect bioassay:

The high expression (T1-1) and low expression (T13-1) plants in T_1 generation plants were tested for insect herbivory using the 1st and 2nd instar larvae of the generalist herbivore, *Spodoptera litura*. The 1st instar larvae, which fed on the leaves of high expression plant (T1-1) caused very little damage (less than 2% damage of the leaf) and consequently gained very less weight (avg. weight of each larvae was 6 mg) when compared to larvae fed on low expression plant (T13-1) as well as controls was 20 mg (avg. weight of larvae) and 39 mg (avg. weight of larvae) respectively, which caused 13% and 20% damage respectively and gained more weight within three days of post feeding (**Fig. 5.III.10**). Simultaneously, 2nd instar larvae were also tested with the same plant. Interestingly, here also the larvae which fed on high expression plant gained very less weight 37 mg (avg. weight of larvae) and the area damaged was about 18% (**Fig. 5.III.11**). But, those larvae which fed on low expression plant was 87 mg (avg. weight of larvae) against the larvae that fed on control which weighed 99 mg (avg. weight of larvae) and caused more damage to the leaf which was more than 70% after post feeding. These observations were represented in graphical diagram (**Fig. 5.III.12**).

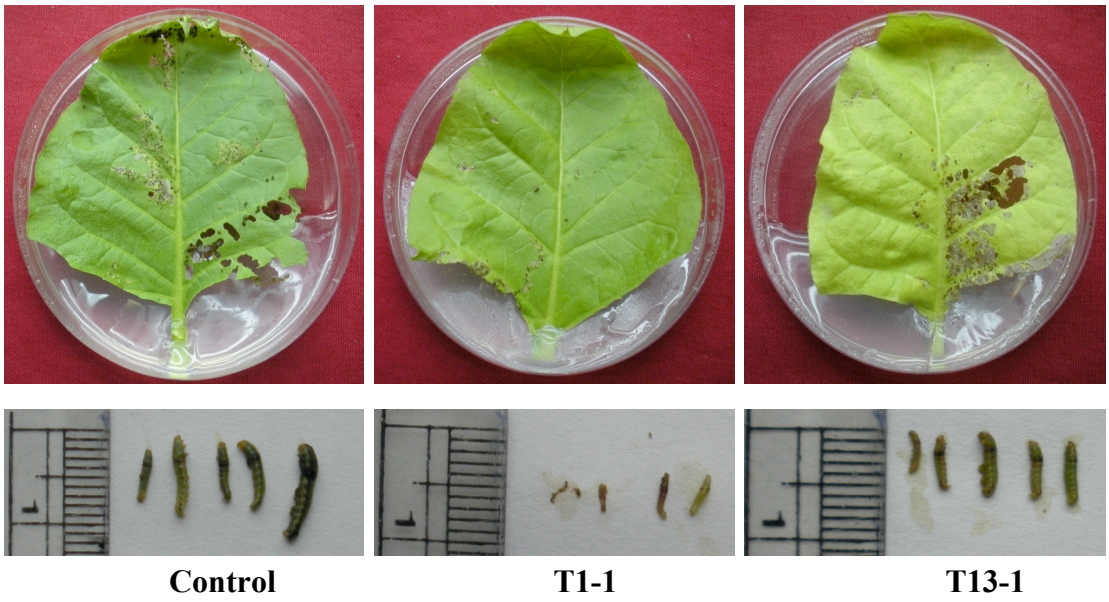


Figure 5.III.10. Insect bioassay using the 1st instar larvae of *Spodoptera litura* (Photographs were taken after three days of feedings)

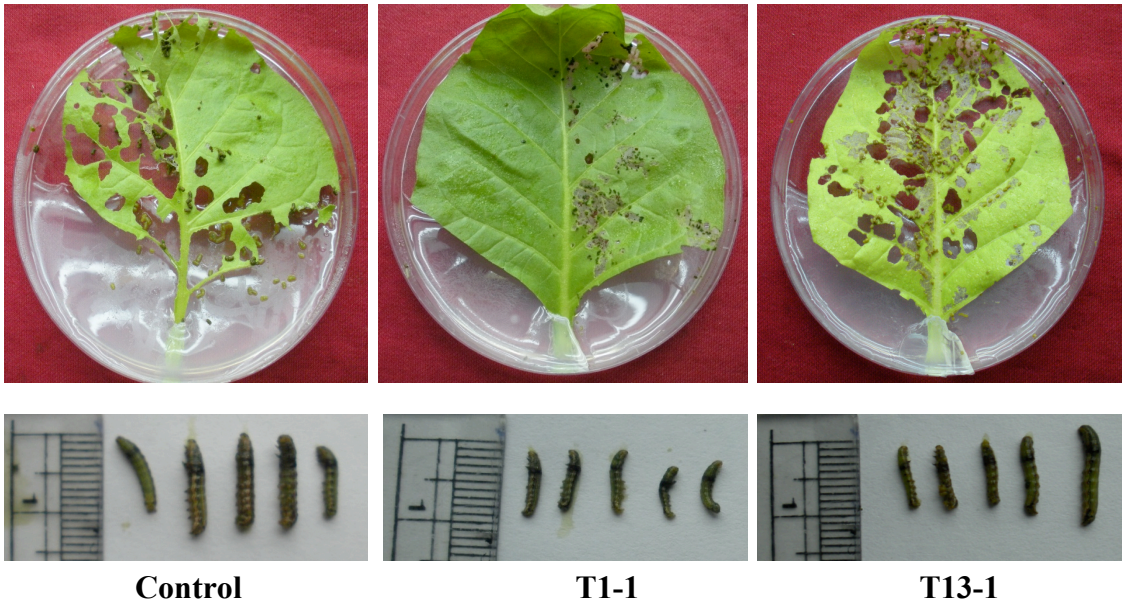


Figure 5.III.11. Insect bioassay using the 2nd instar larvae of *Spodoptera litura* (Photographs were taken after two days of feedings)

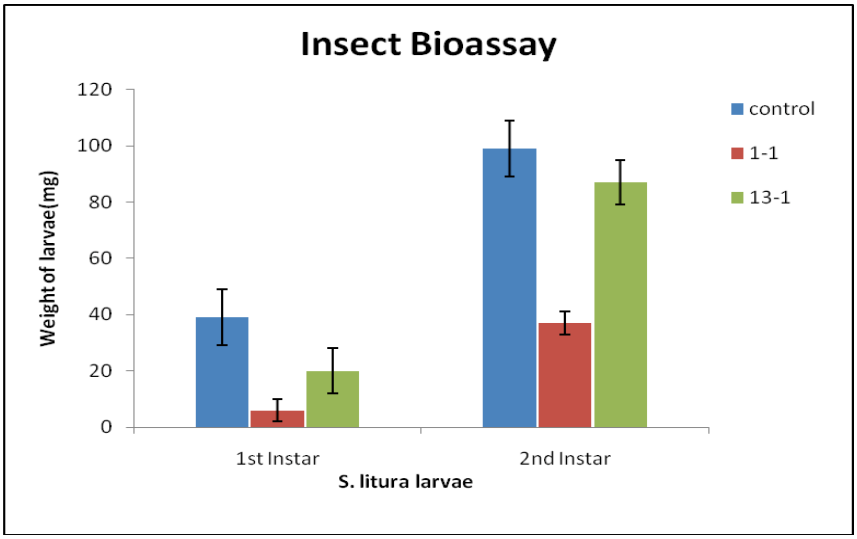


Figure 5.III.12. Graphical representation of the weight gained by 1st instar and 2nd instar larvae of *Spodoptera litura* after post feeding.

Discussion

The importance of plant defensin to host was well documented (Broekart et al. 1995; Terras et al. 1995; De Bolle et al. 1996; Francois et al. 2002). Hence, the gene *TvD1* was cloned in the binary vector pCAMBIA2300 driven by the promoter 35S with a polyA signal and transformed into tobacco for heterologus expression and *in vivo* characterization. There were reports about heterologus expression of plant defensin in plants confering high level of resistance to the host but to a limited pathogens as in tobacco (Terras et al. 1995), Geranium (De Bolle et al. 1996), potato (Bie et al. 1999), rice (Gao et al. 2000; Jha et al. 2009), etc., The independent transgenic plants showed different levels of expression of *TvD1*, this may be due to the position of integration and/ or effects of copy number (Meyer 1998). Through RT-PCR, different expression plants were selected, that is, high (T1) as well as low expression (T13) plant and used for antifungal bio-assay along with wild type control plants.

