

# Prokaryotic expression of a constitutively expressed *Tephrosia villosa* defensin and its potent antifungal activity

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**Abstract** Plant defensins are small, highly stable, cysteine-rich antimicrobial peptides produced by the plants for inhibiting a broad-spectrum of microbial pathogens. Some of the well-characterized plant defensins exhibit potent antifungal activity on certain pathogenic fungal species only. We characterized a defensin, TvD1 from a weedy leguminous herb, *Tephrosia villosa*. The open reading frame of the cDNA was 228 bp, which codes for a peptide with 75 amino acids. Expression analyses indicated that this defensin is expressed constitutively in *T. villosa* with leaf, stem, root, and seed showing almost similar levels of high expression. The recombinant peptide (rTvD1), expressed in the *Escherichia coli* expression system, exhibited potent in vitro antifungal activity against several filamentous soil-borne fungal pathogens. The purified peptide also showed significant inhibition of root elongation in *Arabidopsis* seedlings, subsequently affecting the extension of growing root hairs indicating that it has the potential to disturb the plant growth and development.

**Keywords** *Tephrosia villosa* · TvD1 · Constitutive expression · Prokaryotic expression · Antifungal activity · *Arabidopsis* root growth inhibition

## Introduction

Defensins represent a defense strategy in plants against invading pathogens, particularly in protein rich leguminous

species (Osborn et al. 1995). The characteristic feature of plant defensins is the presence of four disulfide bridges between eight cysteine residues within the mature peptide. Generally, defensins have a net positive charge (Broekaert et al. 1995) and are implicated in the front line of host defense (Thomma et al. 2002). The expression of plant defensin is sometimes tissue specific with varied levels in different tissues like HaDef1 in the roots of *Helianthus* spp. (Zélicourt et al. 2007); SD2 and SF18, in contrast, are localized in the inflorescences of the same plant (Urdangarín et al. 2000). Most of the defensins are seed specific, such as Pa-AMP of pokeweed (Liu et al. 2000), Mj-AMP-1 and Mj-AMP-2 of *Mirabilis jalapa* (De Bolle et al. 1995), SPE10 of *Pachyrrhizus erosus* (Song et al. 2005), Rs-AFP1 and Rs-AFP2 of *Raphanus sativus* (Terras et al. 1992). Some 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* plants express a leaf-specific defensin, PDF1.2 upon pathogen challenge (Thomma and Broekaert 1998).

It has also been demonstrated that defensins are induced by external stimuli such as biotic and abiotic stress signals including pathogen infection and methyl jasmonate treatment (Manners et al. 1998). In addition, some of the plant defensins are induced by environmental stimuli; cold in winter wheat induces the expression of defensin TAD1 (Koike et al. 2002) and the infestation of the parasitic plant *Orobancha* spp upregulates the gene for HaDef1 defensin in the roots of *Helianthus* spp (Zélicourt et al. 2007).

The mode of action of defensins is still not clear. Generally, leguminous defensins do not induce any morphological change, but the defensins of other families like Brassicaceae and Saxifragaceae may induce hyperbranching (Osborn et al. 1995). Defensins like MsDef1 inhibit the growth by blocking the mammalian L-type calcium ion channels, which are dependent on a calcium gradient.

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Although structurally similar to MsDef1, RsAFP2, and MtDef2 do not block the L-type calcium channels (Spelbrink et al. 2004). Defensins like DmAMP1 bind on the site M(IP)2C, which are present as patches on the hyphal membranes or alternatively required for anchoring of other membrane or cell-wall associated proteins, which themselves interact with DmAMP1 (Thevissen et al. 2000). Some similar antifungal peptides bind to the *N*-acetyl glucosylceramides present on the fungal plasma membrane (Thevissen et al. 2004). Fungal mutants lacking glucosylceramides are not sensitive to RsAFP-2. Recently, it was reported that plant defensins have roles in regulating plant growth and development. Defensin like MsDef1, MtDef2, and RsAFP2 inhibit the root growth in the germinating *Arabidopsis* at micromolar concentrations (Allen et al. 2007). In our reports also, we suggest the root growth inhibitory action of the defensin at the same concentration required for antifungal activity. Most of the bacterially expressed plant defensins were shown to be active as antifungal peptides at concentrations ranging from 100–200 µg/ml (Xu and Reddy 1997; Park et al. 2002; Olli and Kirti 2006; Hui et al. 2007; Olli et al. 2007; Solis et al. 2007).

