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Cloning and heterologous expression of *CDef1*, a ripening-induced defensin from *Capsicum annuum*

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Abstract

CDef1, a cDNA clone that encodes a defensin gene, was isolated from a cDNA library of ripening *Capsicum annuum* via differential screening. Sequence analysis of the cDNA clone showed 534 bp encoding a 75 amino acid polypeptide with a predicted pI of 8.1 and a molecular mass of 8.1 kDa. The deduced peptide consists of a signal sequence of 27 amino acids, followed by a defensin domain of 47 amino acids containing 8 conserved cysteine residues with a predicted mature peptide of 5.2 kDa. The deduced peptide has high sequence similarity to the family of plant defensins. *CDef1* is expressed in the mesocarp at the onset of fruit ripening and continued thereafter. Structural analysis on the sequence revealed a Cys stabilized αβ motif (CSαβ) consisting of triple-stranded anti-parallel β-sheets and an α-helix organized in βαββ architecture. The predicted three-dimensional structure of CDef1 reveals two potential receptor-binding sites; one is located on the loop that connects β1 and an α element (Phe37, Lys38, Leu40 and Leu42) and another is located on the interconnecting loop of β2 and β3 (Ile62, Phe64 and Leu66). CDef1 was expressed using the prokaryotic *Escherichia coli* expression system with a 47 kDa fusion peptide.

Keyword: *CDef1* gene, CDef1 protein, Defensin, fruit ripening, 3D structure, recombinant expression.
Abbreviations: CD(M)- Capsicum defensin-maltose, IPTG- Isopropyl β-D-thiogalactopyranoside, MBP- maltose binding protein, MMLV- Moloney Murine Leukaemia Virus, ORF- Open Reading Frame, SDS- Sodium Dodecyl Sulphate, FTIR- Fourier transform infrared spectrophotometry.

Introduction

Fruit ripening is a genetically programmed process that involves changes in fruit flavor, texture, color and aroma (Giovannoni, 2007). These attributes play a significant role in assisting seed dispersal of fleshy fruits. The high content of macromolecules, especially in ripe fruits, makes them more vulnerable to pathogen infection. However, plants have developed a variety of different protective mechanisms to defend themselves against constant abiotic and biotic stress, especially from phytopathogenic fungi. These innate defense mechanisms either form a physical barrier or induce antimicrobial peptides through a signaling compound released in advance of a pathogenic attack (van Loon et al., 2006). Plant defensin, which has antimicrobial properties, has been identified, isolated and characterized from various plant tissues (Thomma et al., 2002). These groups of proteins are ubiquitous throughout the plant kingdom; they have been described in diverse plant species (Carvalho and Gomes, 2009). Plant defensin was originally termed *γ*-thionin because it is similar in size (5 kDa) and has three or four disulfide bridges like *α*- and *β*-thionins. However, it was renamed plant defensin due to its high structural and functional similarities to defense peptides in many organisms, including mammals and insects (Broekaert et al., 1995). The first member of this protein family, *γ*-hordothionin, was isolated from wheat and barley. It can arrest the translation in rabbit reticulocyte extract system (Mendez et al., 1990). Plant defensin is composed of 45-54 amino acid residues that contain eight conserved cysteine residues forming four disulfide bridges to stabilize the protein structure (Thomma et al., 2002). However, overall sequence conservation between the species is relatively limited, except for the eight cysteine residues (Kant et al., 2009). The global fold of plant defensin comprises a cysteine-stabilized αβ motif (CSαβ) consisting of an α-helix and a triple-stranded β-sheet, organized in a βαββ architecture and stabilized by four disulfide bridges (Almeida et al., 2002). Plant defensins have been isolated and characterized from diverse plant species. Although most isolated plant defensins are seed-derived, they have been detected in various plant organs, such as leaves, flowers, pods, seeds, and tubers (Carvalho and Gomes, 2009). The expression of some defensin genes is developmentally regulated, whereas others are elevated in response to biotic and abiotic external stimuli.

