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Preliminary Risk Assessment of a Novel Antifungal Defensin Peptide from Chickpea (*Cicer arietinum* L.)

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Abstract

Risk assessment of an antifungal peptide is a prerequisite before using the corresponding gene to produce broad-spectrum, fungal-resistant GM crops. In this study, a new, antifungal defensin peptide (Ca-AFP) isolated from chickpea (Cicer arietinum L.) was evaluated for biological activity, stability, and range of toxicity to determine risks in using the gene for transgenic crop production. The biological function of Ca-AFP was found to be highly stable in extreme pH (range from 2 to 10) and temperature (up to 100°C) conditions. The peptide also showed unaltered efficiency in a wide range of temperatures (from 15° to 42°C) indicating a wide functional temperature. Despite extreme stability, it was non-toxic to non-target organisms (e.g., bacteria and insect cell lines). When the viability of human cell lines together with erythrocytes were tested, the peptide was also found to be non-toxic. Only one B-cell epitope was determined with this amino acid sequence. Immunological tests with mice failed to develop any antibody, indicating the non-immunogenic nature of the peptide, and thus, the negligible possibility of it being an allergen. All these preliminary assessments suggest that Ca-AFP is a noble peptide worthy of exploring its efficiency in transgenic crops to combat fungal pathogens.

Keywords

Antimicrobial compound, toxicity, biological activity stability, human cell, allergicity

Introduction

Risk assessment is a prerequisite for the commercialization of any genetically modified (GM) crop, as regulatory bodies require information regarding the potential risk of releasing them into the environment (Dutton et al., 2003). With the increasing trend to produce GM crops commercially, concerns over the safety of these crops and crop products have increased very relevantly as demonstrated by several Multilateral Environmental Agreements, such as the Convention on Biological Diversity and the Cartagena Protocol of Biosafety (Young, 2004). The main biosafety concerns at the present time include the safety of human health, non-target organisms, and the environment, as well as the risk of horizontal gene flow (Snow et al., 2005). Therefore, any gene or gene product with potential beneficiary impact must be assessed for its risks before using it to produce any GM crop.

A large portion of research is going on to develop GM crops with resistance to biotic stresses like insects, weeds, viruses, and fungi. Among the pathogens, fungi have been identified as the most notorious group since they cause 70% of the major crop diseases (Agrios, 1997). At present, applying fungicides is the most widely practiced method to reduce crop losses from fungal attack. However, due to an absence of natural resistance, an intensive search is going on to develop a safer and more effective fungus-control method. Consumers' concern over a possible environmental impact associated with exposure to fungicides also acts as a driving factor.

Because of these issues, genetic engineering may provide a good option to protect crops from fungal pathogens by generating fungus-resistant GM crops (Erik & van Der, 2001). Therefore, the search is on to identify new genes that confer durable resistance to broad-spectrum phytopathogenic fungi and that are safe for other organisms, including humans. Since the discovery of chitinase and glucanase in 1988 (Broekaert et al., 1988; Mauch et al., 1988), many antifungal genes and proteins have been identified. These include ribosome-inactivate proteins (Leah et al., 1991), permatins (Vigers et al., 1991), cysteine-rich peptides like thionins (Bohlmann et al., 1991) and defensins (Finkina et al., 2008; Terras et al., 1995). Among these, defensins have generated considerable interest because of their small size and broad antifungal and/or antibacterial activities.

As the spectrum of biological function varies from one antimicrobial molecule to another, performing each and every toxicity assessment for a particular molecule on a case-by-case basis is essential. Defensins and other antimicrobial compounds that are isolated from plants are screened for toxicity while characterizing the biological activity to determine the safety of the new gene and gene product before producing GM crops. The toxicity analyses are commonly performed on insects, bacteria, and sometimes on human cells for the preliminary evaluation of biosafety (Chen et al., 2002; Taylor, 1997; Terras, 1992; Veldhuizen et al., 2008).