From the detached leaf antifungal bio-assay, it was confirmed that the high expression plant (T1) had enhanced resistance which was more than 90% against the pathogens *Phytopthora parasitica* and also against *Rhizoctonia solani*, whereas the low expression plant (T13) showed lesser tolerance and damage was almost equal to the wild type control plant against the fungal pathogen *P. parasitica*. Similarly, it was observed that plant overexpressing *DmAMP*1 in rice showed significantly improved resistance against the pathogens such as *Magnoporthae oryzae* and *R. solani* by 84% and 72%, when compared to non-transgenic plants. And also plants like potato overexpressing the wasabi defensin showed partial resistance against the pathogen *B. cinerea* (Khan et al. 2006). In the present study, the necrotic lesions was initially restricted to the infection site in the high expression plant leaf against both the fungal pathogens whereas in control (non-transformed) and low expression plant, the infection spread along the entire leaf against the pathogen *P. parasitica*, but latter showed partial resistance against the pathogen *R. solani*. This is again in accordance with the observation made by Jha et al. (2009) in rice against the pathogen *M. oryzae*, Zhu et al.(2007) in papaya against the pathogen *P. palmira*.

High expression plants such as T1, T26, T32 and low expression plants such as T13 seeds were germinated and used for T_1 generation analysis. The T_1 transgenic plants were confirmed through PCR and used for Southern hybridization analysis in order to determine the stable integration and copy number. From the Southern analysis, it was confirmed that in independent transgenic plants the integration of one to multiple copies of transgenes indicating that all the plants were regenerated from different independent transformation events. The plant T13 had multiple copies of transgene with the level of expression of the integrated transgene being low. This is in accordance with report by Meyer (1998).

T_1 transgenic plants were also used for whole plant antifungal bio-assays using the fungus *R. solani*. Here, also the high expression plant (T1) showed better tolerance when compared to low expression plant (T13) and wild type control. The latter has wilted symptoms in the lower half of the plant within 5 days of post inoculation whereas the former did not develop any such symptoms. The rice plant overexpressing *DmAMP1* showed resistance against the pathogen *R. solani*, but the control showed necrotic lesion all along the plant (Jha et al. 2009). Similarly, defensin from Chinese cabbage overexpressed in tobacco showed tolerance against the pathogen *Phytophthora parasitica* whereas the non-transgenic control plants showed wilting symptom within 5 days and plants died after 8 days. It was also determined through whole plant bio-assay (Park et al. 2002).

Though plant defensins are meant for conferring resistance against the fungal pathogens, in some cases they acts against the bacteria, some legume defensin appears to possess insecticidal activity also. Wijaya et al. (2000) reported that *Cassia fistula* defensin exhibited Proteinase Inhibitor activity and *Vigna radiata* defensin (VrD1) (Lin et al. 2007), *V. unguiculata* defensin (VuD1) (Pelegrini et al. 2008) showed insect α- amylase inhibitory activity. Hence, the tobacco transgenic plants expressing *TvD1* were checked against the insect *Spodoptera litura* larvae. It was shown that the high expression plant (T1) has resistance against the first as well as second instar larvae whereas the low expression plant (T13) and wild type control showed susceptibility especially against the second instar larvae, and the low expression plant (T13) showed

partial resistance against the first instar larval attack. This may be due to presence of insect α- amylase inhibitory activity in *TvD1* as demonstrated in the later chapter. This is in fact the first report of defensin transgenic plants exhibiting insecticidal property. In order to increase the activity of TvD1, in the forth coming chapter we did mutational analysis and screened the mutants for the improved antifungal activity along with the insecticidal property. Hence, from this chapter, it was concluded that the gene *TvD1* appears to be a potent candidate gene against the several pathogens and it can be used for crop plant transformation against the fungal pathogens as well as insect pests.

IV. Mutational analysis of *TvD1* for enhanced activity

Generation of mutants:

TvD1 appears to be a potent antifungal peptide (Vijayan et al 2008) and hence, is a good candidate protein for studies on mutagenesis aimed at improving its potency against various soil borne filamentous fungal pathogens. In order to increase the activity potential, primers were designed for substitution of single amino acid in and around the loop 3 of the native TvD1such that by changing serine into arginine at position 32 **(S32D mutant)**, and aspartic acid into arginine at 37 **(D37R)**, respectively. For the introduction of a stretch of amino acids at the loop3, the 5 amino acids **-G-M-T-R-T- (AlphaTvD1)** was added by replacing **–D-D-F-R- (nativeTvD1)** in the native protein. In all the mutants, the signal peptide was removed and an acid sensitive dipeptide (Asp-Pro) was introduced between the ~16 kDa histidine tag and the peptide by adding the amino acids aspartic acid and proline through the primers in the pET32a (+) expression vector. Hence, after purification the His-tag would be removed from the mature peptide.

The mutated genes were synthesized (**Fig. 5.IV.1**) using appropriately designed primers with restriction enzyme sites at the end of the amplified gene fragments for subsequent manipulationsand ligate into the pET32a (+) expression vector. The vector was then transformed into *E. coli* BL21 DE3 cells for expression and sequence analyses.

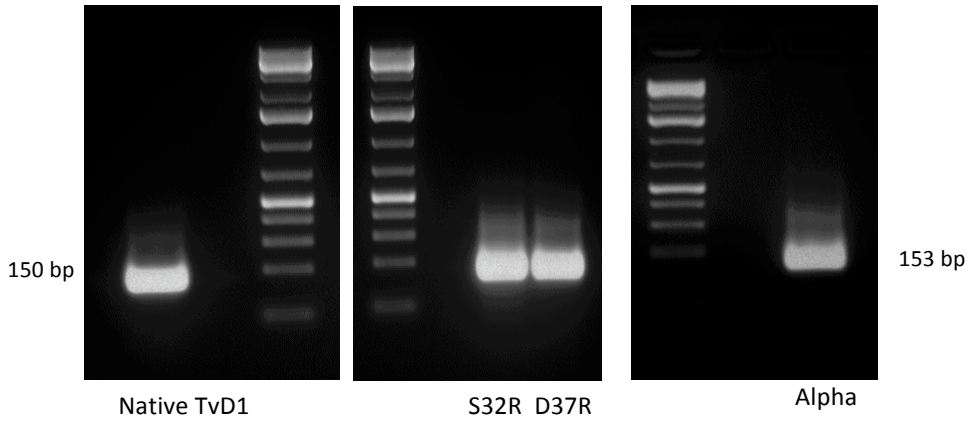


Figure 5.IV.1. Gel picture showing the amplification of different mutant gene sequence along with native protein coding gene

Prokaryotic expression and purification of proteins:

Bacteria were freshly inoculated into the LB medium from the overnight grown culture which was allowed to grow further till the OD_{600} reached 0.6, followed by induction with 1mM IPTG. After 4 hours of induction and expression, protein was purified using the Nickel-NTA agarose column (as per the manufacturer's instruction, Qiagen, USA) and 15% SDS-PAGE was used for purified protein analysis (**Fig. 5.IV.2**). The elution buffer containing the purified protein was adjusted to pH 1.4 and is incubated for 18 hrs at 55^{o}C. This was then followed by neutralization at pH 7.0 with NaOH. Finally, the protein was separated through the fresh column again and concentration was determined through Bradford's method. The purified proteins were used for bio-assay.