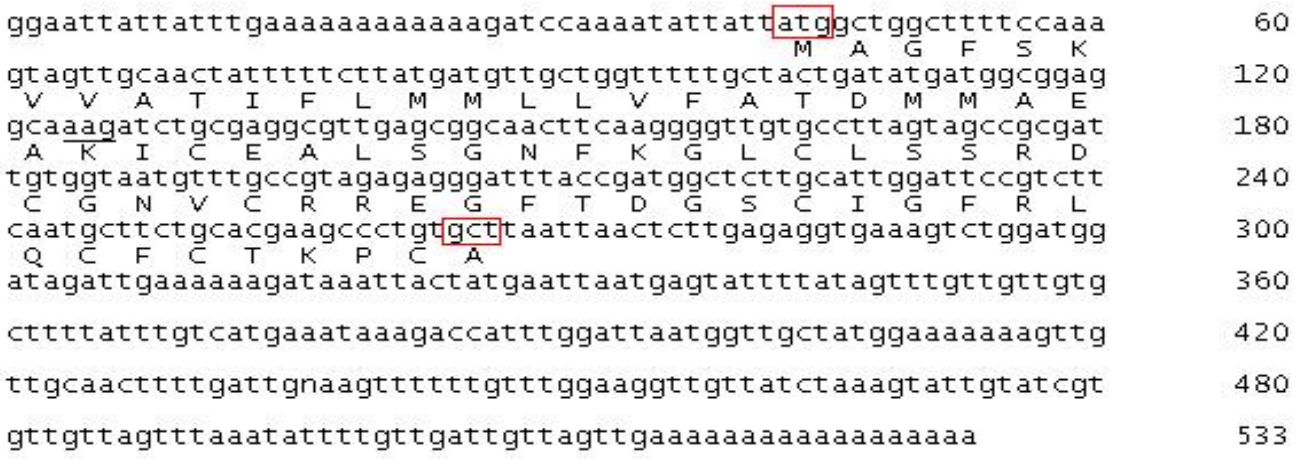

Fig 1. Nucleotide and deduced amino acid sequences of CDef1 of *Capsicum annuum*. The 534 bp cDNA is shown above the deduced 75 amino acid sequence of the CDef1. The start and stop codons are boxed. The first 27 amino acids is a signal peptide followed by the 48 amino acid residue mature peptide that starts from aag (underlined).

In *Gingko biloba,* the defensin gene, *GbD,* belongs to a multigene family and has been shown through expression analysis, to be up-regulated by wounding and methyl jasmonate treatment, thus suggesting its function in protecting against plant pathogen invasion (Shen et al., 2005). In the present study, we report on a cDNA clone corresponding to CDef1, a defensin gene from *Capsicum annuum*, which was isolated through a differential screening of the cDNA library constructed from the mesocarp of ripe fruit. Transcriptional expression of the defensin gene was investigated on Capsicum fruit under normal ripening conditions. We have also predicted the possible tertiary structure of CDef1 via bioinformatics tools. The coding region of CDef1 was ligated into expression vectors, and the recombinant protein was expressed in *E. coli*, purified and confirmed through SDS-PAGE.

Materials and methods

Plant material

Capsicum annuum fruits of MC11 variety were obtained from Malaysian Agricultural Research and Development Institute (MARDI), Klang, Malaysia. Only good quality mature fruits were chosen for experimental work. Samples were collected and grouped into growing stages of ripening (0%, 25%, 50%, 75% and 100% ripening according to coloration). The fruits were cleaned, weighed and immediately frozen in liquid nitrogen and stored at -70°C until use.

Nucleic Acids Extraction

Total RNA from five growing stages of fruit ripening was extracted according to Lopez-Gomez and Gomez-Lim (1992) with a slight modification made to the extraction buffer (4M thiocyanate was included). Poly (A^+) mRNA was isolated using the Poly A tract mRNA Isolation system III purification kit in accordance with manufacturer's instructions (Promega).