Recently, a new defensin peptide, Ca-AFP, was identified from germinating chickpea (*Cicer arietinum* L.) seeds (Islam, 2004; Reddy et al., 2004). Purification, sequence (Gen Bank acc. No. DQ 288897), and structure modeling, along with antifungal activity spectrum and up-regulation of this novel peptide, have been researched. The effectiveness along with broad antifungal activity makes Ca-AFP an attractive candidate to explore its potential to control fungal infection in transgenic crops (Islam, 2004).

Since it is necessary to screen the safety of any gene product before attempting transgenic production, in the present study experiments were conducted to test the extent of the Ca-AFP toxicity to organisms other than the target fungi. These included bacteria, insect cells, and human cells together with blood cells. The possibility of raising allergicity was also considered by analyzing possible epitope sites within the sequence. Prior to all these bioassays, the stability of the biological activity was analyzed against extreme conditions; this was important to determine the activity range of this peptide.

Materials and Methods

Biological Materials

Chickpea seeds (*Cicer arietinum* L.) were collected from the local markets in order to isolate the peptide and perform the assays. *Pythium aphanidermatum* cul-

ture was kindly provided by Prof. M. V. Rajam (University of Delhi, India). Ovarian cells, Sf21 (derived from *Spo-doptera frugiperda*), and *T. ni* cells (derived from *Trichoplusia ni*) were purchased from Invitrogen, (Carlsbad, California) and human cell cultures were purchased from the American Type Culture Collection (Manassas, Virginia).

Stability of Antifungal Activity of Ca-AFP

The stability of antifungal activity with respect to pH, temperature, and reducing agent was tested according to Cammue et al. (1992). Tests for antifungal activity were performed against *Pythium aphanidermatum* by the agar-well diffusion method followed by the microspectrometry measurement of microcultures having 20 μ l test solution diluted five times with potato dextrose broth medium.

pH stability was tested in the following buffers: 20 mM glycine-HCl (pH 2 and 3); 50 mM Tris-HCl (pH 9); and 20 mM glycine-NaOH (pH 10). After 1 hour of incubation in the appropriate buffers, the samples were dialyzed for 16 hours against a 10 mM phosphate buffer (pH 7) to remove the possibility of these extreme pH buffers interfering with the fungal growth.

For heat stability the purified peptide samples were treated at 80°, 90°, and 100°C for different time intervals (e.g., 10, 20, and 30 minutes). Afterwards the heat treated samples were cooled to room temperature before performing the diffusion agar assay.

To determine the optimum temperature for the biological function, the antifungal assay was performed at 15°, 25°, 30°, and 42°C.

As disulfide bonds in antimicrobial proteins and peptides are reported to be essential for biological activity, a bioassay was performed with purified peptide after reducing it by adding DTT at 2.5 mM and incubating at 37°C for 2 hours. Following DTT treatment the peptide sample was dialysed against a 10 mM phosphate buffer (pH 7) before using it in the bioassay. In all the experiments, an untreated purified sample was used for bioassay as the control.

Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Analytical electrophoresis of proteins was carried out in polyacrylamide gels (Laemmli, 1970) in the Mini Protean II apparatus (Bio Rad, Hercules, California) using the Tris-Glycine discontinuous buffer system (Sambrook et al., 1989). 15% gels were prepared and protein samples were prepared by mixing with 4 X sample buffer (100 mM Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 4% β -mercaptoethanol, and 0.01% bromophenol blue). The electrophoresis was carried out at a constant voltage of 90 mA until dye came out of the resolving gel. After the completion of the electrophoresis, the gels were stained with Coomassie Brilliant Blue (Laemmli, 1970).

Toxicity Assay of Ca-AFP

Antibacterial Activity Assay

Antibacterial activity was measured as reported by Broekaert et al. (1990) using absorbance (optical density, OD) of cultures in a microtiter plate as a function of growth. Bacterial suspension cultures at $OD_{600} = 0.1$ were mixed with appropriate concentrations of Ca-AFP in a microtiter plate. A reading at OD_{600} was taken at the beginning of the experiment and 8 hours after incubation. Net bacterial growth was calculated by subtracting the OD_{600} at the beginning of the experiment from the OD_{600} after 8 hours of incubation.