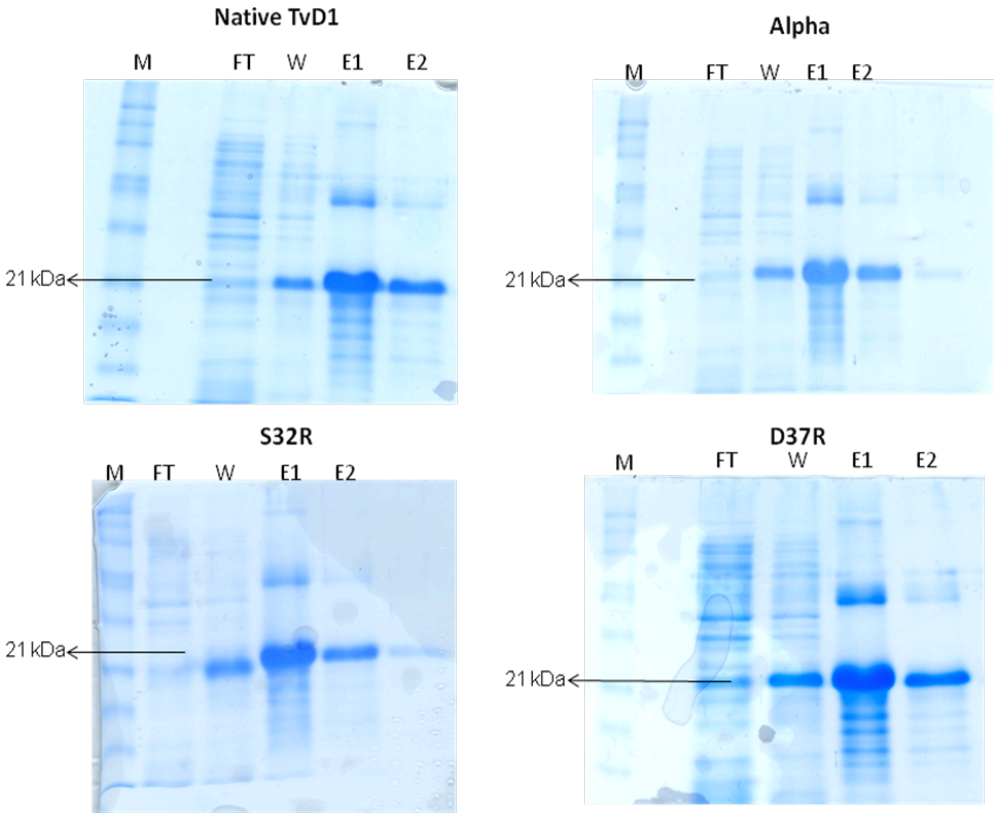


Figure 5.IV.2. SDS-PAGE analysis of the mutant and native TvD1 after expression where, M, FT, W, E1 and E2 represent marker, flow through, first wash, elution1 and elution2 respectively. Alpha, S32R, and D37R are the mutants of TvD1. His-tag was removed by incubating at pH 1.4 at 55^{o}C for 18 hours and purified through the column

Bioassay:

The recombinant protein was used for *in vitro* antifungal and antibacterial assays. It was tested through spore germination using the plant pathogenic fungal pathogen such as *F. culmorum* and *F. oxysporum* (Song et al. 2005). The antibacterial activity of the various peptides was tested using the plant pathogenic bacterium *Pseudomonas syringae* (Makovitzki et al. 2007).

Antifungal bioassay:

From the antifungal bio-assay, it could be observed that the mutant, Alpha TvD1 at 5 μM concentration exhibited 75% and 88% inhibition against the fungal pathogens, *F. culmorum* and *F. oxysporum*, respectively (**Fig. 5.IV.3, 5.IV.4, 5.IV.5 & 5.IV.6; Table 5.IV.1 & 5.IV.2**). However, the other two mutants such as S32R, D37R and the native peptide exhibited comparatively less antifungal activity at 5 μM with 51%, 56% and 35% inhibition respectively against the fungus *F. culmorum.* It was 51%, 49% and 37.5% inhibition respectively, against the fungal pathogen *F. oxysporum* (**Fig. 5.IV.3, 5.IV.4, 5.IV.5 &5.IV.6; Table 5.IV.1 & 5.IV.2**).

The various peptides were tested with lower concentration also, that is at 0.5μM against for both the fungal species (**Fig.5.IV. 4 & 5.IV.6; Table 5.IV.1 & 5.IV.2**). The mutant Alpha TvD1 has 18% and 21% inhibition against the test fungus *F. culmorum* and *F. oxysporum*, respectively. At 0.5 μM, the mutant such as S32R, D37R and native TvD1 showed only 5%, 4% and 3% inhibition respectively against the fungus *F. culmorum* and it was 15%, 14.5% and 4% respectively against the fungus *F. oxysporum.* At 10 μM, all the proteins were inhibitory to both the fungal pathogens, that is, it was more than 90%.

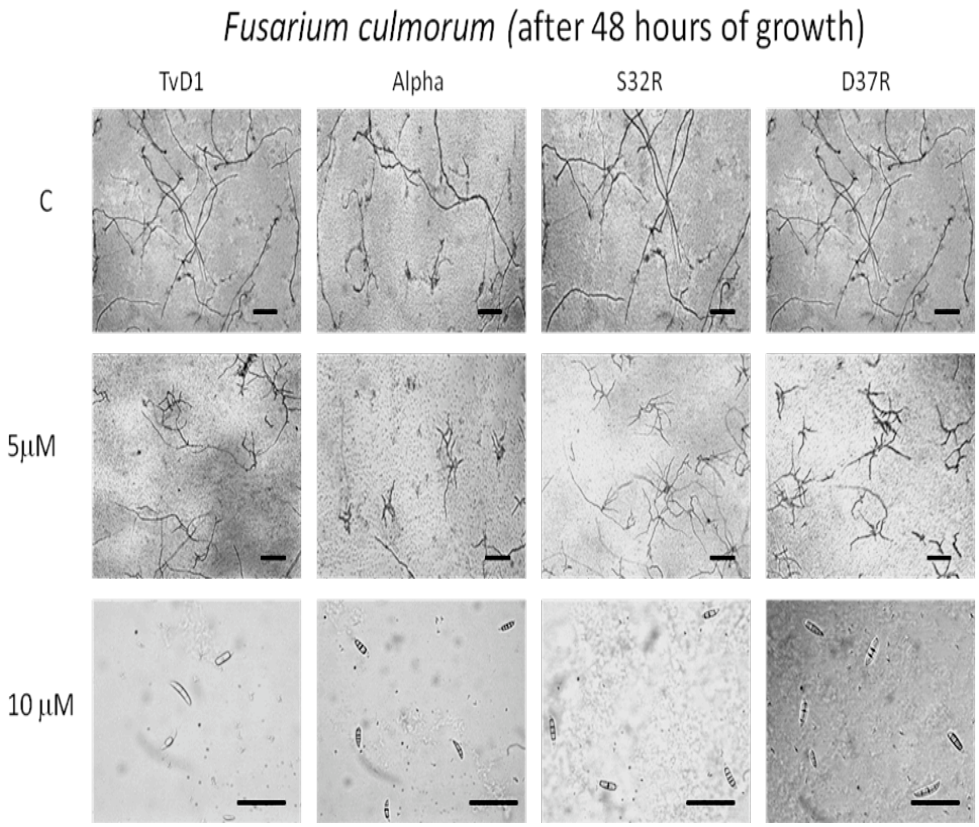


Figure 5.IV.3. Purified protein used for spore germination assay against the fungus *F. culmorum.*

Table 5.IV.1. Percentage inhibition of the native TvD1 and mutant peptides such as Alpha TvD1, S32R, D37R against the fungus *F. culmorum*

		Growth inhibition percentage		
		0.5 µM	5 µM	10 µM
Fusarium culmorum	TvD1	2.7±1.1	34.7±3.1	93.9±0.7
	Alpha	17.6±0.6	75.0±1.1	95.3±0.7
	S32R	5.4±1.8	50.7±2.0	92.2±2.4
	D37R	4.1±1.1	56.3±4.1	93.2±2.1

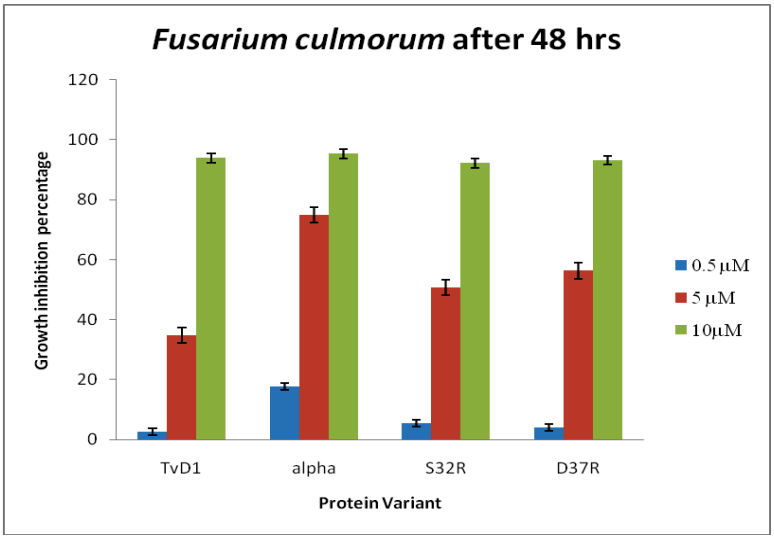


Figure 5.IV.4. Percentage growth inhibition of different mutant as well as native TvD1 peptides.

