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Characterization of defensin (*Tfgd2*) from *Trigonella foenum-graecum*

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Defensins are small cysteine-rich peptides with a size of 5–10 kDa, and some of them exhibit antifungal activity. We have cloned and sequenced a 219 bp coding region of the cDNA of a defensin from *Trigonella foenum-graecum*, designated as *Tfgd2* using primers designed on the basis of a defensin, *AlfAFP* from *Medicago sativa* and reverse transcription-PCR. We have cloned the 701 bp genomic region of the defensin that comprised two exons and one long intron. The deduced amino acid sequence of Tfgd2 was similar to AlfAFP, except for two amino acid substitutions. It has 50% homology with the antifungal defensin *Psd1* from *Pisum sativum*, whose NMR solution structure has been determined. The mature peptide has 45 amino acids, while the signal peptide comprised 27 amino acids. Southern analysis of the genomic DNA blot indicated that the defensins appear to be an oligo-gene family with at least two members in *Trigonella*. Purified peptide from *Escherichia coli* expression displayed inhibitory activity against broad-spectrum fungal pathogens, *Rhizoctonia solani* and *Fusarium moniliforme*.

Keywords: Antifungal activity, cysteine-rich peptide, defensin, *Trigonella foenum-graecum*.

AMONG the antimicrobial peptides (AMPs), plant defensins are particularly important for frontline host defense against fungal pathogens. They are thought to be members of small gene families and are rich in conserved cysteine residues. All members of this family adopt a comparable global fold centred on the CS$\alpha\beta$ motif, but relatively few amino acid residues are absolutely conserved between all members[1]. This motif is also found in insect defensins and scorpion neurotoxins[2–4]. Despite their structural similarity, plant defensins are highly varied in their primary amino acid sequences, with only eight structure-stabilizing Cys residues in common[5]. In many cases, small differences in amino acid sequence can predict the specificity of the role of defense[6]. Variation in the primary sequences may account for the different biological activities reported for plant defensins, including antifungal activity[7], antibacterial activity[8], protease activity[9], and α-amylase inhibitory activity[10].

We have cloned the genomic region of *Tfgd2* from *Trigonella foenum-graecum* using the genomic DNA with

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an amplified fragment of 701 bp (NCBI GenBank accession no. AF535089) using the forward primer 5′-GGGTACCATGGAGAAGAAATCACTAGC-3′ and reverse primer 5′-GGGATCCTTAACATCTTTTAGTACACCA-3′. The primers contained the restriction enzyme sites *Kpn*I and *Bam*HI to facilitate cloning of the amplification product into suitable vectors for further characterization. We have also cloned the coding region of its 219 bp cDNA using total RNA and reverse transcription PCR (AY227192), sequenced it, and expressed it in *Escherichia coli* using an expression vector. We have checked the *in vitro* antifungal activity of the expressed protein carrying the His-tag. These observations are presented in this communication.

Since defensins are expressed after methyl jasmonate treatment[11], total RNA was extracted from the leaf tissue from ten-day old *Trigonella* seedlings after treatment with 30 µM methyl jasmonate for 24 h using the Tri-Reagent (Sigma-Aldrich) following the manufacturer's instructions. The final RNA pellet was dissolved in formamide. Basic molecular biology techniques were derived from Sambrook *et al.*[12]. Isolated RNA was reverse-transcribed and amplified with the above-mentioned oligonucleotide primers for defensin coding region based on *AlfAFP*, a defensin from *Medicago sativa*, whose antifungal nature has been demonstrated[13]. The gel-eluted amplification product was cloned into a T/A cloning vector, pTZ57R (MBI Fermentas) and sequenced, and its nucleotide and deduced amino acid sequence comparisons were made using the BLAST (Basic Local Alignment Search Tool) program on the non-redundant databank of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/). Expasy tool was used to deduce the amino acid sequence of Tfgd2.