cDNA Cloning and Screening

Poly (A^+) mRNA isolated from ripe mesocarp tissue at 25%, 50%, 75% and 100% ripening was used to synthesize double stranded cDNA using the Zap-cDNA synthesis kit (Stratagene). The cDNAs obtained were size fractionated, and selected fractions were ligated into the Uni-Zap XR vector (Stratagene). Recombinant DNA was packaged *in vitro* using Gigapack III packaging extract (Stratagene). The library obtained was amplified once and stored at -70°C until used. About 90,000 recombinant phages were differentially screened by *in situ* plaque hybridization. Plaque lifts on replicate filters were prepared on nylon membranes (Hybond N^+, Amersham). The two cDNA probes used were synthesized from Poly (A^+) mRNA isolated from unripe (green fruits) and ripe (pool of 75% and 100% ripening fruits). The probe was labeled by random priming with 2µL [α-^{32}P]dCTP (300 mCi mmol^{-1}), 3 µL hexanucleotides (240 ng µL^{-1}) and 15 units of Moloney Murine Leukaemia Virus (MMLV) reverse transcriptase, according to the vendor's instructions (Promega). Two rounds of differential screening were performed. After hybridization, the filters were washed with 2X SSC, 0.1%(w/v) Sodium Dodecyl Sulphate (SDS) at 65°C for 10 min, 1X SSC, 0.1% SDS and 0.5XSSC, 0.1% SDS. Filters were exposed to X-Omat X-ray film (Kodak) for 72 h at -70°C with an intensifying screen. Plaques that showed strong differential intensities with both probes were considered positive. The positive plaques were then subjected to secondary screening to purify single isolates. *In vivo* excision of pBluescript SK(-) phagemids were carried out on the isolated plaque according to manufacturer's recommendations (Stratagene).

Sequencing and DNA sequence analysis

The insert size of the cDNA clone was determined through agarose gel electrophoresis, followed by digestion of the plasmid with *PstI* and *XhoI.* Selected clones obtained from the library screening were sequenced on an automated DNA sequencer (PRISM 377, ABI) using vector specific primers. Nucleotide and deduced amino acid sequence comparisons against the databases were done using BLASTX and BLASTP programs (http://www.ncbi.nlm.nih.gov/). Sequence alignment was performed using the T-Coffee (Notredame et al., 2000).

Northern Blot Analysis

Northern blot analysis was carried out on RNA extracted from 5 different stages of fruit ripening similar to the ones used in library construction. Ten micrograms of total RNA was fractionated on a formaldehyde agarose gel and transferred onto a Hybond N^+ membrane (Amersham, UK). The probe

was labeled with the presence of 2 µL [α-^{32}P]dCTP (300 mCi mmol^{-1}) using the Random Prime Labeling System (Amersham Bioscience, Sweden) according to vendor's instructions using the random primer method. Pre-hybridization and hybridization were performed in 50% formamide at 42°C. After hybridization, the membrane was washed at 42°C in 0.5 X SSC, 0.1% SDS.

Construction of expression plasmid

The coding region for the mature defensin peptide was amplified by PCR from CDef1 (AF488780). The forward primer, 5`GGG GGG GAA TTC ATG AAG ATC TGC GAG GCG 3', carrying *Eco*RI and reverse primer, 5´GCC GCC AAG CTT TTA AGC ACA GGG CTT CGT GCA 3', carrying *Hin*dIII were designed from the known cDNA sequence of the aforementioned chili defensin. The PCR product was digested by *Eco*RI and *Hin*dIII (Promega) and purified with the QIAquick gel extraction kit (Qiagen). The purified product was ligated into the pMAL-c2x (New England Biolabs) vector in the same translatable reading frame and the orientation of the insert was confirmed by DNA sequence analysis.

Recombinant Protein Expression and purification, SDS-PAGE and Western blotting

The induction and purification of the maltose binding protein (MBP)–chili defensin fusion proteins were done according to manufacturer instructions (New England Biolabs). Isopropyl β-D-thiogalactopyranoside (IPTG)-induced *E. coli* cells were disrupted by sonication (Sonifer B-12, Branson Sonic Power) in an ice water bath with 15 s pulses for 5 min. The supernatant (crude extract) was removed and diluted in 1:5 ratios with column buffer. Amylose resin (New England Biolabs) was used for affinity chromatography purification of defensin-MBP fusion protein. Chromatography was carried out according to the manufacturer's procedure. The Bradford assay (Bradford, 1976) was employed to verify protein content in the bacterial crude extract and the resultant insoluble material; both fractions were then analyzed by SDS-PAGE and Western immunoblot. The cleavage was carried out using factor Xa. Purified fusion protein (1 mg ml^{-1}) was mixed with 10µg factor Xa in a final volume of 20µL. The reaction mixture was incubated for 24 h at 4°C. Complete cleavage was checked by SDS-PAGE.