Table 5.IV.2. Percentage inhibition of the native TvD1 and mutant peptides such as Alpha TvD1, S32R, D37R against the fungus *F. oxysporum*

		Growth inhibition percentage		
		0.5 µM	5 µM	10 µM
Fusarium oxysporum	TvD1	4.3±1.2	37.5±1.1	90.4±0.8
	Alpha	21.3±4.1	88.3±1.1	94.7±1.1
	S32R	14.9±1.1	51.1±2.1	90.7±0.8
	D37R	14.5±0.1	48.9±0.6	91.5±0.1

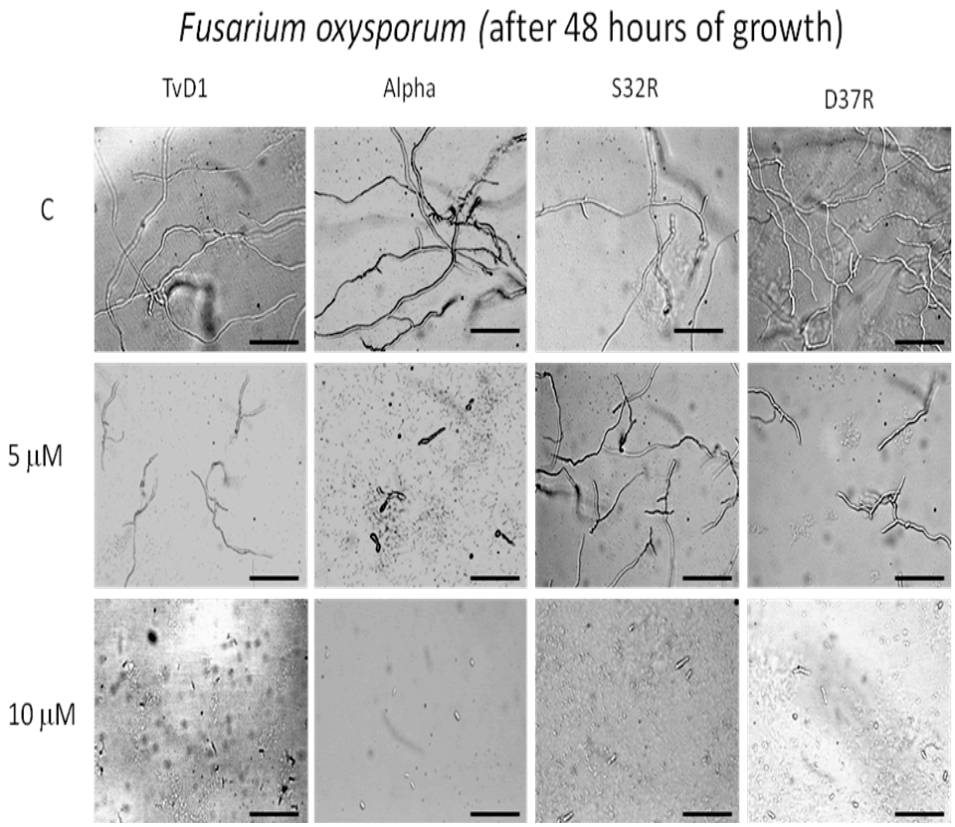


Figure 5.IV.5. Final purified protein used for spore germination assay against the fungus, *Fusarium oxysporum*.

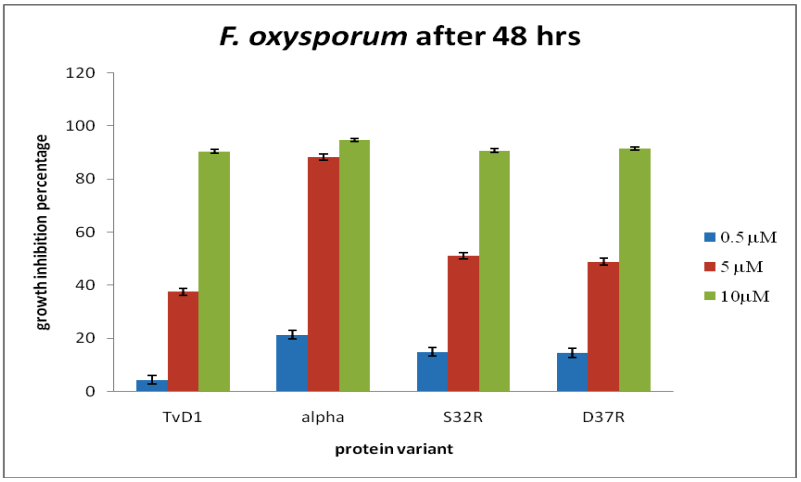


Figure 5.IV.5. Percentage growth inhibition of different mutants as well as the native TvD1 peptide against the fungus *F. oxysporum*.

Antibacterial assay:

When the antibacterial activity was assayed, there was no significant difference between the mutants and native TvD1 in controlling the growth and also the inhibitory concentration was slightly higher than it required for fungal inhibition (**Table 5.IV.3 & Fig.5.IV.6)**. At 1 µM, the assay showed around 16% inhibition for the native TvD1 and mutant Alpha TvD1.There was an increase in inhibition as the concentration of the peptides increased. Nearly 50% inhibition was observed at 5 µM concentration in respect of the native and mutant peptides.At 10 µM, it was 73% and 76% inhibition for both native and Alpha TvD1 respectively.

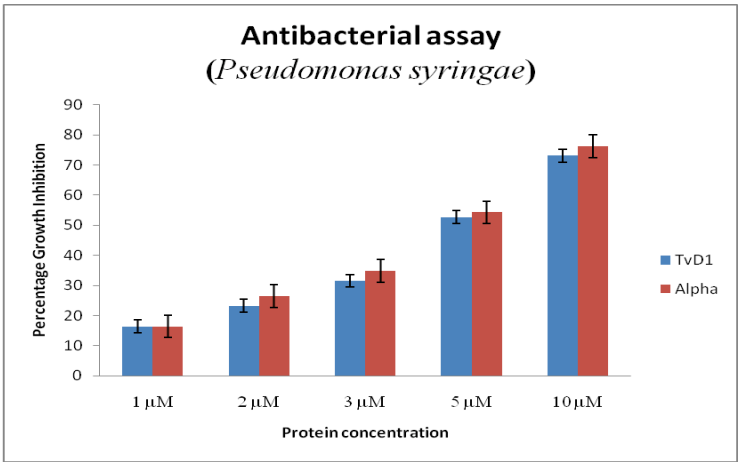


Figure 5.IV.6. Percentage growth inhibition of different mutant as well as native peptides against the bacterium *P. syringae*.

Table 5.IV.3. Percentage inhibition of the native TvD1 and mutant Alpha TvD1 against the bacterium *P. syringae*

Protein concentration	Percentage Growth inhibition	
	TvD1	AlphaTvD1
1 μM	16.39±2.1	16.45±4.2
2 μM	23.29±1.5	26.39±3.6
3 μM	31.54±1.7	34.78±3.1
5 μM	52.73±1.6	54.28±3.1
10 μM	73.1±2.0	76.19±3.5

From the above expreriments, IC_{50} value were calculated and it was tabulated as follows

Table 5.IV.4. IC_{50} values of the native and mutant recombinant peptides *in vitro* against fungal pathogens *F. oxysporum* and *F. culmorum* and the bacterium *Pseudomonas syringae* (where, nd is not determined)

Different mutants of TvD1	IC_{50}		
	F. oxysporum	*F. culmorum*	*P. syringae*
Native (μM)	6.5	6.5	6.9
Alpha (μM)	2.5	3.0	6.6
S32R (μM)	5.0	5.0	nd
D37R (μM)	5.5	4.5	nd

Chitin deposition assay:

Congo red will stain the chitinaceous cell wall and it stains intensely in hyphae with impeded growth. In the Congo red staining assay, the tips of the germinating hyphae *F. culmorum* exposed to native and Alpha TvD1 stained intensely with the dye indicating deposition of chitin and consequent retardation of hyphal growth (**Fig. 5.IV.7**).