The cultivated peanuts suffer seriously from the attack by the late leaf spot pathogen, *Pheoisariopsis personata* in many agro-climatic zones of the world and conventional crop improvement programs could not, so far, offer effective solutions to this problem. A defensin that could fight the late leaf spot infection in peanut is highly pertinent in plant biotechnology.

In the present study, we have observed the potent antifungal activity of the constitutively expressed defensin TvD1 purified from the *Escherichia coli* expression system against the target pathogen, *P. personata* and several other soil-borne fungal pathogens, which cause economically significant diseases such as *Fusarium oxysporum f. sp. vasinfectum* (vascular wilt of tomato), *F. moniliforme* (root rot of rice), *Rhizoctonia solani* (sheath blight of rice), *Phytophthora parasitica f. sp. nicotianae* (leaf rot of tobacco), *Curvularia spp* (leaf spots), *Botrytis cinerea* (grape rot) and *Alternaria helianthi* (black or gray leaf spot of sunflower). These observations are reported in this communication.

## Materials and methods

### Nucleic acid extraction, PCR amplification, and cloning

Genomic DNA was isolated from freshly frozen leaves of *Tephrosia villosa* plants collected from the field using the standard CTAB method (Doyle and Doyle 1990). Total RNA was also isolated from the leaves without any stress or pathogen treatment using Tri-reagent (Sigma-Aldrich, USA). Polymerase chain reaction was carried out for

amplifying the genomic clone and the open reading frame (ORF) of the cDNA of *TvD1* using primers designed on the basis of an alfalfa defensin (MsDef1). The primers used were forward: 5'GGGTACCATGGAGAAGAAATCAGTAGC 3' and reverse: 5' GGGATCCTTTAACATCTTTTAGTACACCA 3'. *KpnI* and *BamHI* sites were included (italicized) for future cloning steps in the desired vectors. For RT-PCR, the first strand of the cDNA was synthesized by reverse transcription with an oligo-(dT) primer and M-MLV reverse transcriptase enzyme (Sigma-Aldrich, USA). Amplification conditions for PCR were: 94°C for 4 min, 35 cycles: 94°C for 1 min, 58°C for 55 s, 72°C for 1 min and final extension was 72°C for 10 min.

The gel-eluted (Gel Clean-up Kit, Eppendorf, Germany) amplification products were cloned into the T/A cloning vector, pTZ57R (MBI Fermentas) and sequenced. Nucleotide and deduced amino acid sequence comparisons were made using the BLAST (Basic Local Alignment Search Tool) programs such as BLASTN (Zhang et al. 2000) and BLASTX (Altschul 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 was used to deduce the amino acid sequence coded by the gene *TvD1*.