Bioinformatics Analysis

ProtParam was used to investigate the physicochemical properties of the *CDef1* sequence. Based on our prior knowledge of plant defensin, this protein usually has a signal peptide attached on mature proteins. We used SignalP 3.0 [40; http://www.cbs.dtu.dk/services/SignalP] to identify the signal peptide and its cleavage site. Once the signal peptide was identified, it was removed from the mature protein. Further bioinformatics analysis was performed only on the mature part of the protein. A domain and motif identification search was done using InterProScan http://www.ebi.ac.uk/Tools/InterProScan/ against various domain databases. Sequence similarity searches were performed using BLAST against NPSA (Network Protein Sequence Analysis: http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_server.html) non-redundant database and the UNIPROT database. In addition to indentifying more plant defensins, this step was necessary to determine the best protein structure

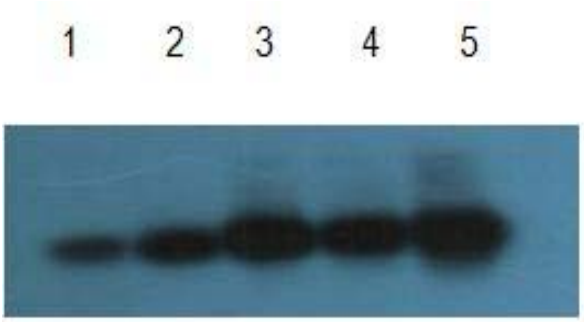

Fig 2. Northern blot analysis of the *CDef1* gene. Twenty µg of total RNA prepared from fruits at different ripening stages (1), mature green fruit (2), 25% ripe fruit (3), 50% ripe-fruit (4), 75% ripe fruit (5), 100% ripe fruit were hybridized with ^{32}P-labeled inserts of *CDef1*. Gels were stained with ethidium bromide before blotting to ensure equal loading of total RNA.

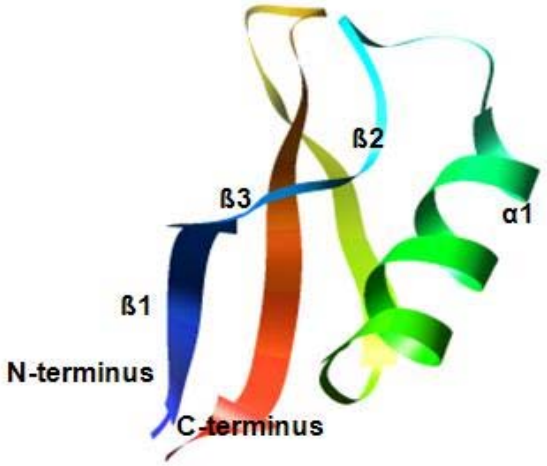

Fig 3. Theoretical 3D structure of CUKM11 was generated using 1GPT as a fold template suggested by Meta Server (http://meta.bioinfo.pl) (Ginalski et al., 2003). Based on COP classification(http://scop.mrc-lmb.cam.ac.uk/scop/) (Andreeva et al., 2004), 1GPT is classified in a small protein class adopting a knottin fold. The 3D model was built using I-Tasser(http://zhang.bioinformatics.ku.edu/I-TASSER/)(Zhang, 2008).

prediction technique to generate a theoretical model for the CDef1 sequence and its 3D template. However, our template search failed to identify suitable templates; hence, the fold recognition protein structure prediction approach was used. For a fold recognition analysis, we used two different fold recognition servers: Bioinfo metaserver [http://www.meta.boinfo.pl/] and Phyre (ver2.0) [http://www.sbg.bio.ic.ac.uk/phyre]. A multiple sequence alignment was generated using the sequences gathered from BLAST analysis and the sequences from the fold recognition output using T-Coffee program [http://www.ch.embnet.org/software/TCoffee.html].
A secondary structure prediction of the sequence was based on a number of secondary structure prediction programs, such as Psipred, Sam-t02 and PROFSEC. The results of these programs were gathered from Bionfo.pl-MetaServer. The resulting suggested fold was used as a template to generate a crude structure of the CDef1 sequence.