Insect Cell Viability Assay

Toxicity on insect cell lines was evaluated on Sf21 and *T. ni* cells (High Five, Invitrogen) in terms of viability following exposure to the peptide. During the experiment 1×10^5 cells were seeded in each well of the 96-well cell culture plate (Costar, Corning, Bath, UK) and cultured in 80 μ l of SFII 900 serum-free medium (for Sf21 cell) and Express Five Serum Free medium (for *T. ni* cell) in the presence of a 20 μ l test solution. The viability of cells was analyzed microscopically by Trypan Blue staining (Invitrogen) until 72 hours with a gap of 24 hours. All the experiments were performed in triplicate.

Human Cell Viability Assay

HEK293 (Human Embryonic Kidney cell line), Huh-7, and HepG2 (human hepatoma) cell lines were used to analyze the human cell viability assay. All three cell lines were grown in DMEM (Dulbecco's Modified Eagle's Medium) with 10% FCS (Fetal Calf Serum). They were incubated in a humidized chamber at 37 °C in 5% CO₂. For the assay 1×10^5 cells were seeded in each well of the plate and allowed to adhere for 18 hours in their medium in the humidized CO₂ chamber. The old medium was replaced with the 80 μ l serum-free medium containing the 20 μ l test solution. The viability of cells was analyzed microscopically by Trypan Blue staining (Invitrogen) until 72 hours with a gap of 24 hours. All the experiments were performed in triplicate.

MTT Assay

The toxicity effect of Ca-AFP on the human cell line HEK 293 was analyzed at enzymatic level through MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. In 96-well cell culture plates (Costar) 1×10^5 cells were seeded and allowed to adhere to the surface for 12-14 hours. After settling of the cells, a fresh serum-free medium containing various concentrations of Ca-AFP was added to the wells. In the control no test solution was added. The cells were allowed to grow for 24 hours and to each well 10 μ l of MTT reagent (5 mg/ml stock concentration) was added to give final concentrations of 0.5 mg/ml. The reaction was stopped after observing purple precipitates in the control wells. The

media were carefully removed without disturbing the cell layers and dried. In the dried wells DMSO was added and incubated overnight to solubilize the precipitates. The absorbance of the solubilized material was taken at 550 nm using Versa Max Microplate Reader (Molecular Devices, Sunnyvale, California). The experiments were performed in triplicate.

Haemolytic Activity Assay

Haemolytic assay was performed according to the Terras protocol (Terras et al., 1992). Human erythrocytes (blood group A) were washed several times with phosphate-buffered saline following 150 mM myo-inositol solution. Finally, a 1% (volume-packed cells/volume) suspension of red blood cells was made in 150 mM myo-inositol. Tests were performed by incubating 80 μ l of erythrocyte suspension with 20 μ l of the test solution in a microplate well by incubating the plate at 25 °C for 1 hour. A sample containing 20 μ l of 1% SDS was included to yield values for 100% lysis (positive control). After centrifugation of the microplates (5 minutes at 800X g), 20 μ l of the supernatants were transferred to a new microplate and diluted with 180 μ l distilled water. Absorbance was then read at 415 nm. The lytic activity percentage was calculated as 100 times the ratio of the absorbance of the test sample over the positive control sample (1% SDS). Morphology of the cell following exposure to the peptide for 72 hours was also observed under a microscope.

Statistical Analyses

Two-way ANOVA (analysis of variance) was performed on time-series toxicity data considering concentrations and time as two factors. One-way ANOVA was performed to analyze the MTT and haemolytic assays. All statistical analyses were performed using the GraphPad Prism version 5.00 (www.graphpad.com). P-value was considered at the 5% level of significance to deduce inference of the significance of the data.

Immunogenicity Test to Detect Allergens

The B cell epitope prediction was done using Bcepred (www.imtech.res.in/raghava/bcepred). The immunogenicity test was performed using this peptide in a number of adjuvants. The immunogenicity test of the Ca-AFP peptide was performed on laboratory mice using various combinations of complete and incomplete adjuvants following standard protocol. Bleeds were collected after the second and third boosts. Western Blot analysis was performed to determine the titre of the raised antibodies.