### Expression and purification of *Tephrosia* defensin protein

The coding region of the *TvD1* gene was cloned between *KpnI* and *BamHI* sites in pET32a vector (Novagen, USA). The cloned vector was transformed into *E. coli* BL21 (DE3) pLys S and the transformed cells were grown at 37°C in LB Broth (Himedia, India) overnight at 200 rpm in a shaker. A 10-ml aliquot from the overnight culture was added into 1 l of fresh LB medium for 1 h till the OD<sub>600</sub> reached 0.6–0.8. These cells were induced using 0.4 mM IPTG (Fermentas, Germany). After 6 h incubation, the cells were harvested by centrifugation at 5,000 rpm for 5 min at 4°C. The pellet was washed with fresh 1× PBS buffer. Subsequently, the first lysis (prolysis) buffer (300 mM NaCl, 50 mM NaHPO<sub>4</sub>, 10 mM imidazole, 15% glycerol, and 0.1% SDS) was added and mixed homogeneously. The cells were lysed by sonication at 6× pulse for 5 min at 4°C (every 20-s pulse, a 10-s interval was given). The lysate was centrifuged at 12,000 rpm for 20 min to remove the debris. The supernatant was mixed with the second lysis buffer (300 mM NaCl, 50 mM NaHPO<sub>4</sub>, and 10 mM imidazole) along with 2 ml Ni-NTA (Qiagen, Germany), and the volume was made up to 50 ml/l culture of cells and mixed for 1 h at 4°C at 60 rpm in a rocker. The supernatant mixture was loaded on to the column (Sigma-Aldrich, USA) and the flow-through was collected at the rate of 0.5 ml/min, which was maintained till the end. Finally, the

column was washed with ten volumes of wash buffer (300 mM NaCl, 50 mM NaHPO<sub>4</sub>, 20 mM imidazole, and 15% glycerol). The recombinant peptide rTvD1, was eluted with elution buffer (300 mM NaCl, 50 mM NaHPO<sub>4</sub>, and 250 mM imidazole), according to the manufacturer's instructions (Qiagen, Germany).

#### Antifungal activity assay

Antifungal activity of rTvD1 was tested by microspectrophotometry 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 was pipetted into a well of a 96-well microtiter plate containing 90 µl of the test fungal spore suspension (~2.5×10<sup>4</sup> spores/ml) in potato dextrose broth (PDB), which was placed in an incubator at 28°C. Each protein concentration was tested for its antifungal activity in triplicate. Fungal spore germination and growth were observed microscopically and the optical density (OD) was measured with a microplate reader at a wavelength of 595 nm after inoculation for 30 min and 48 h. Controls were tested identically except that the protein was omitted. Value of the growth inhibition lower than 10% was 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 hr minus that at 30 min. IC<sub>50</sub> is defined as the protein concentration at which 50% inhibitions was 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)×100.

A graph plotting percent inhibition of fungal growth against the concentration of protein was used to determine the IC<sub>50</sub> of antifungal activity, i.e., the concentration producing 50% inhibition.

For the in vitro plate assay, fungal discs of uniform size were 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 in diameter, four sterile Whatman no.1 filter paper discs (1 cm diameter) were placed on the plate at equal distance from the center. Purified rTvD1 was added at various concentrations, (ranging from 25–100 µg/µl) 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 was placed at four places at equal distance from the centre of the PDA plate. The protein at respective concentration was added directly over the sclerotia and its effect on their germination and growth was observed.

#### *Arabidopsis* germination assay

The purified protein was diluted to desired concentration in 10 mM Tris-HCl pH 7.6. This dilution was then mixed with an equal volume of Murashige and Skoog basal salts (Murashige and Skoog 1962) with B<sub>5</sub> vitamins (Gamborg's vitamins) in a 96-well assay plate. A single sterilized Columbia seed was placed in each well and grown at 24°C under a 16 h photoperiod for 7 days (Allen et al. 2007). The experiment was carried out in triplicates.

## Results

#### 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 acids serving as signal peptide and the mature peptide of about 5.2 kDa (NCBI GenBank accession number AY907349 with an *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. 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.

