



Cocculus hirsutus Trypsin Inhibitor Confers Resistance to *Ralstonia solanacearum*: *In silico* Analysis

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Abstract

Genetic engineering has proven to be an efficient strategy for the development of Disease resistant crops. Gram negative bacteria *Ralstonia solanacearum*, the causative agent of wilt has been causing devastating economic losses in tomato crop yield worldwide. In this study *Cocculus hirsutus* trypsin inhibitor has demonstrated for its bactericidal activity. Transgenic plants expressing ChTI were developed conferring resistance to Gram negative bacteria, particularly *R. solanacearum*. *In vitro* and *In vivo* studies revealed the bactericidal activity of ChTI with mortality rate upto 42% and 48% in *E. coli* and *R. solanacearum* at 500 TIU/mg respectively. Transgenic plants expressing ChTI also developed resistance to the wilt. Whereas non transgenic plants developed symptoms of the disease. Further Strong interaction patterns of ChTI-Lipid and ChTI-membrane proteins revealed the possible mechanism of action of these PIs. These results demonstrate the effectiveness of ChTI as bactericidal agent and is able to enhance resistance in transgenic plants against bacterial pathogens.

Keywords: *R. Solanacearum*; Cell Membrane Binding Protein; *Cocculus hirsutus* Trypsin Inhibitor; Evans Blue Assay; Spot Dilution Bacterial Assay; In Silico Protein-Lipid Interactions

Abbreviations

LB: Luria-Bertani; TZC: 2,3,5-Triphenyl Tetrazolium Chloride; TLA: Trypsin Like Activity; TIA: Trypsin Inhibitory Activity; TIU: Trypsin Inhibitory Units; TU: Trypsin Units; MIC: Minimum Inhibitory Concentration

Introduction

Multitudinous factors are responsible for the bacterial pathogenesis development, among which proteinases play a pivotal role. Contribution of some conserved proteinases, to the pathogenesis, has been reported in both gram positive and negative bacteria. Conserved proteinase domains in gram positive bacteria control the virulence factor production, whereas proteinase in gram negative bacteria alter the biological process across the bacterial envelope thus contribute to host cell infection [1]. Many gram negative bacterial pathogens use type III secretion system to inject viru-

lence factors to induce pathogenesis in host cell. *Pseudomonas syringae* is known to inject AvrRpt2 protease to promote pathogen virulence [2,3]. *P. syringae* uses a different strategy, secret serine proteinases AvrA which misguides the plant flagellin mediated immune response in tomato [4]. Bacteria are known to secret proteinases to degrade cell wall components that make their way to enter plant cell. Prt1 isolated from *Pectobacterium carotovorum* was able to degrade potato lectin [5] and Yop J proteinases target plant hormone system interfering with the jasmonic acid and salicylic acid signalling pathway, by attenuating the expression of defence response genes [6-8].

Plants up-regulate number of genes which include antimicrobial peptides (AMPs) which are distributed in all plant tissues. They are the primary components of defence barrier in plants and show lethal effects on different microorganisms. AMPs are cationic, amphipathic, with helical structure having broad spectrum anti-

microbial activity. Plant AMPs are grouped based on the sequence homology and mode of action into defensins, thionins, lipid transfer proteins, cyclotides, snakins and hevein-like proteins [9]. These molecules target microbes at extracellular levels through receptor or non-receptor mediated interactions and intracellular host immune system modulation [10]. Proteinase inhibitors, isolated from various plant sources, are found successful in inhibiting the growth of phytopathogens. PIs extracted from leaves of *Coccinia grandis* showed antibacterial activity against *Staphylococcus aureus*, *Proteus vulgaris* [11]. PI from seeds of *Lavatera cashmeriana* inhibited the growth of *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* [12]. PIs have gained major attention in clinical field as potent antibiotics [13, 14], anti-carcinogenic agents [15,16] and therapeutic drugs [17,18]. Considering the importance of PIs in medicine and in agriculture as biocontrol agents, potentiality of serine proteinase Inhibitor, ChTI [19] was evaluated.

Material and Methods

Extraction of inhibitor from transgenic tomato plants expressing ChTI

Transgenic tomato plants harbouring ChTI was developed conferring resistance to *Helicoverpa armigera*. Resistant lines were selected based on PCR, expression of ChTI, inhibitory activity and in gel activity assay [20]. Leaf acetone powder from positive plants was stirred in extraction buffer (1:5 w/v; 50 mM Tris-HCl, 50 mM EDTA, 25 mM ascorbic acid and 10 mM β -mercaptoethanol at pH 7.4), centrifuged at 12,000 rpm for 20 minutes at 4°C. The supernatant was incubated at 70°C for 10 minutes; snap chilled, centrifuged at 12,000 rpm for 20 minutes at 4°C to get Heat soluble proteins (HSPs). ChTI in HSPs was purified using trypsin sepharose affinity column and assayed for trypsin inhibitory as well as antibacterial activity.