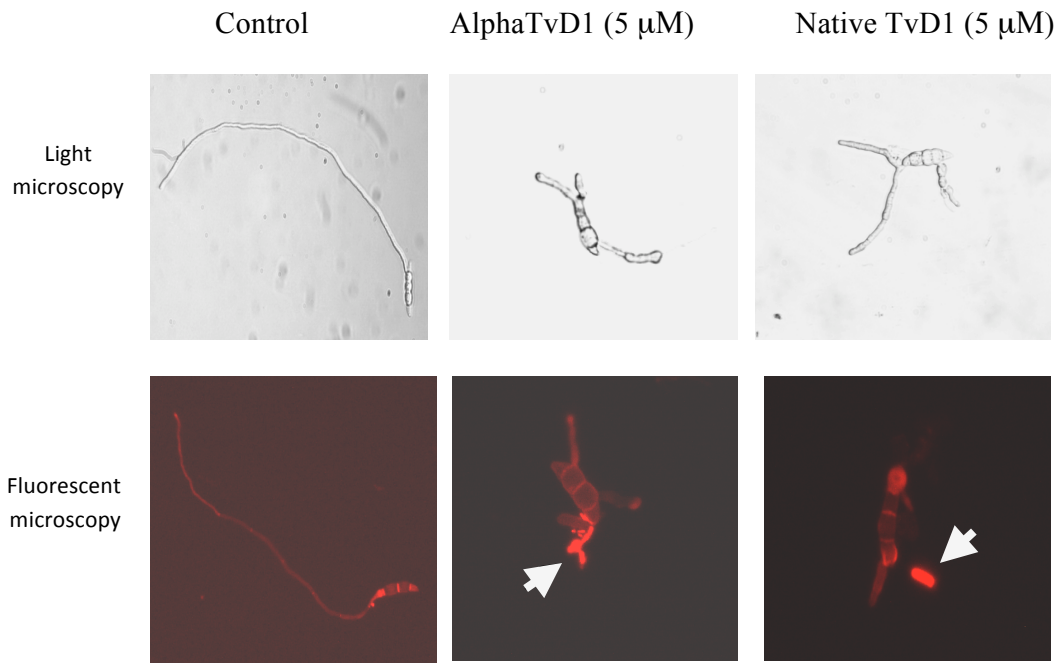


Figure 5.IV.7. Chitin deposition assay for the germinating *F. culmorum* spores in the presence / absence of protein at 5 μM (native and Alpha TvD1) after 18 hours of growth.

Membrane permeabilization assay:

Fusarium culmorum spores at a concentration of ~ $4x10^4$ were incubated in 1/4-strength potato dextrose broth for 14 – 18 hours at 25°C. Afterwards, the germinated spores with hyphae were incubated with the recombinant proteins (native TvD1 and Alpha TvD1) at a concentration of 2.5 and 5 μM respectively for 3 hours with gentle agitation. Fluorescence of the spores with hyphae were visualised using the fluorescence microscope with excitation and emission wavelengths of 488 and 538 nm, respectively after 10 min of adding SYTOX Green dye (0.5 μM).

At lower concentration (2.5 μM), the nucleus of the fungus got stained and it was visible for both the native and Alpha TvD1 in the fluorescent field of the microscope **Fig. 5.IV.8**. And at higher concentration, that is at 5 μM, the nucleus is disorganized completely and DNA became fragmented **Fig. 5.IV.9**.

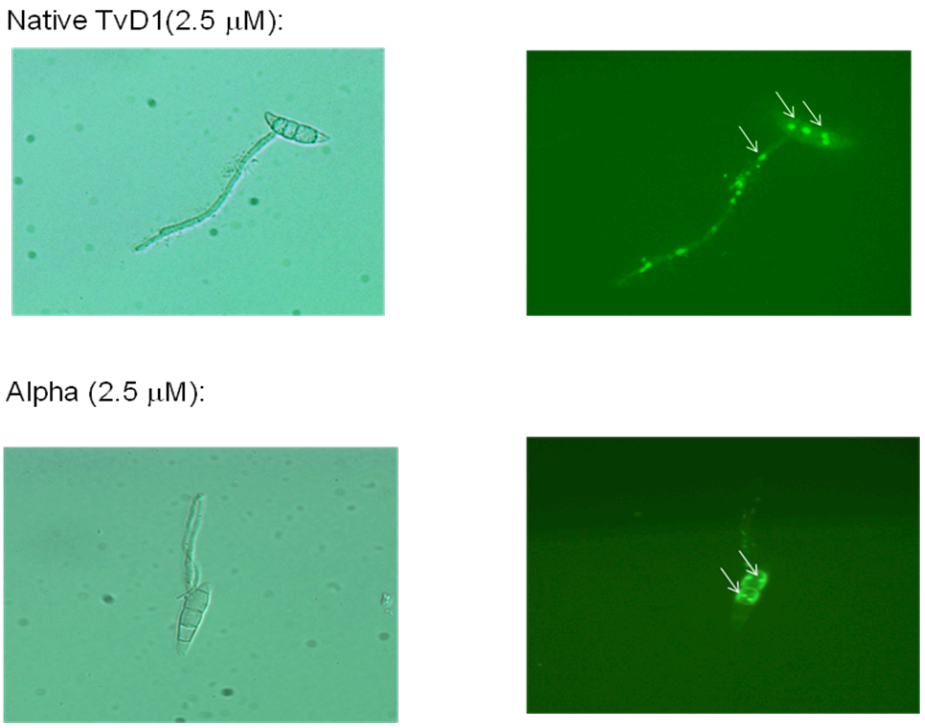


Figure 5.IV.8. SYTOX Green membrane permeabilization assay with the fungus *F. culmorum* in the presence of native and Alpha TvD1 peptide at a lower concentration that is at 2.5 μM.

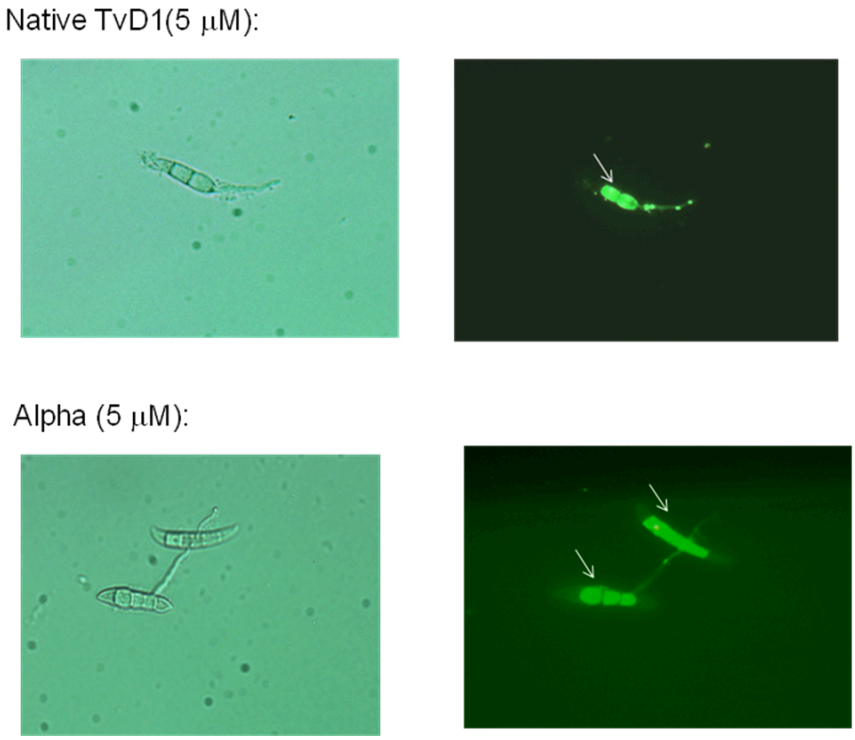
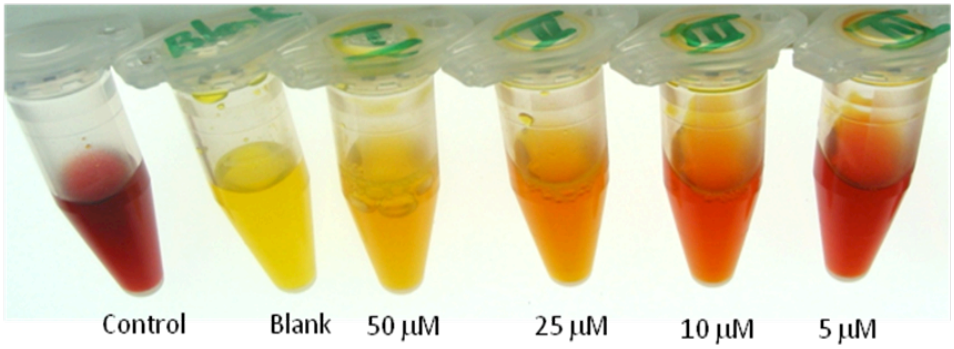