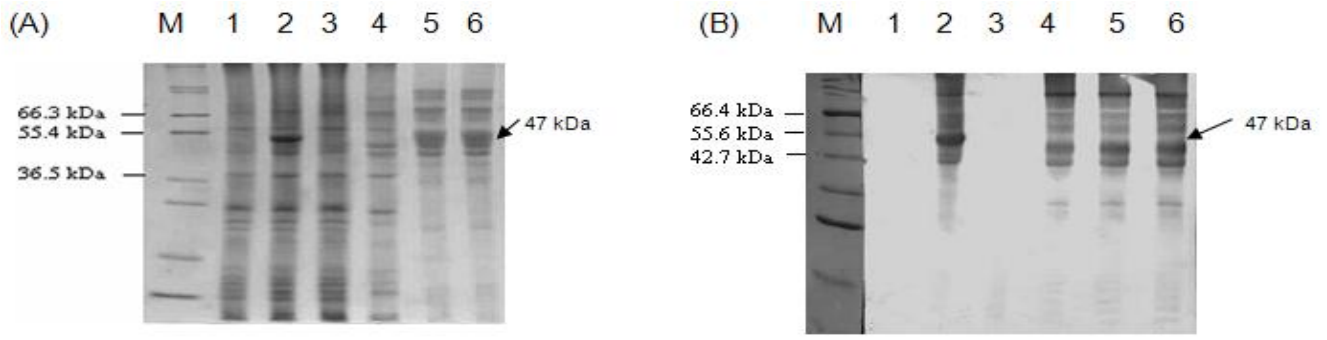

Fig 4. SDS-polyacrylamide gel electrophoresis (A) and Western blot (B) analysis of expressed CDM-MBP proteins. *Lane 1*, control cell lysates from *E. coli* cells harboring pMALc2x vector alone (uninduced); *Lane 2*, induced control cells; *Lane 3*, cell lysates from *E. coli* cells harboring pMALc2x-CDM vector before induction; *Lane 4-6*, cell lysates from *E. coli* cells harboring pMALc2x-CDM vector 1, 2 and 3 hours after induction, respectively. Arrows indicate the expressed CDM-MBP protein with the respective size of approximately 47 kDa; *Lane M*, molecular mass markers in kDa.

Results and discussion

Most defensins characterized to date have been isolated from seed tissues. In 2008, Games et al. (2008) purified a defensin from *Phaseolus vulgaris* seeds with inhibitory activity against the growth of phytopathogenic fungi *in vitro*. This finding suggests that seed-produced defensin has a protective role in seedling tissue and may contribute to its survival. In this study, using a differential hybridization technique, a *Capsicum annuum* cDNA clone encoding a putative defensin was isolated from a previously constructed cDNA library of ripe chili fruit (Zainal et al., 2002). The isolated full length cDNA clone was designated CDef1. The nucleotide sequence of CDef1 has been deposited in the GenBank database with accession number AF44238 (Fig. 1). The cDNA clone is 534 bp long and encodes a predicted full-length protein with an open reading frame (ORF) of 75 amino acids and a predicted molecular mass of 8.1 kDa. The mature transcript of *CDef1* encodes a polypeptide that can be divided into two parts, the amino signal peptide from amino acid 1-27 and the mature chain from amino acid 28-75. Hypothetically, the signal peptide will direct the mature CDef1 to the apoplastic region of the plant cell. A typical signal peptide has been found on most of the reported plant defensins, indicating that all of these proteins are targeted to the apoplast (de Beer and Vivier, 2007). The putative translation of the ORF indicates that the encoded peptide contains eight conserved cysteine residues that form four disulfide bonds, a common feature in defensin (Thomma et al., 2002). Northern blot analysis was performed on five different stages of ripening, where the transcript was first detected on green mature fruit and followed at 25% intervals until 100% ripening (Fig. 2). This observation suggests that *CDef1* may play a significant role in ensuring that ripe fruits are protected from pathogenic invasion. To investigate the evolutionary relationships between *CDef1* and other plant defensins, a phylogenetic tree was constructed based on the deduced amino acid sequences of predicted CDef1 and the members of the defensin family from other plant species.