#### Constitutive expression of TvD1

Using RT-PCR, the expression of *TvD1* gene was analyzed in seeds, roots, stems, young, and mature leaves,

<i>Tephrosia villosa</i>	-KTCENLADTYRGPFCFTTGSDDHCKNKEHLLSGRCRDDFR---CWCTKRC	100
<i>Cicer arietinum</i>	---CENLADTYRGPFCFTTGSDDHCKNKEHLVSGRCRDDFR---CWCTKNC	95
<i>Arachis hypogaea</i>	-ATCENLADTYRGPFCFTTGSDDHCKNKEHLLSGRCRDDFR---CWCTRNC	93
<i>Vigna radiata</i> (VrD2)	-KTCENLANTYRGPFCFTTGSDDHCKNKEHLRSGRCRDDFR---CWCTRNC	91
<i>Medicago sativa</i>	-KTCENLADTFRGPFCFTNGACDDHCKNKEHLVSGRCRDDFR---CWCTRNC	87
<i>Trigonella foenum-graecum</i>	-KTCENLADKYRGPFCF--SGCDTHCTTKEHAVSGRCRDDFR---CWCTKRC	82
<i>Medicago truncatula</i>	-KTCENLADKYRGPFCF--SGCDTHCTTKENAVSGRCRDDFR---CWCTKRC	80
<i>Arachis diogeni</i>	-ATCENLADTFRGPFCFGNSNCFHCKTKEHLLSGRCRDDFR---CWCTKRC	80
<i>Pisum sativum</i>	-KTCEHLADTYRGPFCFTNASCDDHCKNKAHLISGTCHN-WK---CFCTQNC	69
<i>Vigna radiata</i> (VrD1)	-RTCMIKKEGW-GKCLIDTTCAHSCK--NRGYIGGNCKGMTR--TCYCLVNC	19

**Fig. 1** Comparison of the mature peptide sequence of TvD1 with mature defensin peptides characterized in legumes. Alignment of the sequences of some of the legume defensins sequences available in the GenBank like 2GL1\_A (*V. radiata* VrD2); DQ296045.1 (*A. hypogaea*); DQ342338.1 (*C. arietinum*); AY907349.1 (*T. villosa*); AAG40321 (*M. sativa*); AY313166.1 (*Medicago truncatula*); AY227192.1 (*T. foenum-graecum*);

AY288448.1 (*Arachis diogeni*); P81929.1 (*Pisum sativum* PsD1) NM\_128160.1 1TI5\_A (*V. radiata* VrD1) was performed using CLUSTAL-W program available at <http://www.ncbi.nlm.nih.gov>. TvD1 shows 95% sequence identity with *Cicer* defensin, 92% identity with *A. hypogaea* defensin and 91% identity with VrD2 of *V. radiata*

and flowers of the plant (Fig. 2). Interestingly, this defensin is constitutively expressed in all the organs, however, with higher levels of expression in young and mature leaves.

#### In silico characterization of TvD1 peptide

We constructed the three-dimensional structure of the mature portion of TvD1 (29–75 amino acid residue region) using standard protein modeling. The NMR structure of the defensin, VrD2 from *V. radiata* identified with an *E* value =  $5e-11$  (PDB\_ID: 2GL1, Lin et al. 2007) shared 91% pairwise 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  $\beta$ -strands and one  $\alpha$ -helix held together with four cysteine disulphide bridges as in the case of VrD2 (Fig. 3).

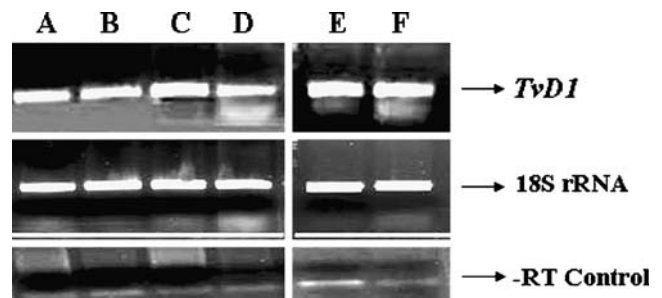
#### Prokaryotic protein expression and purification

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. 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.