Figure 5.IV. 9. SYTOX Green membrane permeabilization assay with the fungus *F. culmorum* in the presence of native and Alpha TvD1 peptide at a concentration of 5 μM.

α-Amylase inhibitory activity using the insect *Tenebrio molitor*:

Insect α-amylase inhibition is one of the approaches conceived in developing transgenic crops, particularly in legumes, for resistance against insect attack. Native and Alpha TvD1 peptide were tested for the insect (*Tenebrio molitor*) gut α-amylase inhibitory activity. At 10 μM, it was 32% and 16% inhibition respectively for native and Alpha TvD1, it was 75% and 60% respectively at 25 μM and it was 85.5% and 78% respectively at 50 μM **(Figure 5.IV.10 & 5.IV.11; Table 5.IV.5**). At the same time, the native and Alpha TvD1 did not show any inhibitory effect on the human saliva α-amylase even at 50 μM recombinant peptide as well barely α-amylase (**Fig. 5.IV.12**& **5.IV.13**).

Alpha TvD1 :



Native TvD1:

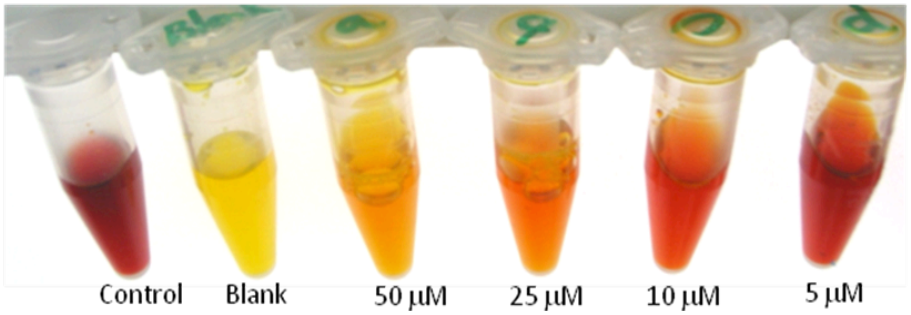


Figure 5.IV.10. Insect (*Tenebrio molitor*) α-amylase inhibitory activity with recombinant protein (native TvD1 and Alpha TvD1) at different concentrations (Bernfold 1955)

Table 5.IV.5. Percent inhibition of the insect *Tenebrio molitor* gut α-amylase by native and Alpha TvD1.

Protein concentration	Alpha TvD1	Native TvD1
50μM	85.53 ± 2.3	78.05 ± 2.7
25μM	74.83 ± 3.1	60.0 ± 1.5
10μM	32.45 ± 1.2	16.04 ± 1.7

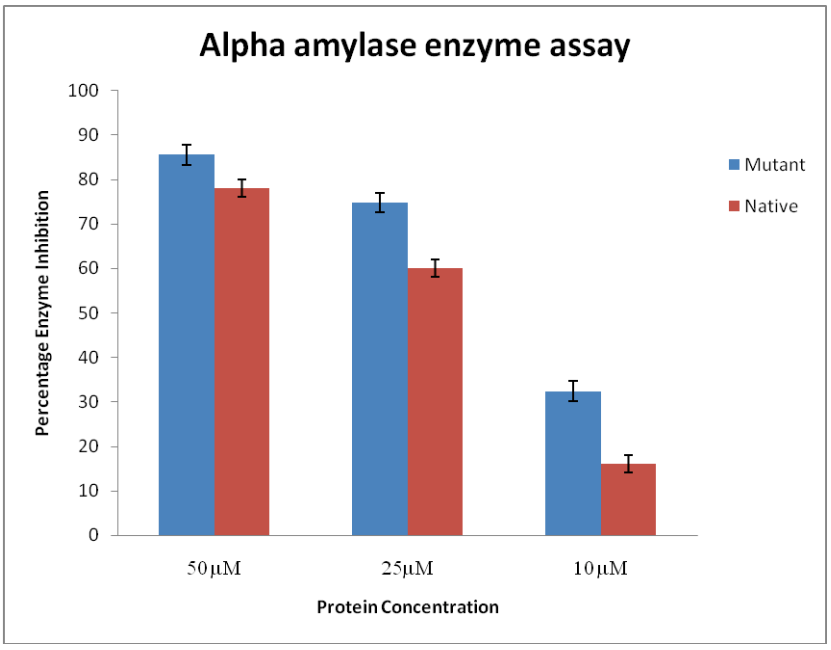


Figure 5.IV.11. Percent inhibition of the insect α-amylase with the recombinant protein (native and Alpha TvD1).

