Chapter 5

Characterization and heterologous expression of a legume defensin, TvD1 in tobacco for amelioration of biotic stress tolerance
Results

I. Isolation, cloning and functional characterization of the gene \textit{TvD1}:

**Cloning of \textit{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).

\textit{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 \textit{TvD1} (Fig. 5.I.1) with some of the well characterized legume defensin mature peptides using alignment tool (CLUSTALW) showed that \textit{TvD1} had significant similarity to the defensins from \textit{Cicer arietinum} (95%), \textit{Arachis hypogaea} (93%), \textit{Vigna radiata} defensin-2 (91%), \textit{Medicago sativa} (87%), \textit{Trigonella foenum-graecum} (82%), etc.

![Comparison of mature TvD1 with other mature peptides from legume](image)

**Figure 5.I.1.** Comparison of mature \textit{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. 5.1.2). Interestingly, this defensin was constitutively expressed in all the organs, however, with higher levels of expression in young and mature leaves.

Figure 5.1.2. 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. 5.1.3) 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.
Figure 5.1.3. 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. 5.1.4). The use of prolysis buffer has increased the protein concentration to 1.8 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 5.1.4. 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. 5.1.5), 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 5.1.1). 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. 5.1.5). 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. 5.1.6; Table 5.1.1) 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 5.1.5. 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. 5.1.7).
Figure 5.1.6. 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 5.1.7. 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) *Phytophthora parasitica f. sp. nicotianae*, B) *Fusarium moniliforme*, C) *Botrytis cinerea* and D) *Rhizoctonia solani*
Table 5.1.1. Antifungal assay with the recombinant peptide TvD1 against some fungal pathogens

<table>
<thead>
<tr>
<th>Fungus</th>
<th>% Inhibition at different concentrations of TvD1 (µg/ml)</th>
<th>IC$_{50}^a$ µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10</td>
<td>25</td>
</tr>
<tr>
<td>Botrytis cinerea</td>
<td>44.16±1.3</td>
<td></td>
</tr>
<tr>
<td>Curvularia sp</td>
<td>42.10±3.3</td>
<td></td>
</tr>
<tr>
<td>Fusarium moniliforme</td>
<td>44.67±0.3</td>
<td></td>
</tr>
<tr>
<td>F. oxysporum</td>
<td>45.50±1.0</td>
<td></td>
</tr>
<tr>
<td>Alternaria helianthi</td>
<td>26.98±3.7</td>
<td></td>
</tr>
<tr>
<td>Pseudomonas persicata</td>
<td>56.00±3.9</td>
<td></td>
</tr>
</tbody>
</table>

Data represent mean ± S.D., n = 3; *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. 5.1.8), and it was inhibited in a dose-dependent manner (Table 5.1.2). 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 5.1.8. Seed germination assay of *Arabidopsis* showing that the growth of the roots was severely affected by rTvD1.

Table 5.1.2. *Arabidopsis* seed germination assay

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

Data represent mean ± S. D., n = 3.
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 phylogenetic 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. montiliforme (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 EcoRI and BamHI 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).

The vector was mobilized into Agrobacterium tumefaciens 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 medium 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).

![Images of tobacco regeneration](image)

**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.II.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.
Results

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 *nptII* 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)
**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 T₀ putative transgenic plants showing the amplification of 700 bp nptII 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, T₀ 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 (nptII). This analysis showed the expected amplification of 700 bp nptII marker gene (Fig. 5.III.3).

**Expression analysis of the T₀ 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 T₀ 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.](image)

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

**Figure 5.III.5B.** Detached leaf fungal bioassay using the fungus *Rhizoctonia solani* (Photograph was taken after 5 days post inoculation).
**T₁ 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 T₁ 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 T₁ 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 T₁ transgenic plants showing the amplification of 700 bp *nptII* 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 *EcoRI*, which did not have any site in the T-DNA. After blotting, the membrane was probed with the PCR amplified fragment of *nptII* 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 T1 generation transgenic plants after restriction digestion with EcoRI and probed with nptII 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 nptII fragment was used as a probe.

**Expression analysis of the T1 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 T0 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 T1 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.

![Antifungal bioassay](image)

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 T1 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 larva 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 1\textsuperscript{st} instar larvae of \textit{Spodoptera litura} (Photographs were taken after three days of feedings)

Figure 5.III.11. Insect bioassay using the 2\textsuperscript{nd} instar larvae of \textit{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 heterologous expression and *in vivo* characterization. There were reports about heterologous expression of plant defensin in plants conferring 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 *DmAMP1* 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 T1 generation analysis. The T1 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).

T1 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 $\alpha$- 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.
Results

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 TvD1 such 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 manipulations and 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°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.