The coding region of *Tfgd2* from *Trigonella* has a length of 219 bp. The deduced sequence has 72 amino acids. The N-terminal portion of the peptide has a 27 amino acid residue-long signal peptide and the mature protein has 45 amino acids. The cleavage site for the signal peptide was predicted using the SignalP program (signalp@cbs.dtu.dk). The signal peptide of Tfgd2, predicted using the TargetP program (http://www.cbs.dtu.dk/services/TargetP/) is a secretory signal peptide.

Genomic DNA was isolated from *Trigonella* using the CTAB method[14]. Using the genomic DNA for amplification with the above PCR primers, a 701 bp fragment of the genomic region of the defensin gene has been amplified. Upon cloning and sequencing, it was observed to contain two exons spanning 1–58 and 541–701 bp, while the internal region between these two exons represented the intron. The intron was characterized by conserved splice sites at both ends. Southern hybridization analysis of the genomic DNA digests was done using DNA samples digested with *Hin*dIII and *Eco*RV to assess the number of gene copies for defensin in *Trigonella* (Figure 1). Two fragments were observed to hybridize to the probe in *Trigonella* indicating that the defensin belonged to an oligo-gene family with at least two members.

In order to identify defensin sequences of known tertiary structure, we searched the Protein Data Bank (PDB)[15] with the WU-BLAST program (available at www.ebi.ac.uk). Multiple sequence alignment (Figure 2) corresponding to defensins, including Tfgd2 and the sequence corresponding to the defensin of known tertiary structure (Psd1) was carried out using the CLUSTAL W program[16]. The signal peptide region of the protein was identified using SignalP program[17]. The comparative modelling methods in the MODELER program[18] were used for constructing the three-dimensional models[19]. Homology searches using the WU-BLASTP program against the PDB database identified the NMR structure of pea defensin Psd1 from *Pisum sativum*[20], with an e-value corresponding to $9.7e^{-12}$ and sharing about >50% sequence homology.

The overall three-dimensional fold of 1JKZ comprised three β-strands and one α-helix held together by four disulphide bridges to form a cysteine stabilized α,β-fold. This fold belongs to the knot1 super family (InterPro database)[21]. We therefore used the three-dimensional structure of 1JKZ for modelling structure of Tfgd1, Tfgd2 and AlfAFP, whose antifungal activity has been demonstrated in transgenic plants[13]. These models were evaluated with the PROCHECK program[22]. This program identifies amino acid residues in the most favoured region of the Ramachandran map[23]. From the multiple sequence alignment as indicated in Figure 2, we observed that the position of cysteine residues was highly conserved in this protein family. Comparison between Tfgd1 and Tfgd2 from *Trigonella*, AlfAFP of *M. sativa* and pea seed

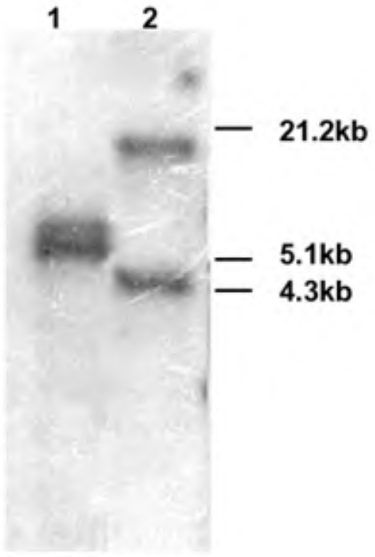

Figure 1. Southern analysis showing the number of defensin genes present in *Trigonella foenum-graecum* DNA samples were digested with *Eco*RV (lane 1), and *Hin*dIII (lane 2). Blot was probed with the radioactive $^{\alpha\text{-}32}$P-labelled RT-PCR-amplified 219 bp fragment.