Sequence and structural analysis of defensin CDef1

Following a sequence similarity search, thirty-eight sequences with e-value ranges from $1e^{-06}$ to $1e^{-23}$ were chosen for further analysis. Pair wise alignment of CDef1 and its closest relative (O65740) showed 66.2% sequence identity and important conserved residues, such as eight cysteines (Cys30, Cys41, Cys47, Cys51, Cys61, Cys68, Cys70, and Cys74), one serine (Ser34), one glycine (Gly59) and one glutamic acid (Glu54). This observation is consistent with the findings of Lay et al (2003), who suggested that the conservation of these residues may be due to their roles in providing stability and in the folding mechanism, especially the cysteines that are involved in the formation of disulfide bridges. In our generated 3D model (Fig. 3), three disulfide bridges maintain the specific CSαβ motif. Cys47-Cys68 and Cys51-Cys70 linked the β3-strand to the α-helix, and Cys41-Cys61 linked the interconnecting loop of β1 to the β2-strand. The fourth disulfide bridge (Cys30-Cys74) clasped the N and C terminal regions of the molecules, thus further enhancing the rigidity of the polypeptide backbone. This behavior is believed to create a pseudo-cyclic protein (Lay and Anderson, 2005). These disulfide bridges are correctly bonded, with an average spacing of 2.1Å. The conservation of all eight cysteine residues is important for the stabilization of the globular structure of this protein. Multiple sequence alignments also revealed the consensus cysteine arrangement in this sequence responsible for the forming of the CSαβ motif arranged as C(..10x)C(..5x)C(..3x)C(..9x)C(..6x)CxC(..3x)C.

The secondary structure of this protein conformed to the CSαβ motif, which consisted of triple-stranded anti-parallel β-sheets and an α-helix organized in a βαββ architecture. This motif has also been reported to occur in charybdotoxin (scorpion venom) and insect defensin A (Bontems et al., 1991). Domain analysis was performed using InterProScan [http://www.ebi.ac.uk/InterProScan/], which runs an integrated search against databases such as PROSITE (Attwood et al., 1999), Pfam (Bateman et al., 2002), PRINTS (Attwood et al., 2002), and SMART (Letunic et al., 2004). More than one database is used to collate consensus results. The SMART database detected a Knot 1 domain (SM00505) at position 2 to 47 in the CDef1 sequence with a calculated e-value of 3.77^{e}-13 and another domain, belonging to the Gamma thionin family (PF00304), with a lower e-value. The Knot 1 domain or knottins represent plant lectins/antimicrobial peptides, plant proteinase/amylase inhibitors, plant gamma thionins and arthropod defensin. The gamma-thionin family (PF00304) was identified from the Pfam database; it is a member of the Scorpion toxin-like knottin superfamily. In the mature form, these proteins generally consist of 45 to 50 amino acid residues. These peptides contain 8 conserved cysteines involved in disulphide bond. The folded structure of gamma-purothionin is characterized by 3 stranded anti-parallel β sheets and a short α-helix. The features found in both

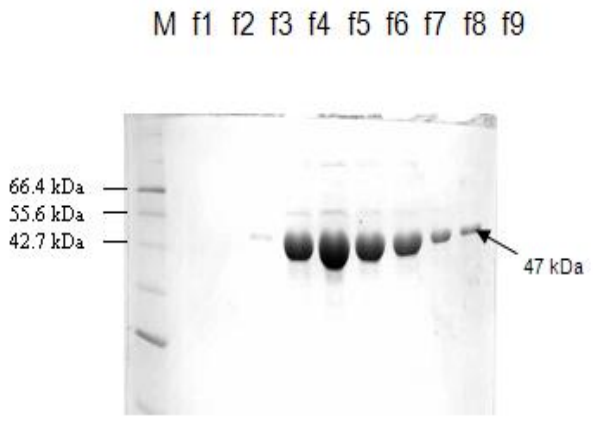

Fig 5. Protein sedimentation profile of the CDM-MBP protein after amylase affinity chromatography. The fractionated CDM-MBP protein was separated by SDS-PAGE and stained with Coomassie brilliant blue. The arrow indicates the position of the purified fusion proteins (~ 47 kDa). *Lane M*, molecular mass markers in kDa.

