Isolation and In Silico Characterization Of Plant Defensin, Ctd1, From The Tropical Forage Legume (Clitoria Ternatea L.)

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ABSTRACT

A plant defensin, ctd1, was isolated from the tropical forage legume, Butterfly Pea (Clitoria ternatea L.). The full-length cDNA consists of a 228 bp open reading frame with 75 amino acids, of which the N-terminal 28 amino acids constitute the signal peptide and 47 amino acids constitute the mature peptide. A characteristic of this protein is eight cysteine residues forming four disulfide bridges (Cys14-Cys35, Cys20-Cys41, Cys24-Cys43 and Cys3-Cys47) to stabilize the mature peptide. The phylogenetic based on the mature peptide was inferred using neighbor-joining method found Ctd1 clustered with other Phaseoleae species. The γ-core motif identified in this protein highlights the important molecules of Ctd1 peptides and hence appears as a suitable candidate for deployment in transgenic crops for disease resistance.

INTRODUCTION

Plants have developed diverse mechanisms for defense against pathogenic organisms and environmental stresses to enable continued survival. These mechanisms include the production of secondary metabolites, the rapid oxidative burst and inducible physical barriers as well as the activation of defense-related genes [35,1,3]. In plants, three main groups of antimicrobial peptides are produced: defensins, thionins and lipid transfer proteins [31]. These small antimicrobial peptides act as a natural defense system and contribute to a wide spectrum of host defenses against pathogens [34,3].

The first research into plant defensins revealed proteins with antimicrobial activity, isolated from the radish (Raphanus sativus L.) seed, Rs-AFP1 and Rs-AFP2 [43]. Defensins are small in size (5-6 kDa), basic, cysteine rich peptides containing 45-54 amino acids, stabilized by eight cysteine residues that form four conserved disulfide bridges [4,41,34]. Plant defensins have been identified, isolated and characterized in various plant species from a broad range of plant tissue such as leaves [41], pods [6], tubers and floral organs [26,13,21]. Furthermore, plant defensins are highly prevalent in seeds as a protection for vulnerable germinating seeds against pathogens [7,25,4,20].

Plant defensins are described to have a varied array of biological activities such as antifungal and antibacterial activities [1,26,11,45,9], protein translation inhibition [5], ion channel blocker activity [17] anti-cancer activity [22,23] and they have been noted to reduce in vitro HIV1 reverse transcriptase activity [27]. Plant defensins also have the potential to be used as a plausible gene source for genetic manipulation for crop improvement. Genetic modification for disease resistance in rice using the Brassica oleracea and B. campestris defensin gene demonstrated high levels of resistance against both rice blast and bacterial leaf blight caused by Magnaporthe grisea and Xanthomonas oryzae respectively [14]. Additionally, the expression of novel defensin genes isolated from the seeds of alfalfa (Medicago sativa) in transgenic potato delivered a high level of field resistance towards the fungal pathogen Verticillium dahliae [10].

Clitoria ternatea, commonly known as Butterfly pea, Clitoria, Kordofan pea (Sudan), Aparajit (India) or Cunda (Brazil) is a species of legume in the Fabaceae family [12]. These plants are native to tropical equatorial Asia and have been introduced and distributed in the East and West Indies, South and Central America, China and India. These plants are well known in traditional medicine, particularly in Ayurveda as a “brain tonic” to increase overall functioning of the brain [12]. Research into these plants suggests there is a large potential for

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pharmacological application such as anti-microbial [42,15], antipyretic [30], anti-inflammatory [29], antidiabetic [40], anti-epileptic [39] and anthelmintic uses [8,17].

In a previous study, a cysteine-rich antimicrobial protein (Ct-AMP1) was isolated from the seed of *Clitoria ternatea* [28] and was shown to possess antifungal and antibacterial activity towards a range of pathogens. Due to the high potential of *Clitoria ternatea* defensins, in this study we report the isolation and *in silico* characterization of the defensin protein (Ctd1) from *Clitoria ternatea* leaves treated with methyl jasmonate for application in the transgenic expression leading to protection against pathogen attack.

**MATERIAL AND METHODS**

**Extraction of Total RNA:**
Ten day old germinating seeds of *Clitoria ternatea* were treated with 0.05% methyl jasmonate (Sigma-Aldrich, USA) for 24 hours to induce expression of defensins [31]. Total RNA was extracted from the leaf tissue of the seedlings using an RNA Extraction Kit (Qiagen, Germany) according to the manufacturer’s protocol. The RNA pellet was dissolved in sterile DEPC water and the total RNA was then used for cDNA synthesis using SuperScript III<sup>®</sup> first strand synthesis System for RT-PCR (Invitrogen, USA).