defensin, Psd1 indicated conservation of several charged, neutral and hydrophobic residues. Significant amino acid residue changes were also noticed, such as His5 in Psd1 was mutated to Asn in Tfgd2, Thr9 to Lys, Asp22 to Thr, Lys25 to Thr, Ala28 to Glu, Thr34 to Arg, Asn37 to Asp, Gln44 to Lys, and Asn45 to Arg. The structural overlay of these proteins as shown in Figure 3 indicates that the structures are highly similar and the four disulphide bridges are highly superimposable with an overall RMSD of 0.6 Å. However, there appeared to be significant difference in loops 1 and 3 of the superimposed structure and this appeared to be because of the amino acid substitutions as described earlier. These observations indicate that in spite of high sequence similarity and conservation of the overall fold, the specific amino acid residue mutations mentioned above might be responsible for the specific biological action of proteins[7–10]. Almeida *et al.*[20] reported that two amino acid residues, His29 and Phe41 in Psd1 might distinguish between the plant defensins with and without antifungal activity. We observed that the His29 residue was conserved in Tfgd2 and Psd1, whereas Phe41 of Psd1 had been replaced by Trp(W) in Tfgd1, Tfgd2 and AlfAFP. It is interesting to note that His29 in Psd1 was also substituted by Asn in *M. sativa* and still the latter retained the antifungal activity[13].

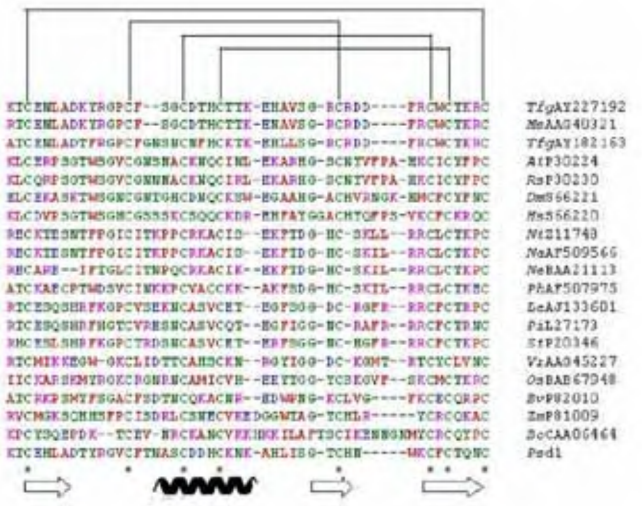

Figure 2. Multiple sequence alignment of amino acid sequence of *Trigonella* defensins (Tfgd2–*Tfg*AY227192, Tfgd1–*Tfg*AY182163) with other known defensins from *Pisum sativum* (Psd1), *Medicago sativa* (*Ms*), *Arabidopsis thaliana* (*At*), *Raphanus sativus* (*Rs*), *Dahlia merckii* (*Dm*), *Heuchera sanguinea* (*Hs*), *Nicotiana tabacum* (*Nt*), *Nicotiana alata* (*Na*), *Nicotiana excelsior* (*Ne*), *Petunia hybrida* (*Ph*), *Lycopersicon esculentum* (*Le*), *Petunia integrifolia* (*Pi*), *Solanum tuberosum* (*St*), *Vigna radiata* (*Vr*), *Oryza sativa* (*Os*), *Beta vulgaris* (*Bv*), *Zea mays* (*Zm*) and *Brassica oleracea* (*Bo*). Gaps have been introduced to maximize sequence similarity. α-Helices and β-strands (hollow arrows) are indicated below the sequences. * indicates 100% conservation of amino acid residue. The eight cysteine residues are highly conserved. The disulfide connectivity pattern is indicated.