Results and Discussion

Biological Function Stability of Ca-AFP Against pH and Temperature

Ca-AFP was functionally active against *Pythium aphanidermatum* in all bioassays following treatments

with various pH buffers (pH 2-10) (Figure 1A). The IC_{50} value for *P. aphanidermatum* culture was 4 $\mu\text{g}/\text{ml}$ for all the treated samples, which is also the same for the untreated control sample in the phosphate buffer. This indicates that the biological activity of this peptide remains unaltered even after exposure to extreme pH buffers.

In the temperature stability investigation on Ca-AFP, the peptide showed 100% activity following heat treatment at 100°C for 30 minutes (Figure 1B). Thus, it was determined that Ca-AFP is a highly thermostable peptide. Following both extreme pH and temperature treatments, each treated sample was analyzed through SDS-PAGE for molecular integrity. The size of the treated pep-

ptide remained unaltered, showing the presence of only one band corresponding to the untreated control sample. No fragmented molecule was present which eliminates the possibility of mimicking the biological activity of the native molecule by a fragment (Figure 2).

The peptide was found biologically active at all temperatures ranging from 15° to 42°C. However, at 15°C the growth of *P. aphanidermatum* was slow (Figures 1C and 1D). At this stage it was thought that Ca-AFP was more effective at lower temperatures. However, in a micro-spectrometry measurement of the microculture, it was revealed that at all temperatures the inhibition was the same with respect to the control (IC_{50} values for 15°,

Figure 1

Stability of Ca-AFP in response to pH and temperature. Stability was studied through biological activity:

- A.** Assay after treating purified Ca-AFP with different pH such as pH 2(a), pH 7.4 (b), pH 9 (3) and pH 10 (d).
B. Assay after heat treatment (a) untreated control, (b) denatured Ca-AFP, (c) 90°C and (d) 100°C for 30 mins.
C and D. Assay at 15 and 30°C (a = Ca-AFP in 50mM phosphate buffer; c = buffer). Photographs taken two weeks following treatments. Note that the overall growth of *P. aphanidermatum* is less at 15°C compared to 30°C.

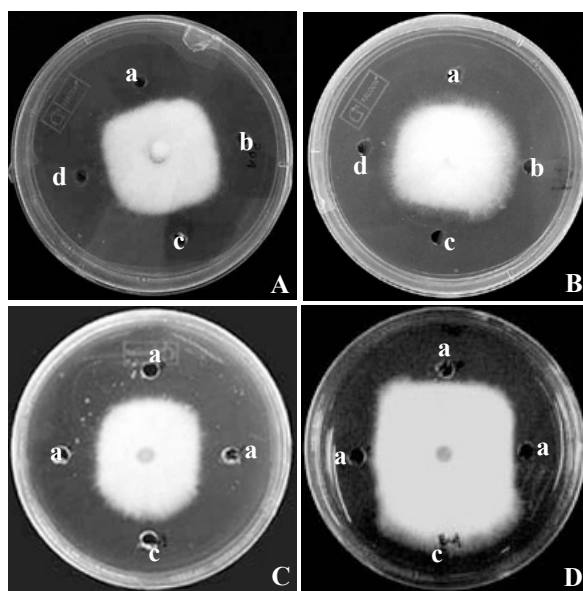
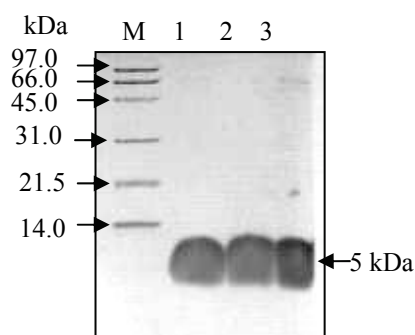


Figure 2

SDS-PAGE analysis of treated peptide with untreated control peptide. Lane1= Un-treated control Ca-AFP, Lane2= Treated Ca-AFP at pH 2, Lane3= Ca-AFP following heat treatment at 100°C for 30 mins.