#### In vitro antifungal assay

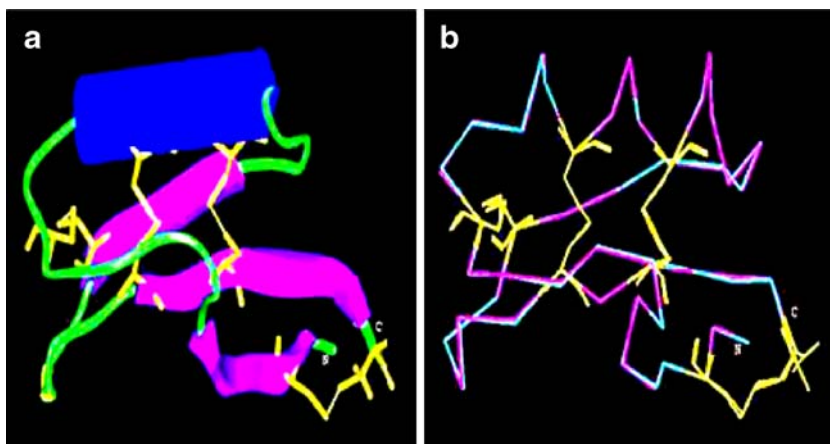
Antifungal activity of the rTvD1 was studied using various assays. It was observed that the antifungal activity of this protein was strongly dependent on the target fungus. Even though the peptide showed superior antifungal effect in comparison to the known defensins, the concentration at which the peptide inhibited the growth of the fungus was not uniform and varied with the fungal pathogen under consideration. In the spore germination assay (Fig. 5), the growth of some fungal pathogens was inhibited at very low concentrations (10–25  $\mu\text{g/ml}$ ) and a moderately higher concentration was needed for inhibition of other pathogens (50  $\mu\text{g/ml}$ ) (Table 1). Among the fungal species tested, the peanut late leaf spot fungus *P. personata* appeared to be the



**Fig. 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 the cDNA of *TvD1* using total RNA as template without reverse transcription



**Fig. 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  $\alpha$ -helix, pleated sheets represent  $\beta$ -strand and yellow-colored structures denote the cysteine bridges. In the stick model (b), the yellow-colored structures represent the cysteine bridges



most sensitive with  $IC_{50} < 10 \mu\text{g/ml}$ . The antifungal activity was assessed in other fungal pathogens like *F. oxysporum*, *F. moniliforme*, *P. parasitica*, *B. cinerea*, *A. helianthi*, and *Curvularia sp* with  $IC_{50} < 25 \mu\text{g/ml}$ . Hyperbranching of the mycelium was also observed, when the spores of *F. oxysporum* were germinated in the presence of  $10 \mu\text{g/ml}$  of rTvD1 (Fig. 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  $38 \mu\text{g/ml}$  (Fig. 6, Table 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.

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

#### *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. 8), and it was inhibited in a dose-dependent manner (Table 2). At  $10 \mu\text{g/ml}$  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  $50 \mu\text{g/ml}$  and above. It has also been noticed that the protein affected the number and extent of lateral root formation.