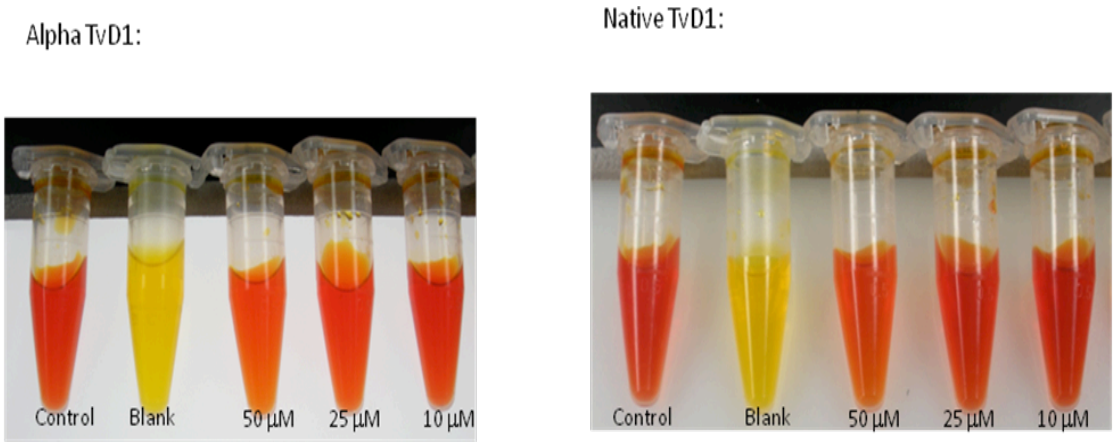


Figure 5.IV.12. Barely α - amylase inhibitory activity

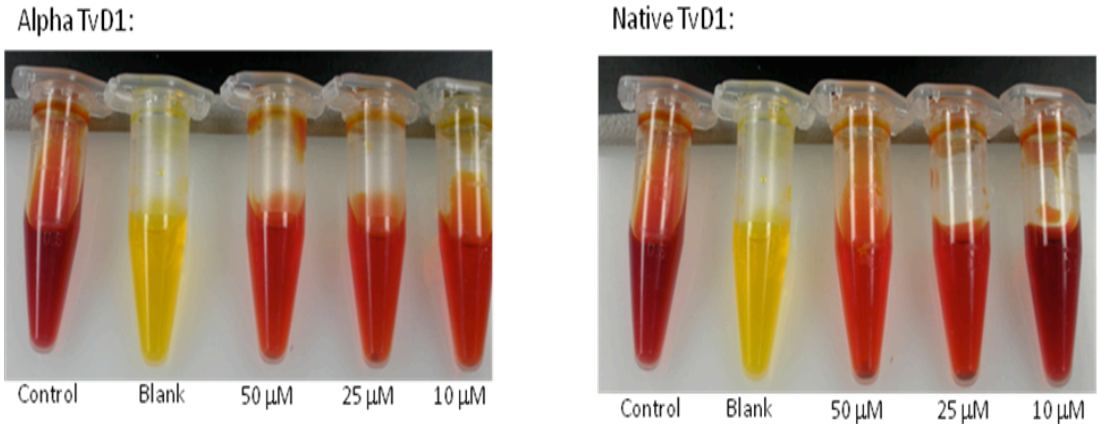


Figure 5.IV.13. Human saliva α - amylase inhibitory activity

Discussion

The data presented here demonstrated that the defensin TvD1 has potent antifungal property against some of the soil pathogens. In order to enhance its antifungal property, mutational analysis was carried out in the present study in TvD1 by modifying some aminoacid residues in the loop 3 that connects the b-strand 2 and b-strand 3. Possible enhancement in the antimicrobial activity would help in generating transgenic crops with enhanced resistance against microbes and insects. Mutational analysis of the plant defensin was first carried out by De Samblanx et al. (1997) in Rs-AFP2 and, this has led to the determination of the specific aminoacids responsible for antifungal activity. Similarly, Spelbrink et al. (2004) determined some amino residues through mutational analysis in MsDef1 and MsDef2 responsible for antifungal activity. In the present investigation, we substituted the aminoacid residue at 32 by replacing the serine with arginine (S32R), aspartic acid with arginine at 37 (D37R) and looked for enhanced antifungal activity. Similarly, it was shown that in the Rs-AFP2 changing the positive charge potential by replacing the existing aminoacid at 9 and 39 into arginine has increased its antifungal activity by two fold (De Samblanx et al. 1997). In VrD2, insertion of 5 aminoacids such as –G-M-T-R-T- by replacing the existing 4 amino acid such as -D-D-F-R- enhanced the insect α – amylase inhibitory activity when compared to VrD1(Liu et al. 2008). Hence, some of these replacements/ insertions were carried out in the native TvD1 to develop mutants with possible enhancement in antimicrobial and insecticidal activity.

Through PCR, the mutants such as S32R and D37R were synthesized (Ke and Madison 1997), whereas Alpha TvD1 and native TvD1 were synthesized by using overlapping primers and in all the cases the signal peptide was removed. Since the peptide is pH stable, an acid sensitive dipeptide D-P (Aspartic acid-Proline) was included between the His-tag and the mature peptide in the bacterial expression vector pET32a. The inclusion of dipeptide did not show any drawbacks in the activity of the peptide (Lin et al. 2007; Zorko et al. 2009). And also after acid digestion, the proline will be the first amino acid residue in the mature peptide and it would not have any suppressive role in the peptide (Zorko et al. 2009).

All the proteins were expressed in *E.coli* BL21 DE3 cells (Olli and Kirti 2007; Vijayan et al. 2008) and purified as per protocol mentioned in the materials and methods. Then, they were incubated at pH1.4 for dipeptide digestion and separation of the His – tag from the mature peptide (Lin et al. 2007). The purity was assessed using 15% SDS-PAGE as shown in **Fig. 5.IV.2**, which was followed by dialysis and the purified peptide was used for bioassays.

From the fungal spore germination assay, it could be clearly seen that the mutant Alpha TvD1 has enhanced activity against the fungal pathogens such as *F. culmorum* and *F. oxysporum* when compared to two other mutants such as S32R and D37R, which possessed comparatively better activity than the native TvD1. The mutant Alpha TvD1 with an extra amino acid and the substitution of 4 other amino acids in the loop3 possessed significantly increased its activity potential against the fungal pathogens especially *F. culmorum* and *F. oxysporum*. The relative antifungal activity of the TvD1 by arginine substitution depends upon the test fungus. The same was observed in the case of RsAFP2 (De Samblanx et al. 1997), confirming that the composition and structure of the putative receptor on the fungal hyphal membrane between different fungal species. This would be applicable to plant pathogenic bacteria also. The peptide modification in the loop3 did not result in any significant effect between the mutant and native peptide, and also the required concentration for antibacterial activity was little more than it was required against the fungal pathogens (**Table 5.IV.3**).

In general, animal defensins possess antibacterial activity preferentially when compared to plant defensins. The plant defensins that displayed antibacterial property were designated as antimicrobial peptides (AMP) eg., Dm-AMP1, Ah-AMP1, etc., instead of antifungal protein (AFP) eg., RsAFP2, RsAFP1, etc. Bacterial membranes have negatively charged structures exposed at their surfaces such as teichoic acid in Gram positive bacteria and lipopolysaccharides in Gram-negative bacteria. In their membranes, negatively charged phospholipids such as lipidII, phosphatidyl serine, phosphatidyl glycerol and cardiolipin spread into both the membrane leaflets and these may serve as docking point for the plant defensins (Carvalho and Gomes 2009).

Congo red has a strong affinity for β-glucans and thus binds to chitin in the fungal cell wall. Actively growing hyphae do not have much chitin deposit at the tips and show little Congo red staining. Sometimes, the growing hyphal tips would appear as balloon- like structure due to the impeded growth and deposition of chitin. It was shown that in the presence of peptides such as Alpha and native TvD1, the growing hyphal tips appeared as balloon- like structures and it induced hyperbranching in the fungus *F. culmorum*. Similarly, it was observed in the fungus *Magnoporthae griseus* with intense stain at the growing tip due to the deposition of chitin in the presence of the *Aspergillus giganteus* antifungal peptide (Moreno et al. 2006).

To determine the mechanism of action, we have studied the effect of TvD1 on plasma membranes and its localization within the treated *F. culmorum* hyphae using SYTOX green fluorescent techniques. In general, microbial cell death would occur in the presence of antimicrobial peptides in two ways: (i) through disruption of the plasma membrane leading to leakage of cytoplasmic contents or (ii) through interaction with intracellular targets (Brogden 2005). It was demonstrated that TvD1 permeabilized the plasma membrane of susceptible fungal hyphae in a dose-dependent manner that correlates with growth inhibition. At non-inhibitory concentrations (2.5 μM) of native TvD1 as well as mutant Alpha TvD1, some permeabilization was detected because of the presence of nicks in the DNA of the nuclei, but the cytoplasm of permeabilized hyphae appeared normal under the microscope. However, at higher inhibitory concentrations of both peptides (5 μM), permeabilized hyphae displayed significant cytoplasmic granulation, and the SYTOX green fluorescence pattern was much more diffuse across the cell, indicating the disruption of nuclei. This suggests that the peptides-induced permeabilization is required for growth inhibition, although it may not be sufficient to induce cell death. Although permeabilization of membranes has been reported for many antimicrobial peptides, the mechanisms of permeabilization could be very different, and in many instances, remained elusive (Van der weerden et al. 2008). Various models have been suggested, including the barrel- stave model, which involves the formation of a pore by the oligomerization of amphipathic peptides to form a hydrophilic channel; the toroidal pore model, in which the pore includes lipid

head groups to stabilize the high positive charge of the peptides; and the carpet model, is which layering of the plasma membrane with positively charged protein causes destabilization in a detergent-like manner (Brogden 2005).