25°, 30°, and 42°C were calculated to be 3.9, 4.0, 4.1, and 4.0 µg/ml, respectively). So, the observed slow growth at 15°C was due to incubation of the cultures at a sub-optimal temperature. In the DTT-treated Ca-AFP experiment, the peptide failed to inhibit the fungal growth while the control untreated unreduced Ca-AFP was effective against *P. aphanidermatum* in all the experiments.

The functional temperature of plant defensins has been classified into two groups by Osborn et al. (1995). Ah-AMP1, Ct-AMP1, and Dm-AMP1 are more effective at low temperatures while Hs-AFP1 and Rs-AFPs are not temperature-sensitive in their biological activity. In the present study, the functional temperature for Ca-AFP puts it into the second group, as its activity remains unchanged at a wide range of temperatures. The pH and temperature stability of Ca-AFP is similar to Rs-AFPs reported by Terras et al. (1992, 1993). But the antifungal activity of defensins isolated from plants, insects, and humans varies with the change of the ionic strength of the medium (Aerts et al., 2008). Thus, further study is needed to know precisely the active range.

The stability of the plant defensins was reported to be caused by the disulfide bonds that maintain the 3D structure. These bonds are also reported to be essential for biological functions (Kragh et al., 1995; Kristensen et al., 1999; Liu et al., 2000; Segura et al., 1998; Terras et al., 1992, 1993). A similar importance of the disulfide bonds was found in cases of Ca-AFP. Comparable reports are also there for non-defensin, cysteine-rich antimicrobial peptides like Mj-AMPs. Cammue et al. (1992) reported that after the reduction of disulfide bonds, the Mj-AMPs completely lost its antifungal activity against the susceptible fungus *Fusarium culmorum*. In contrast to these reports, Varkey and Nagaraj (2005) reported that the structure-function relationship of defensins is not applicable to all defensins or the importance of the cysteine bonds is not always important for antimicrobial activity. They reported that a peptide corresponding to human defensin HNP-1 sequence that was devoid of cysteines showed the same effectiveness against the susceptible microbes as the native cysteine-rich defensin HNP-1.

Antibacterial Activity

Ca-AFP (concentrations up to 200 µg/ml) showed no effect on the growth of several gram-negative (e.g., *E. coli* and *Agrobacterium tumefaciens*) and gram-positive (e.g., *Bacillus subtilis* and *B. thurigiensis*) bacteria in the assay.

According to Osborn et al. (1995), plant defensins that are temperature-insensitive for biological function show no antibacterial activity. Ca-AFP is in agreement with this. Plant defensins like Dm-AFP1, Ah-AFP1, and Ct-AFP1, which show functional temperature sensitivity, are reported to have antibacterial activity against *Bacillus subtilis* at 150 µg/ml, 100 µg/ml, and 15 µg/ml concentrations, respectively (Osborn et al., 1995). However,

these defensins did not affect the growth of other bacteria such as *E. coli*. On the contrary, when mung bean defensin (VrCRP) was screened against *E. coli*, it was found to rupture the bacterial cells (Chen et al., 2002). This probably reflects the relative importance of infection pressure from fungi as opposed to bacterial pathogens on plants (Thomma et al., 2002). In contrast to plant defensin peptides, defensins isolated from insects, mussels, and humans are mainly active against several bacteria and occasionally against fungi (Dimarcq et al., 1998; Veldhuizen et al., 2008). Human defensin, pBD-2, is reported to have very strong inhibition capabilities against pathogenic bacteria at very low concentrations (4-8 µM) with action time less than 3 hours.

Insect Cell Viability Assay

When toxicity on insects was analyzed, Ca-AFP showed no toxicity effect on both types of lepidopteron cell lines, namely, *Sf21* and *T. ni*, in terms of growth and viability. Cells in the controls as well as in the test samples grew uniformly and made monolayers. The Trypan Blue exclusion method revealed viability comparable to that of the control even in the presence of 200 µg/ml Ca-AFP (Table 1). The viability data of *Sf21* (F (4, 40) = 0.1028, P = 0.981) revealed no significant difference among the concentrations. Similar results were found in the *T. ni* cell line (F (4, 40) = 0.033, P = 0.999). Although viability at all the concentrations (0-200 µg/ml) was significantly affected in both cell lines with respect to time (P < 0.001), it can be concluded that Ca-AFP may not have any toxic effect on insect cells.