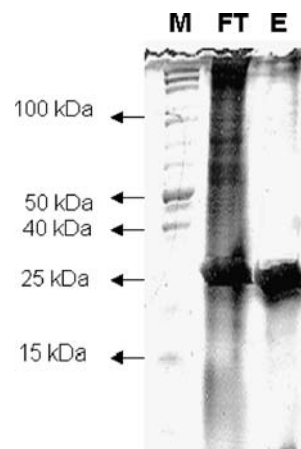
## Discussion

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

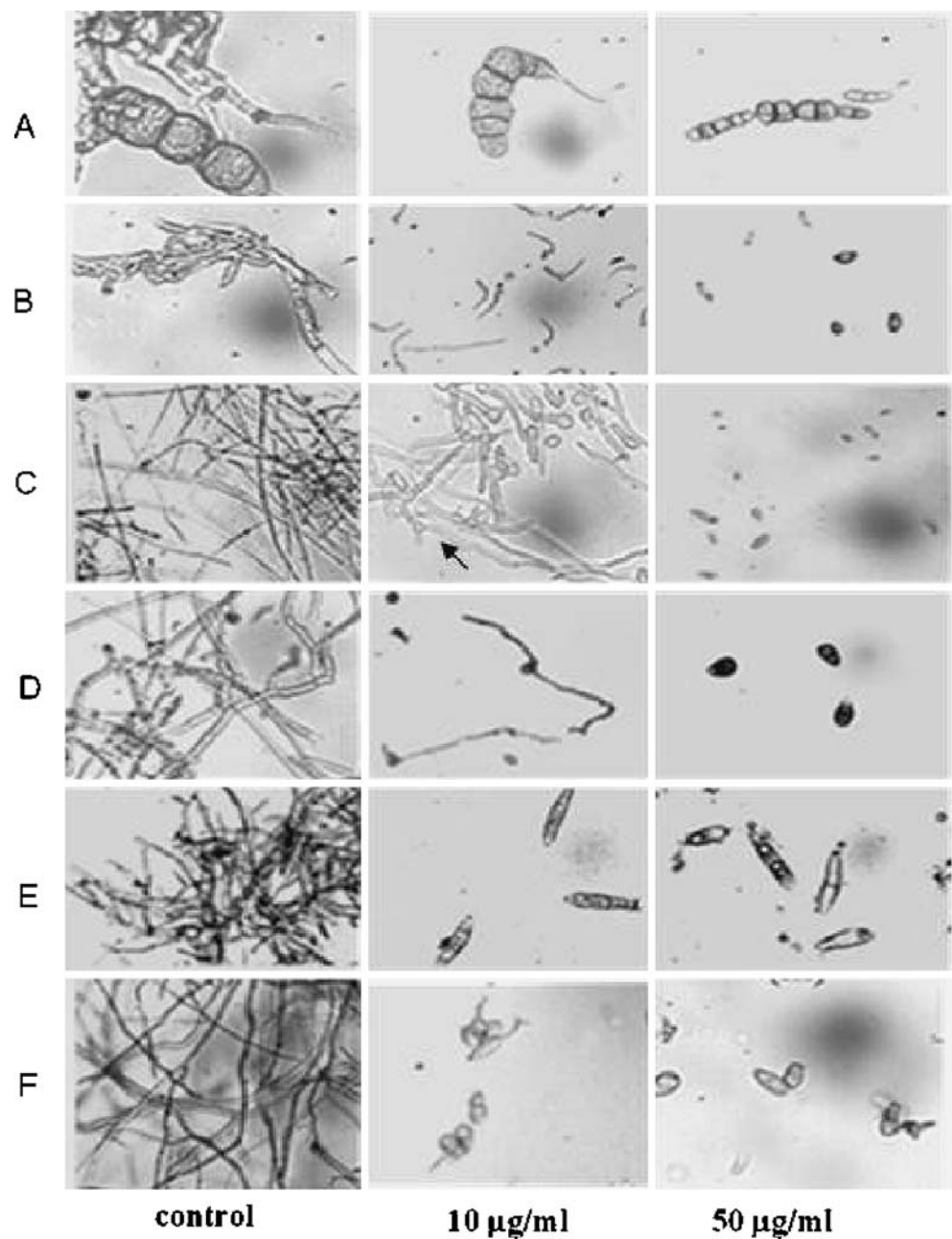
biotechnology for enhancing disease resistance in crop plants (Osusky et al. 2000; Punja 2001).

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  $\alpha, \beta$ -fold. This fold belongs to the Knot-1 superfamily (INTERPRO ID: IPR003614). Examination of the model structure indicated that more than 95% residues were 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  $0.15 \text{ \AA}$  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 can be observed that His residue at position 29 in Psd1 is also conserved in TvD1 and the conserved Phe residue at position 41 is conservatively

**Fig. 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



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



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 secretion 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

**Table 1** Antifungal activity assay of the recombinant protein TvD1 using spores/conidia of some fungal pathogens

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

The IC<sub>50</sub> value for *Rhizoctonia solani* (38  $\mu\text{g/ml}$ ) was calculated from the graph plotting percentage growth inhibition vs. protein concentration in the in vitro plate assay. Data represent mean $\pm$ S.D.,  $n=3$ ;