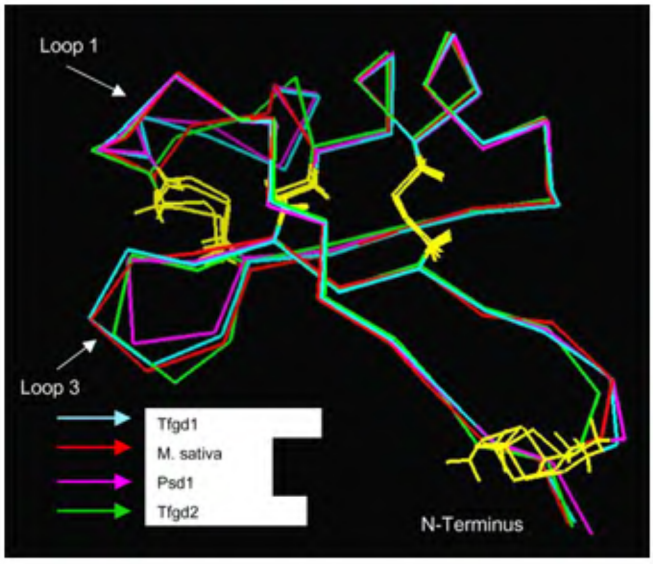

Figure 3. The backbone superposition of Tfgd1, Tfgd2, AlfAFP and 1JKZ structures. The four disulphide bridges shown in stick representation are highly conserved.

The 219 bp cDNA was isolated from *Tfgd*/pTZ57R-T plasmid by digestion with *Kpn*I and *Bam*HI and ligated into the *Kpn*I/*Bam*HI digested pET32a (Novagen, USA) in frame to the T7 promoter, His-tag and S-tag. This plasmid was then transformed into *E. coli* BL21 (DE3) pLysS cells for protein expression and purification. Tfgd2:His-tag protein production was induced by adding 1 mM IPTG to *E. coli* culture. The cleared supernatant was purified on a 5 ml Ni-NTA His-Bind resin column (Novagen). Then, 1 ml fractions were collected and the purification profile was checked on 18% denaturing polyacrylamide gel[24]. Further purification of the partially purified protein was performed by gel filtration chromatography using a Sephadex G-50 column (Amersham Biosciences, UK) according to the manufacturer's instructions. Purity

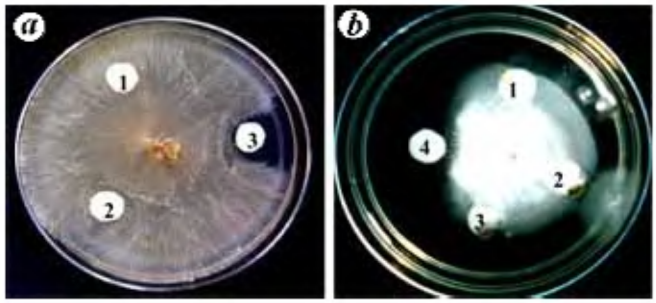

Figure 4. ***a***, *In vitro* antifungal assay of Tfgd showing inhibition of mycelial growth of the broad host-range fungus, *Rhizoctonia solani*. Disc 1, Control [extraction buffer (50 mM Tris pH 7.5; 150 mM NaCl)]; Disc 2, 100 µg Tfgd2; Disc 3, 150 µg Tfgd2. ***b***, *In vitro* antifungal assay of Tfgd showing inhibition of mycelial growth of *Fusarium monoliformae*. Disc 1, Control extraction buffer (50 mM Tris, pH 7.5; 150 mM NaCl); Disc 2, 50 µg Tfgd2; Disc 3, 100 µg Tfgd2; Disc 4, 150 µg Tfgd2.

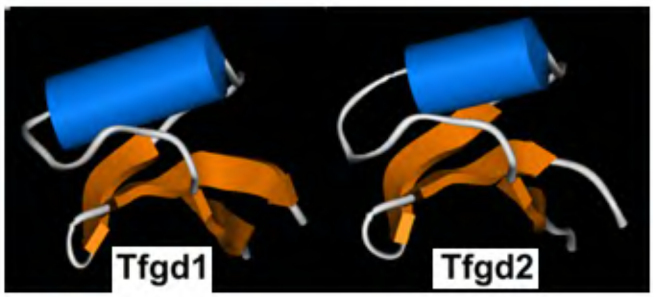

Figure 5. Three-dimensional structure of Tfgd1 and Tfgd2. Note longer α-helix in Tfgd1.

of the fractions was checked by SDS–PAGE. Protein concentration was determined using Lowry's method and the protein was used in the antifungal assay.