A similar study was done with mung bean defensin, VrCRP (Chen et al., 2002). However, in that study the complete arrest of growth and rupture of *Sf21* were found with LC₅₀ 1.7 µM for the cell line. No other insect cell lines were assayed against this defensin. Chen and his colleagues (2005) studied larval development in the presence of azuki bean defensin, VaD1, to study toxicity, and they reported inhibition of larval development under *in vitro* conditions. So far all the toxicity assessments on insects are confined to only five families (Lovei & Arpaia, 2005). There is no study on insects belonging to the Diptera order. More studies need to be done in a wider spectrum in an ecologically realistic background (Wraight et al., 2000).

Human Cell Viability Assay

Trypan Blue staining assays revealed that the viability of two human hepatoma (Huh-7 and HepG2) and one human kidney cell line (HEK 293) were comparable to the control (without Ca-AFP) experiment (Table 1). Among the various concentrations, the viability data of HEK 293 (F (4, 40) = 0.078, P = 0.989) revealed non-significant differences. Similar results were found in the two hepatoma cell lines tested, namely, Huh-7 (F (4, 40) = 2.196, P = 0.087) and HepG2 (F (4, 40) = 0.106, P = 0.9798).

However, over time viability was significantly affected ($P < 0.001$) in all the concentrations in all three cell lines. As the change in treated samples is comparable to the change in the control, it can be deduced that Ca-AFP may not have any toxic effect on the human cell lines.

The viability of HEK 293 cells were further analyzed through MTT assay (Table 2). The data indicated no significant difference among different concentrations of Ca-AFP and also without Ca-AFP (one-way ANOVA: $F(4,15)$

$= 1.253$, $P = 0.331$). Therefore, Ca-AFP might not have any effect on the viability of human cell lines.

When the morphology of HEK 293 and Huh-7 cells was examined microscopically, they were found to grow uniformly into monolayers without exhibiting any morphological abnormalities (Figure 3). Both uncompromised viability and unaltered morphology indicate that Ca-AFP might not have any detrimental effect on human cell lines.

Table 1

Means values (\pm SD) viability of cultured (*Sf21*, *Tni*, Huh-7, HepG2, HEK 293) cell line ($n = 3$) in presence of various concentrations of Ca-AFP.