databases shared common characteristics with plant defensin. CDef1 has a similar fold to the 3D structure of other defensin protein, i.e. 1GPT as suggested by a consensus fold recognition programs on Meta Server and Phyre (with a RMSD value of 0.9 and z-score of 4.7 based on the Combinatorial Extension (CE) structural superimposition between 1GPT and the model. Having a low RMSD value and a z-score above 4.0 indicate the significant structural similarity (Shindyalov et al., 2000) and some residues are well conserved throughout the animal and plant kingdoms, we can assume this protein has similar electrostatic interactions with the receptors. In contrast to human and insect defensin, plant defensin binds to a specific membrane receptor with the involvement of electrostatic interactions (Thevissen et al., 1997). These interactions would be disturbed by high ion strength in solution (Song et al., 2005). Unfortunately not much information is known on the residues involved in such interaction. In this study, two receptor-binding sites were predicted; one is located on the loop that connects β1 and an α element (Phe37, Lys38, Leu40 and Leu42) and the other are located on the interconnecting loop of β2 and β3 (Ile62, Phe64 and Leu66). Similar hydrophobic patches were found in the Rs-AFP2 structure, and mutation studies proved that these patches are involved in receptor-binding activity (de Samblanx et al., 1997). The structural and functional analysis of CDef1 was performed to answer these important questions and to elucidate the potentially diverse mechanisms of plant defensin family.

Production of recombinant defensin protein in E.coli and Purification of CDM-MBP fusion protein

The pMALc2-CDM plasmid encoding the CD(M)-MBP (Capsicum defensin-maltose fusion, containing mature peptide) protein was constructed and introduced into *E. coli* strain TB1 cells to express the *Capsicum* defensin CDef1. SDS-PAGE analysis of cell lysates revealed an abundant protein with molecular weight 47 kDa (Fig. 4A). The CDM peptide contains 50 amino acids with a calculated molecular mass of 5 kDa. When CD(M) fused with MBP (42 kDa), the resulting protein had a molecular mass of 47 kDa. This is in agreement with the calculated molecular mass of the predicted amino acid sequence of the recombinant proteins. A time course study on the production of CDM-MBP proteins in *E. coli* TB1 showed a maximum yield of fusion proteins was achieved after 3 hours of induction. Further analysis of the same cell lysates by Western blotting with an anti-MBP rabbit serum probe showed a protein band of approximately 47 kDa (Fig. 4B). The results show that the CDM-MBP fusion proteins were successfully expressed in *E. coli*. The respective purified proteins were fractionated, and the total protein content in each fraction was determined by Bradford assay. The purity and apparent molecular mass of the eluted proteins were checked by SDS-PAGE. The purified CDM-MBP migrated on SDS-PAGE as a protein with a molecular mass of 47 kDa (Fig. 5). Kant et al (2009) demonstrated that corn PDC1 expressed in *P. pastoris,* when analyzed under Fourier transform infrared spectrophotometry (FTIR), produced peptides with more β-sheets and a less random unordered structure than when it was expressed in *E. coli*. Furthermore the protein expressed in *P. pectoris* was more efficient in inhibiting *F. gramienarium* growth.

Conclusion

In the present study, a cDNA encoding Capsicum defensin was isolated and characterized. Structural analysis on the sequence revealed that it had a Cys stabilized αβ motif (CSαβ) with triple stranded anti-parallel β-sheets and an α-helix organized in a βαββ architecture, which is similar to most of the characterized plant species defensins. However, functional analysis on the expressed peptide was not performed in this study due to the inefficiency in cleaving the recombinant product. The diverse biological activities posed by defensin make them very attractive and useful as a gene source for biotechnology. Since the peptides are not toxic to mammalian and animal cells, plant defensin genes could be employed to produce transgenic crops with improved resistance against phytopathogens. In order to further investigate the ability of *CDef1* to control fungal pathogens in plants, we have produced a transgenic tomato harboring chimeric gene construct, consisting of constitutively over expressing *CDef1* coding region driven by a 35S promoter. Relative to untransformed plants, transgenic tomato plants showed reduction in lesion size when infected with several pathogenic fungal (Zainal et al*.,* 2009).

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