![SDS-PAGE analysis](image)

**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°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*.

<table>
<thead>
<tr>
<th></th>
<th>Growth inhibition percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5 µM</td>
</tr>
<tr>
<td><em>Fusarium culmorum</em></td>
<td></td>
</tr>
<tr>
<td>Tvd1</td>
<td>2.7±1.1</td>
</tr>
<tr>
<td>Alpha</td>
<td>17.6±0.6</td>
</tr>
<tr>
<td>S32R</td>
<td>5.4±1.8</td>
</tr>
<tr>
<td>D37R</td>
<td>4.1±1.1</td>
</tr>
</tbody>
</table>
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*

<table>
<thead>
<tr>
<th>Protein Variant</th>
<th>Growth inhibition percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5 µM</td>
</tr>
<tr>
<td><em>Fusarium oxysporum</em></td>
<td></td>
</tr>
<tr>
<td>TvD1</td>
<td>4.3±1.2</td>
</tr>
<tr>
<td>Alpha</td>
<td>21.3±4.1</td>
</tr>
<tr>
<td>S32R</td>
<td>14.9±1.1</td>
</tr>
<tr>
<td>D37R</td>
<td>14.5±0.1</td>
</tr>
</tbody>
</table>
Figure 5.IV.5. Final purified protein used for spore germination assay against the fungus, *Fusarium oxysporum*.

**Fusarium oxysporum (after 48 hours of growth)**

<table>
<thead>
<tr>
<th>Tvd1</th>
<th>Alpha</th>
<th>S32R</th>
<th>D37R</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
</tr>
<tr>
<td>5 μM</td>
<td><img src="image4.png" alt="Image" /></td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
</tr>
<tr>
<td>10 μM</td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
<td><img src="image9.png" alt="Image" /></td>
</tr>
</tbody>
</table>

**F. oxysporum after 48 hrs**

![Bar chart showing growth inhibition percentage for different protein variants](chart.png)

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*

<table>
<thead>
<tr>
<th>Protein concentration</th>
<th>Percentage Growth inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TvD1</td>
</tr>
<tr>
<td>1 µM</td>
<td>16.39±2.1</td>
</tr>
<tr>
<td>2 µM</td>
<td>23.29±1.5</td>
</tr>
<tr>
<td>3 µM</td>
<td>31.54±1.7</td>
</tr>
<tr>
<td>5 µM</td>
<td>52.73±1.6</td>
</tr>
<tr>
<td>10 µM</td>
<td>73.1±2.0</td>
</tr>
</tbody>
</table>

From the above experiments, 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)

<table>
<thead>
<tr>
<th>Different mutants of TvD1</th>
<th>IC$_{50}$</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F. oxysporum</td>
<td>F. culmorum</td>
<td>P. syringae</td>
<td></td>
</tr>
<tr>
<td>Native (µM)</td>
<td>6.5</td>
<td>6.5</td>
<td>6.9</td>
<td></td>
</tr>
<tr>
<td>Alpha (µM)</td>
<td>2.5</td>
<td>3.0</td>
<td>6.6</td>
<td></td>
</tr>
<tr>
<td>S32R (µM)</td>
<td>5.0</td>
<td>5.0</td>
<td>nd</td>
<td></td>
</tr>
<tr>
<td>D37R (µM)</td>
<td>5.5</td>
<td>4.5</td>
<td>nd</td>
<td></td>
</tr>
</tbody>
</table>

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.
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).
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 \(\alpha\)-amylase by native and Alpha TvD1.

<table>
<thead>
<tr>
<th>Protein concentration</th>
<th>Alpha TvD1</th>
<th>Native TvD1</th>
</tr>
</thead>
<tbody>
<tr>
<td>50(\mu)M</td>
<td>85.53 ± 2.3</td>
<td>78.05 ± 2.7</td>
</tr>
<tr>
<td>25(\mu)M</td>
<td>74.83 ± 3.1</td>
<td>60.0 ± 1.5</td>
</tr>
<tr>
<td>10(\mu)M</td>
<td>32.45 ± 1.2</td>
<td>16.04 ± 1.7</td>
</tr>
</tbody>
</table>

Figure 5.IV.11. Percent inhibition of the insect \(\alpha\)-amylase with the recombinant protein (native and Alpha TvD1).
Figure 5.IV.12. Barely $\alpha$-amylase inhibitory activity

Figure 5.IV.13. Human saliva $\alpha$-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 \( \beta \)-strand 2 and \( \beta \)-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 \( \alpha \)– 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 weerd 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 α-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 α- 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. unguiculata (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 (Ignacioimuthu 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.
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, Phytophthora 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, Phaeosariopsis personata, B. cinerea, Curvularia sp. Hence, the IC50 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 bioassay 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, T₀ seeds were collected and used for T₁ generation analysis. T₁ 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 activity 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.