<sup>a</sup> Protein concentration required for 50% growth inhibition (IC<sub>50</sub>) after 48 h of incubation was determined from the dose–response curves (percentage growth inhibition vs. protein concentration)

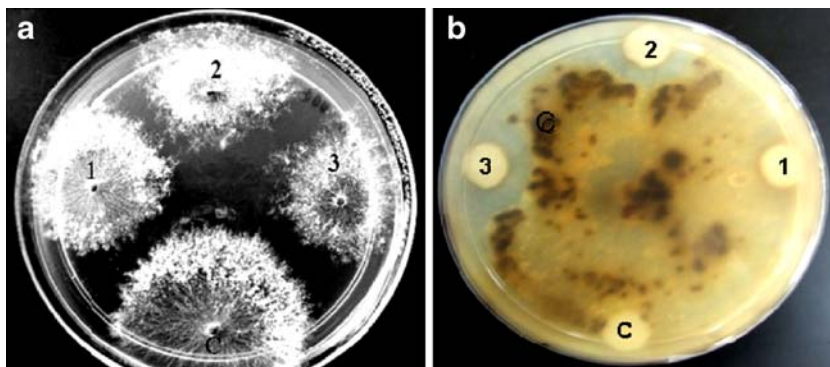
signal. However, the antifungal activity of the expressed protein was very low, requiring a dose up to 250  $\mu\text{g/ml}$  of TDEF1 to have an effect on *F. oxysporum* (Hui et al. 2007). In the present study, the *E. coli*-expressed TvD1 defensin protein with its signal peptide was purified efficiently as a recombinant protein with significant antifungal activity at comparatively lower concentrations.

The assay of antifungal activity of the rTvD1 has shown that the 100- $\mu\text{g/ml}$  concentration was sufficient to form an inhibitory zone for all the tested fungi. It was 100 and 150  $\mu\text{g/ml}$  for Tfgd1 and Tfgd2 defensins, respectively, from *T. foenum-graecum* against *R. solani* and *F. moniliforme* (Olli and Kirti 2006; Olli et al. 2007). The IC<sub>50</sub> value of Lm-Def was observed to be 100  $\mu\text{g/ml}$  against *P. infestans* (Solis et al. 2007). The other *E. coli*-expressed PR proteins such as a chitinase from *Sorghum* required higher concentration such as 300  $\mu\text{g/ml}$  against five 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  $\mu\text{g/ml}$  concentration. It was observed that 100  $\mu\text{g/ml}$  concentration of the recombinant protein (Tfgd1) was an inhibitory factor for spore germination and hyphal growth even after 48 h for the conidia of *P. personata* (Olli and Kirti 2006).