Time (hrs)	Ca-AFP Concentrations ($\mu\text{g/ml}$)				
	0 (Control)	10	50	100	200
Sf21					
0	99.00 \pm 0.00	99.00 \pm 0.00	99.00 \pm 0.00	99.00 \pm 0.00	99.00 \pm 0.00
24	98.17 \pm 0.48	98.13 \pm 0.65	98.13 \pm 0.51	98.07 \pm 0.15	98.13 \pm 0.31
48	96.48 \pm 0.11	96.40 \pm 0.35	96.33 \pm 0.25	96.37 \pm 0.38	96.33 \pm 0.21
72	95.10 \pm 0.96	95.03 \pm 0.23	95.07 \pm 0.21	95.00 \pm 0.56	94.93 \pm 0.12
Tni					
0	97.50 \pm 0.00	97.50 \pm 0.00	97.50 \pm 0.00	97.50 \pm 0.00	97.50 \pm 0.00
24	97.21 \pm 0.32	97.27 \pm 0.21	97.12 \pm 0.22	97.11 \pm 0.30	97.09 \pm 0.33
48	97.08 \pm 0.47	97.10 \pm 0.23	97.14 \pm 0.46	97.04 \pm 0.44	97.05 \pm 0.55
72	97.00 \pm 0.56	97.00 \pm 0.26	97.11 \pm 0.42	97.16 \pm 0.39	97.06 \pm 0.41
HepG2					
0	97.67 \pm 0.58	97.67 \pm 0.58	97.67 \pm 0.58	97.67 \pm 0.58	97.67 \pm 0.58
24	97.33 \pm 2.08	97.00 \pm 2.00	97.33 \pm 0.58	97.33 \pm 1.15	97.00 \pm 1.73
48	97.33 \pm 1.53	97.33 \pm 1.53	97.33 \pm 0.58	97.33 \pm 1.15	97.00 \pm 1.00
72	95.00 \pm 2.65	94.67 \pm 2.52	94.67 \pm 0.58	95.33 \pm 1.53	94.67 \pm 1.15
Huh-7					
0	97.33 \pm 0.58	97.00 \pm 0.00	97.00 \pm 0.00	96.33 \pm 1.53	96.67 \pm 1.53
24	96.67 \pm 0.58	96.33 \pm 0.58	96.33 \pm 0.58	95.67 \pm 0.58	96.00 \pm 1.00
48	96.00 \pm 1.00	95.67 \pm 0.58	95.33 \pm 0.58	95.00 \pm 1.00	95.33 \pm 1.53
72	95.33 \pm 0.58	95.00 \pm 0.00	95.00 \pm 1.00	94.33 \pm 0.58	94.67 \pm 1.15
HEK293					
0	97.67 \pm 0.58	97.67 \pm 0.58	97.67 \pm 0.58	97.67 \pm 1.53	97.67 \pm 0.58
24	97.00 \pm 1.00	96.83 \pm 0.29	96.70 \pm 1.25	97.00 \pm 1.00	97.00 \pm 1.00
48	95.17 \pm 0.76	95.00 \pm 1.00	95.33 \pm 1.15	95.00 \pm 1.00	94.67 \pm 0.76
72	94.00 \pm 1.00	94.00 \pm 1.00	93.83 \pm 1.04	93.67 \pm 0.58	93.67 \pm 1.26

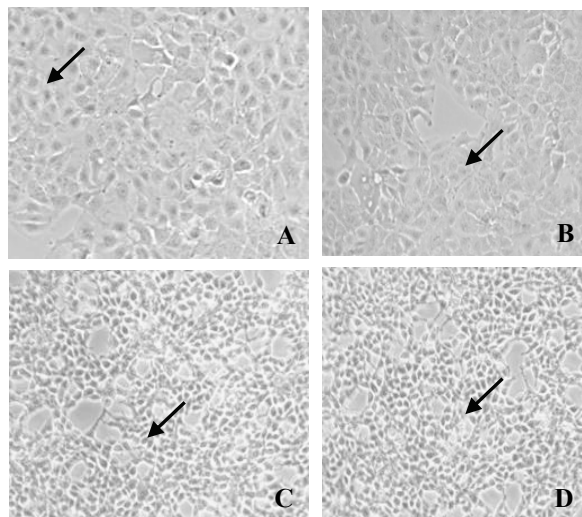
Table 2

Means values (\pm SD) of optical density (OD) during MTT assay in human HEK 293 cell line ($n = 4$) and Haemolytic activity assays ($n=3$) in presence of various concentrations of Ca-AFP.

Assays	Ca-AFP Concentrations ($\mu\text{g/ml}$)				
	0	10	50	100	200
MTT	3.360 \pm 0.046	3.211 \pm 0.320	3.276 \pm 0.320	3.298 \pm 0.031	3.085 \pm 0.256
Haemolysis	0.1390 \pm 0.008	0.136 \pm 0.008	0.140 \pm 0.005	0.138 \pm 0.003	0.138 \pm 0.006

Figure 3

Morphology of human cell cultures in presence and absence of Ca-AFP. Huh-7 cells growing on medium without Ca-AFP; **A.** In presence of Ca-AFP; **B.** HEK 293 cells growing on medium in absence of Ca-AFP; **C.** And in presence of Ca-AFP; **D.** Note that the cell morphology is similar irrespective to the presence of Ca-AFP in all the cell lines. Photographs taken on the third day of the treatments.