Addition of five amino acid such as –G-M-T-R-T- from VrD1 by replacing the existing four amino acid such as –D-D-F-R- enhanced insect a-amylase inhibitory activity in the defensin VrD2. It was also reported that the amino acids in the loop3 in VrD2 determines the insect α- amylase inhibitory activity (Lin et al. 2007). Similarly, the mutant Alpha TvD1 with the five aminoacid inserted in the loop3 has enhanced a- amylase inhibitory activity against the insect *Tenebrio molitor*, when compared to the native TvD1. In contrast to VrD2, which does not exhibit any insect α- amylase inhibitory effect due to the presence of –D-D-F-R- in the loop3, the native TvD1 has interestingly same amino acid residue in the loop3 and exhibited the inhibitory activity. Hence, from these results, it could be clearly envisaged that the amino acids in the loop3 are not exclusively responsible for insect α- amylase inhibitory activity, but some other amino acids in the peptides are also involved in its activity as in VuD1, a defensin from *V. ungiculata* (Pelegrini et al. 2008). At the same time, the native and Alpha TvD1 did not show any effect on human saliva α-amylase even at 50 μM recombinant peptide. Similarly it doesn't have any effect on barely α-amylase. Hence, it is safe for human consumption as well as plant transformation and overexpression.

TvD1 did not possess any inhibitory effect on human as well as plant enzymes as efficiently as the insect a-amylases. Pelegrini et al. (2008) used molecular modeling to assist in identifying the mechanism that VuD1 uses to present such a characteristic. TvD1 has an asparagine residue at position 40 in the loop3; the presence of a positively charged residue in this position seems to be important for inhibition of mammal α-amylases in VuD1. The fact that VuD1 does not possess present a charged residue in this region might explain why it did not able to inhibit PPA (porcine pancreas amylase) and has so well as it inhibit insect amylases. Furthermore, it was observed that the longer loops of PPA could impede the binding of VuD1 (Pelegrini et al. 2008) (**Fig. 5.IV.13**).

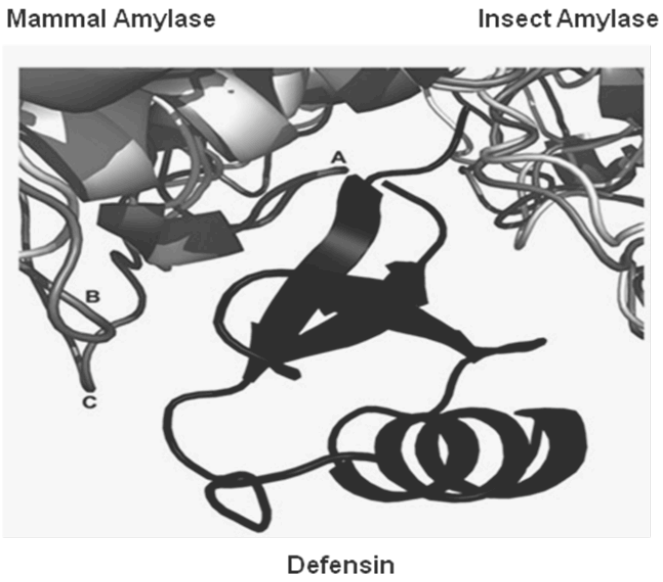


Figure 5.IV.13. 3-D model showing the complex formed between the defensin, insect amylase and mammal amylase where, A, B and C are the extended loop in the mammal amylase that prevent the interaction of defensin at the catalytic site, but it is absent in the insect amylase (modified after Pelegrini et al. 2008).

This was also observed in a study carried out with wheat inhibitors, which showed that steric impediment could also be responsible for the specificity pattern (Franco et al. 2000). Earlier studies have reported the production of transgenic plants containing extra α-amylase inhibitors isolated from the common bean *Phaseolus vulgaris* in pea (Prescott et al. 2005), chickpea (Ignacimuthu and Prakash 2006) and *Vigna radiata* (Sonia et al. 2007) to enhance plant resistance towards the insect pests. In the same way, TvD1 could be useful for pest management programs as an alternative strategy against the insect larvae *T. molitor*. Moreover, the utilization of genetic engineering for the development of transgenic plants resistant to insect pests in addition to fungal pathogens might be an option for application of Alpha TvD1 in Agriculture.

Summary

The 228 bp length *TvD1* gene was isolated from the legume, *Tephrosia villosa* and cloned in the vector pTZ57R for DNA sequencing, which was used for amino acid sequence prediction. Through BlastP program, the amino acid sequence was predicted. The predicted peptide possess 75 amino acid residues, of which first 28 amino acids were determined as signal peptide and the remaining 47 amino acids constitute the mature peptide. The mature peptide was used in homology modeling studies. It showed that the TvD1 shared 91% pairwise similarity with that of *Vigna radiata* defensin 2 (VrD2). Simultaneously, it was cloned in the prokaryotic expression vector pET32a (+) for recombinant peptide expression and purification. The purified protein was used for antifungal bioassay. The purified peptide (rTvD1) was effective against several fungal pathogens, *viz.*, *Rhizoctonia solani*, *Phytopthora parasitica*, *Fusarium moniliforme* and *Botrytis cinerea* in the PDA plate assay. And also by fungal spore germination assay, it was shown that it was more effective against the fungal pathogens such as *Alternaria helianthi*, *F. oxysporum*, *F. moniliforme*, *Phaesariopsis personata*, *B. cinerea*, *Curvularia* sp. Hence, the IC_{50} value was less than 25 μg ml^{-1} for them, for *P. personata*, it was less than 10 μg ml^{-1} and for *R. solani*, it was 38 μg ml^{-1}. The peptide possessed an inhibitory effect on for the *Arabidopsis* root growth also and hence, with a possible role in plant growth and development.

In order to determine the sub-cellular localization of the peptide, *TvD1* was cloned with GFP a as fusion gene in the binary vector pEGAD, which was driven by 35S promoter and t-nos terminator with *basta* resistance gene as a selection marker. Transgenic tobacco plants were raised through *Agrobacterium* mediated transformation. From the confocal microscopy, it was confirmed that the peptide localized to the apoplastic region of the cell for confering resistance against the pathogens, which usually approach the apoplastic region after cell wall degradation.

For *in vivo* characterization, the gene *TvD1* was cloned in the binary vector pCAMBIA2300 driven by 35S promoter with t-nos terminator. We have generated 32 putative transgenic plants, which were confirmed through PCR.

Expression analysis was performed through RT-PCR and high expression as well as low expression plants were subsequently used in detached leaf antifungal bio-assay with the fungal pathogens such as *R. solani* and *P. parasitica*. The high expression plant T1 showed significantly enhanced resistance against the two test fungal pathogens. At maturity, T_0 seeds were collected and used for T_1 generation analysis. T_1 plants were confirmed through PCR. Southern hybridization was performed to determine the copy number and stable integration of the transgene. The high expression plant T1 showed resistance against the pathogen *R. solani* in the whole plant bio-assay also. The plant was also used for insect bioassay using the 1st and 2nd instar larvae of *Spodoptera litura*. It showed tolerance against both the stages of the larvae. But, the wild type control as well as low expression plant was susceptible to the larvae especially for the 2nd instar larvae, the latter showing enhanced tolerance over the former. This is in fact the first report of defensin transgenic plants exhibiting resistance towards an insect, *Spodoptera litura* larvae which are a generalist herbivore.

To enhance the activity of the TvD1, site directed mutagenesis was performed. By PCR, different mutants such as S32R, D37R and Alpha TvD1 were synthesized along with native TvD1. They were cloned in the prokaryotic expression vector pET32a (+), protein was expressed and purified. The purified protein was used for antifungal bio-assays using the fungal pathogens such as *F. oxysporum* and *F. culmorum*. From the fungal spore germination assays, it was concluded the mutant Alpha TvD1 possessed enhanced activity against the both the pathogens, when compared to other mutants and native TvD1. The peptide also showed antibacterial property against the plant pathogenic bacterium *Pseudomonas syringae* but the required inhibitory concentration was slightly higher than that is required for fungal inhibition. Also there was no significant difference between the Alpha mutant and native peptide in the antibacterial activity. The mutant Alpha TvD1 and native TvD1 exhibited insect α-amylase inhibitory activity against the larvae, *Tenebrio molitor*. But at the same time, it did not show any inhibitory activity against the human α-amylase as well as barely α-amylase. Hence, it is safe for human consumption and plant transformation.

It was concluded that the mutant Alpha TvD1 has enhanced antifungal activity with insect α-amylase inhibitory activity. It did not have any inhibitory against the human α-amylase as well as barely α-amylase. It is one of the efficient candidate genes which could be used for plant transformation in crop plants against the biotic stress especially against the fungal pathogens as well as insect pests.

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