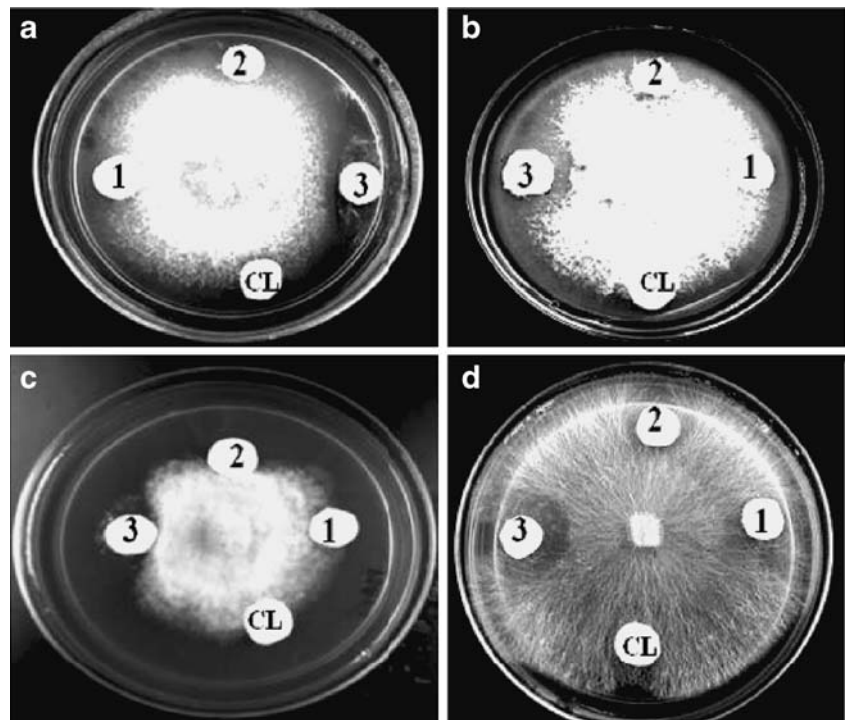
However, similar growth with slight inhibitory effect was observed in the case of *F. oxysporum*, *B. cinerea*, *F. moniliforme*, *A. helianthi*, and *Curvularia sp.* at 10  $\mu\text{g/ml}$  of TvD1, but higher concentrations were needed for 50% inhibition. The IC<sub>50</sub> value of *R. solani* was 38  $\mu\text{g/ml}$  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 against

**Fig. 6** Effect of TvD1 on growth of *R. solani* sclerotia. C, 1, 2, and 3 represent control, 25  $\mu\text{g/ml}$ , 50  $\mu\text{g/ml}$ , and 100  $\mu\text{g/ml}$ , respectively. **a** Protein at respective concentration was directly added over the sclerotium and photograph was taken after 48 h of growth and **b** protein was added on discs at respective concentrations and formation of sclerotia was observed after 3 weeks



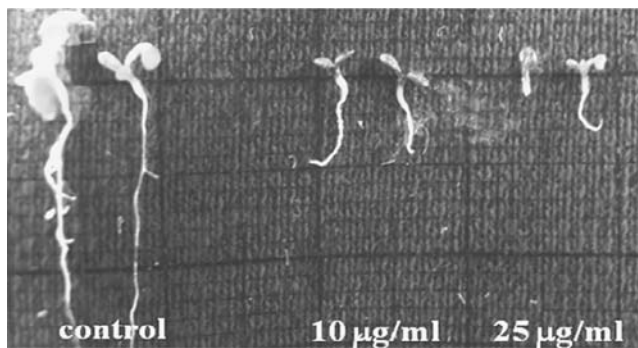


**Fig. 7** Effect of TvD1 defensin on growth of the fungus, where CL, 1, 2, 3 are control (buffer), 25  $\mu\text{g/ml}$ , 50  $\mu\text{g/ml}$ , and 100  $\mu\text{g/ml}$ , respectively. **a** *Phytophthora parasitica* f. sp. *nicotiana*, **b** *F. moniliforme*, **c** *B. cinerea*, and **d** *R. solani*



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 (Schieffelbein et al. 1992; Felle and Hepler 1997). Recombinant TvD1 was able to inhibit root elongation and lateral root formation at 10  $\mu\text{g/ml}$ . The effect was more prominent at higher



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

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 halted the extension of growing root hairs (Allen et al. 2007). Hence, the present defensin has the potential to control the development and growth of the plant.

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  $\mu\text{g/ml}$  against some fungal pathogens, *TvDI* appears to be a potent defensin gene for fungal disease resistance in transgenic crops.

**Table 2** *Arabidopsis* seed germination assay using rTvD1

Sl. no.	Concentrations of the protein ( $\mu\text{g/ml}$ )	Length of the Root (cm)
1	Control (buffer)	1.7 $\pm$ 0.2
2	10	0.5 $\pm$ 0.1
3	25	0.3 $\pm$ 0.1
4	50	No germination

Data represent mean $\pm$ S.D.,  $n=3$



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