**PCR Amplification, cDNA Cloning and Sequence Analysis of Ctd1 Gene:**
PCR primers were designed based on the complete sequence of the defensin gene from six legumes available in the GenBank database (Cicer arietinum [DQ288897], Trigonella foenum-graecum [AY182163], Medicago sativa [AF319468], Arachis diogoi [AY288448], Cajanus cajan [AY244556], Tephrosia villosa [AY907349]), Cld1<sup>TM</sup> (GAATTCTCAACTCTGATGCTGCAATATAAAGCAGTAACAGAAGCA). PCR amplification was carried out in 25 μl reactions for 40 cycles using thermostable DyNAzyme<sup>TM</sup> EXT DNA polymerase (Thermo Scientific) in a PTC-200 thermal cycler (MJ Research, USA). The reaction mixture consisted of 1X PCR buffer; 2.0 mM of MgCl₂, 0.2 mM of each dNTP, 2 μM of forward and reverse primers, 0.5 μg of cDNA as a template and 2.5 U of the enzyme mix. The amplification reaction was conducted using the following cycle conditions: initialization step of 3 min at 95°C, followed by 35 cycles of 30 seconds at 94°C for the denaturation step, 1 min at 45°C for the annealing step, 1 min at 72°C for the elongation step, and a final extension of 10 min at 72°C. PCR products were run on 1.3% agarose gel and purified with QIA Quick Gel Extraction Kit (Qiagen, Germany). The purified PCR product was cloned directly into a vector using TOPO TA Cloning<sup>®</sup> Kit (Invitrogen, USA). Plasmids DNA of recombinant clones were isolated using QIAprep Spin miniprep kit (QIAGEN, Germany). The clones were sequenced commercially.

**Bioinformatic Analysis:**
Sequence similarity searches were completed using Basic Local Alignment Search Tool (BLAST) on the non-redundant data bank of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov.BLAST/). SignalIP 4.1 tool was utilised to deduce the amino acid sequence of the defensin [32]. The deduced amino acid sequence of Ctd1defensin and other defensin genes obtained from the GenBank database were aligned by clustalW2 and analysed by the MEGA5 program ([33](http://www.clustal.org/clustalw/)). Phylogenetic trees were inferred using the Neighbor-Joining (NJ) method [37] according to evolutionary distances generated using the p-distance method. Further bioinformatic analysis was performed to generate the theoretical three dimensional structure of Ctd1 deduced amino acids using Phyre [38](http://www.sbg.bio.ic.ac.uk/phyre) [16] and additional bioinformatics analysis of the model generated was done using RAMPAGE [24].

**RESULTS AND DISCUSSION**

The defensin gene, *ctd1*, from *C. ternatea* leaves treated with methyl jasmonate was successfully isolated by PCR using primers corresponding to six plant defensins in the Fabaceae family. The PCR products were then purified, ligated, transformed into E. coli TOP 10 and sequenced. The coding region of *ctd1* was determined to be 228 bp in length, encoding 28 amino acid residues for the signal peptide and 47 amino acid residues for the deduced mature Ctd1 peptide (Figure 1). Comparative analysis of Ctd1 deduced amino acid sequences with other members of tribe Phaseoleae (Faboideae: Fabaceae) found eight cysteine residues, forming four disulphide bridges conserved in the various plant defensins (Figure 2).
Fig. 1: Nucleotide sequence of ctd1 gene (JQ314215). The predicted amino acid sequence is shown below the corresponding codons. The predicted signal peptide is underlined, the mature peptide is highlighted in grey and the γ-core motif is highlighted in red.

Fig. 2: Comparison of the mature peptide region of the Ctd1 protein with mature defensin peptides characterized in tribe Phaseoleae (Fabaceae). The eight cysteine residues conserved among legume defensins that form four disulphide bridges are shown.

Phylogenetic analysis of Ctd1, based on its deduced amino acid sequence, was conducted in MEGA 5 using Neighbor Joining (NJ) analysis computed with p-distance methods together with the bootstrap test (10 000 replicates). The NJ tree of the deduced amino acid of 10 Ctd1-like sequences appearing in the family of Fabaceae is presented in Figure 3 (sum of branch length 1.20839) and Capsicum annuum (Accession No: 1200228) was used as an outgroup. The tree topology from the NJ showed that three tribes of subfamily Faboideae (Fabaceae) (Phaseoleae, Trifolieae and Fabeae) were distinguished. The tree also demonstrates that the Phaseoleae and Trifoliae tribes form two monophyletic lineages and have tribe-specific manner among each other (Figure 3). C. ternatea defensins were found clustered with other tribe Phaseoleae species such as Phaseolus vulgaris, Vigna radiata, Glycine max and Psophocarpus tetragonolobus.

Fig. 3: Neighbor Joining tree of deduced amino acids of the three tribes of subfamily Faboideae (Fabaceae) was generated. Numbers above the branches indicates bootstrap values (10 000 replications).
acid sequence of Ctd1 is comprised of an α-helix and three anti-parallel β-sheets forming a cysteine-stabilised αβ motif (CSαβ) [1,21]. Based on our generated Ctd1 model, three disulphide bridges (Cys14-Cys35, Cys20-Cys41 and Cys24-Cys43) sustain the specific CSαβ architecture and the fourth disulphide bridge (Cys3-Cys47) links the amino and carboxyl terminal to form a particularly stable protein [21].

Fig. 4: Three-dimensional structure of the mature peptide Ctd1 defensin using Phyre 2 constructed with 100% confidence. The four disulfide bridges are presented.

The occurrence of the conserved motif γ-core GXC(X3-9)C in Ctd1 (GRCRDDLRC) containing the hairpin loop connecting β-strands 2 and 3, suggests this region might be important for the activity of plant defensins (Figure 1) [46,36,33]. Saragam et al., [36] found that γ-core motif express the unique antifungal characteristic of each defensins. Unlike mammalian and insect defensins, most of the plant defensins display antifungal activities and are less active against bacteria [2,42].

Conclusions:
The defensin gene from Clitoria ternatea L. (ctd1) was successfully cloned and in silico characterized. These invaluable plant defensin should be exploited for the development of resistance traits in transgenic crops with the aim of improving plant disease resistance. Efforts are currently aimed at transferring Ctd1 into rice to reduce crop losses caused by fungal pathogens.

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REFERENCES