In the haemolytic assay performed on human blood cells, the percentage of erythrocyte lysis in the presence of different concentrations of Ca-AFP (e.g., 10 µg/ml, 50 µg/ml, 100 µg/ml, and 200 µg/ml) was found similar (15.06%, 15.46%, 15.24%, 15.24%, respectively) to the control having only PBS (15.36%). The data indicated that there is no significant difference among different concentrations of Ca-AFP peptide-treated blood cell lysis with the control (one-way ANOVA: $F(4,10) = 1.253$, $P = 0.9619$). Thus, the results demonstrated that Ca-AFP might not have any detrimental effects on human blood cells as well.

Similar observations with human cell cultures and erythrocytes have been reported for Rs-AFPs and HvAMP1, plant defensins isolated from Radish and Australian legume (Harrison et al., 1997; Terras et al., 1992). The smallest cysteine-rich antimicrobial peptide lb-AMPs are also reported non-cytotoxic towards human cell culture and erythrocytes (Tailor et al., 1997). This harmless effect can be due to its small size. However, in contradiction to these molecules, γ -thionin and β -purothionin, which are also small and cysteine-rich peptides, were found to have cytotoxic effects on human diploid culture cells at a concentration as low as 25 µg/ml (Thomma et al., 2002). On the other hand, cysteine-rich human defensin, hBD-2 on MTT assay, showed a dose-dependent effectiveness on human cell lines M-HeLa and A431 (Markeeva et al., 2005). The hBD-2 caused a 1.5-1.7-fold increase in viability in comparison to the control when tested at a concentration 0.01-2.0 µg/ml; however, the viability decreased at higher concentrations (3-5 µg/ml), and at a concentration of 20-40 µg/ml, a lytic effect was found.

Immunogenicity Test for Allergen

To a large part, regulations by most countries ensure that GM plants entering the food chain do not have any negative health effects (Alexander et al., 2004). However, plant foods derived from genetically modified plants might represent new allergen sources, and therefore it is necessary to evaluate them as potential allergens (GM Compass, 2006). This evaluation is carried out by finding the degree of similarity to other allergens (i.e., amino acid sequence comparison to know whether the new sequence provides epitope for antibody, animal tests, tests with blood from individuals who are sensitive to allergies, etc.). In the present study, a B cell epitope search was performed with the amino acid sequence of Ca-AFP using an online facility at www.imtech.res.in/raghava/bcepred. This analysis revealed that Ca-AFP carries only one B cell epitope, HLVSGRCR. This finding indicates that this epitope might make the peptide less immunogenic or may be less than the threshold to be immunogenic. When Ca-AFP peptide was injected into laboratory mice, antibody was not generated even after the third boost, supporting the previous prediction. Approximately 25% of plant-derived allergens belong to the plant defense molecules (Hoffmann-Sommergruber, 2002). Their small size, high stability at low pH, and resistance to proteolysis make these molecules probable candidates for evoking an immune response in predisposed humans. So far, plant-derived allergens have been identified with sequence similarities to these molecules. However, no such study with plant defensin is reported. Many plant defense molecules such as glucanase, chitinase, lipid-transfer protein, etc. have been reported to have the potential to be allergenic through such epitope searches.

With the recent explosion of gene identity and functional information about transgenes, the diversity of GM crops is increasing very rapidly. This is also making predicting the possible detrimental effects of GM crops more and more complex since transgenes can influence the ecology of the recipient and associated organisms immensely (Wilkinson et al., 2003). The toxicological studies of the novel anti-fungal defensin, Ca-AFP, performed in the present investigation gives a preliminary idea of its biosafety in humans and other organisms. This is much-needed information before considering any gene to transfer into a crop plant. All the studies showed that Ca-AFP is safe for humans and insects as well as harmless for helpful bacteria. These features along with broad-spectrum antifungal activity make Ca-AFP an attractive candidate to use as a transgene to improve many of our crop plants such as tomato, cabbage, and other vegetables that are infected by susceptible fungi. Although the present study shows that Ca-AFP is safe, this preliminary study needs to be taken to a level of extensive analysis for actual assessment of risk in order to justify conducting field trials under the framework of each country. Even more wide-ranging analyses of all the biosafety issues need to be performed before the commercialization of transgenics harboring the Ca-AFP transgene.

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