Standard assays were carried out in petri dishes containing about 20 ml of potato dextrose agar. For the assay, a piece of agar containing frontal mycelia of *Rhizoctonia solani* and *Fusarium moniliforme* was placed in the centre of the plate. The plates were incubated for 6 h at 24°C. After the first incubation period, sterile paper discs (3 mm, Whatman paper) were placed at a distance of 0.5 cm around the frontal mycelia. Different concentrations of the protein sample were added to each disk and the plates were incubated at 24°C for approximately 36 h until mycelial growth had enveloped peripheral discs containing control buffer and had formed crescents of inhibition around discs containing an effective concentration of Tfgd2. The extraction buffer (50 mM Tris, pH 7.4; 150 mM NaCl) was used as control. It is apparent from Figure 4 that the recombinant protein inhibited mycelial spread of the phytopathogenic fungi at a concentration of 150 µg.

We have earlier reported on the cloning and antifungal activity of *Tfgd1* from *Trigonella* with a coding region of 225 bp and a predicted peptide having 74 amino acids[25]. Similarity between Tfgd1 and the presently investigated Tfgd2 is 80%, while Tfgd2 shares 95% homology with AlfAFP from *M. sativa*. When the three-dimensional structure of Tfgd1 and Tfgd2 were modelled (Figure 5), it was observed that the former possessed a longer α-helix compared to the latter. It could be observed that while the crescent of fungal growth inhibition was observed at 100 µg/ml concentration for Tfgd1 (ref. 25), Tfgd2 exhibited similar fungal growth inhibition at 150 µg/ml concentration, indicating that Tfgd1 exhibits more antifungal activity compared to Tfgd2. It is interesting to see if this could be related to the longer α-helix.

Further work is in progress with regard to the action of the present defensin in fungal growth inhibition and a detailed comparison of both the defensin proteins from *Trigonella*.

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RAPD pattern of *Costus speciosus* Koen ex. Retz., an important medicinal plant from the Andaman and Nicobar Islands

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***Costus speciosus*, an important medicinal plant species found in the Andaman and Nicobar Islands, was collected from 14 localities through recurrent survey. RAPD–PCR analysis involving 12 decamer random primers was used to assesses the quantum of genetic variation at genomic level. Four primers showed appreciable intra-species variation or molecular polymorphism at amplicon levels. Despite morphological identity, a great deal of polymorphism was observed among the accessions. UPGMA analysis showed ~35% variation in the collections, which is deemed to be useful in formulating sound conservation strategies for this precious medicinal plant species under the humid tropics of Bay Islands.**

Keywords: *Costus speciosus*, conservation strategies, primers, RAPD–PCR analysis.

THE Andaman and Nicobar Islands represent a biological paradise. Biological diversity in this territory is reported to be immense, be it in flora, fauna or microbes. About 2500 angiosperm species are available in these islands, of which 10% (245 species) is said to be endemic[1]. A large number of plants have medicinal values. Since time immemorial, such plants are being used by the tribes and aboriginals in this remote region. Modern medicine could hardly reach the people, especially those dwelling in the far-flung remote villages amidst deep forests. Among the endemic species, 52 plants are used in medicaments to cure diverse ailments. They are of interest, being unique and not available elsewhere. Primitive aboriginals inhabiting these islands use a host of medicinal plants[2–4] for sustaining their livelihood.

Costus speciosus Koen ex. Retz. (Figure 1) belongs to family Zingiberaceae. It is known as 'Keu' or 'Kust' locally, 'Keukand' in Hindi and 'Kustha' in Sanskrit. The name *Costus* has been derived from Sanskrit. It is a succulent perennial herb, growing up to 2.7 m high and having an erect stem. The plant possesses horizontal rhizomatous rootstock. It generally grows luxuriantly on clayey loam soil near inland forest under moderate shade. The plant propagates vegetatively through rhizomes or via seeds dispersed by birds. Rhizome is the plant part used as medicine. In ayurveda the rhizomes were ascribed to be bitter, astringent, acrid, cooling, aphrodisiac, purgative, antihelminthic, depurative, febrifuge, expectorant and tonic.

Figure 1. *Costus speciosus* plant (above) and closer view of flower (below).

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