In vitro antibacterial assay

Logarithmic phase bacterial cultures were assessed on different concentrations of ChTI (100 - 1500 TIU). Bacterial cultures were grown in LB (*E. coli*) and TZC (*R. solanacearum*) media to a density of 1.5×10^5 cells/ml and were incubated with different concentrations of ChTI for 2h at 37°C. Serial dilutions up to a final density of 1.5×10^1 cells/ml were performed. Five μ l of each was spotted on agar plates and incubated at 37°C. Growth pattern was observed after 18h. 500 μ l cell suspensions were incubated with 0.1%, Evan's blue dye (w/v, 0.1g in 100 ml ethanol) for 1h at 37°C, Percent mortality of cells was assessed by measuring the bound dye. Bacterial suspensions incubated with distilled water and non-transgenic tomato extracts were taken as control.

Trypsin like activity

R. solanacearum/*E. coli* culture was grown till late log phase or early stationary phase. Cells were pelleted by spinning at 6000 rpm for 10 minutes, cells were suspended in 10 mM Tris buffer pH 7.4 and sonicated for 10 minutes. Cell pellets were washed several times with 10 mM Tris buffer pH 7.4 before stirred in same buffer containing 0.2% TritonX-100 for 20 minutes at 4°C, spinned down at 12000 rpm for 5 minutes at 4°C. The supernatant obtained was washed thrice with 5 volumes of ice cold ethanol and 2% acetic acid and finally dissolved in 10 mM Tris buffer pH 7.4 with 0.2% TritonX-100. TLA/TIA was assayed as per the method described by Kunitz casein digestion method [19,20].

Binding studies of bacterial cell membrane protein

Trypsin inhibitor affinity column was prepared as per protocols of March., *et al.* [21] with slight modifications. 20 ml CNBr activated sepharose 4B slurry was washed with 150 ml of 100 mM sodium bicarbonate (pH- 8.5) repeatedly for several times. After a brief water wash, the slurry was stirred with 50 mg/ml of ChTI solution for 20h at 4°C. Unbound inhibitor was removed by washing with bicarbonate buffer and stirred with 300 mg of glycine, to mask unreacted groups if present. The slurry was washed with double distilled water several times till neutral pH and suspended in 100 mM potassium phosphate buffer (pH 7.6). The column was saturated with repeated application of bacterial cell wall proteins, column was thoroughly washed to remove unbound proteins and cell wall protein bound fraction was eluted with 2% SDS. The eluted fractions were analysed on SDS-PAGE followed by western blot using anti ChTI-IgY.

In situ evaluation of ChTI transgenic plants against R. solanacearum

Transgenic Plant response to *R. solanacearum* was assayed with 25 days seedlings. The roots of both control (non-transformed) and ChTI expressing transgenic tomato plants were inoculated with 1×10^5 CFU/ml bacterial suspension, the roots of which were injured prior to inoculation. Minimum of triplicate samples were maintained in each treatment. Disease incidence was evaluated weekly and scaled according to infection, 0 (no wilting) - 4 (wilted dead plant).

In silico analysis of ChTI interactions with cell membrane proteins of R. solanacearum

ChTI protein sequence (NCBI No: EF207318) was deduced using BLASTp program. HDock [22] was used to examine protein-protein interaction and protein - lipid interaction with ChTI and phospholipid binding protein (PLP) from *R. solanacearum* (PDB ID: 2QGU) and Di-Palmitoy-3-Sn-Phosphatidylethanolamine. A total of

100 low energy ChTI-PLP structures were generated. Using information on conserved residues, docking scores and ligand RMSD as main criteria, interacting models were filtered. Data on interacting residues was generated using the DIMLOT tool [23] and complex was analysed using PyMOL1.3 [24].

Results

In vitro evaluation of transgene ChTI against bacterial pathogens

Tomato transgenic leaf extracts (ChTI) were evaluated for antibacterial activity by serial dilution and Evans blue test (Figure 1). Reduction in *E. coli* growth was initiated at 500 TIU/ml and at 1500 TIU/ml caused complete reduction in growth. The results of Evan's blue dye binding assay is also on par with this result (Figure 1C). TLA activity of *E. coli*, 350.0 ± 5.0 TU (100 µg protein per ml) reduced to 42% in presence of 500 TIU/ml of ChTI, with an IC_{50} of 800 TIU. The result of the effect of ChTI on *R. solanacearum* showed growth reduction in ChTI treated bacterial suspensions ranging from 500 - 1500 TIU/ml (Figure 2A). Further Evan's blue assay indicated a mortality rate of 42% in cell suspensions with 500 TIU (ChTI) which enhanced to 74% at 1500.0 TIU (ChTI) (Figure 2B). Trypsin like activity of bacterial cell wall extract was 473.4 ± 3.44 (100 protein µg/ml), reduced to 48% in presence of 500 TIU/ml of ChTI, with an IC_{50} value of 550.0 TIU.

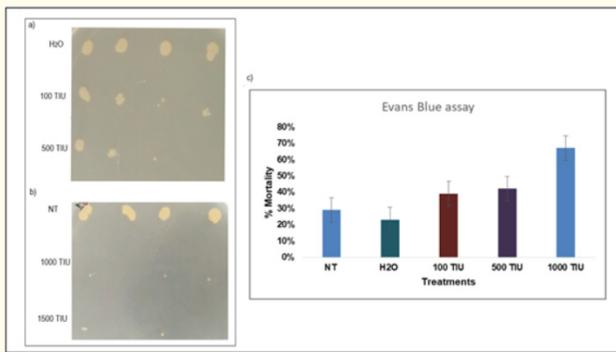


Figure 1: *In vitro* evaluation of bactericidal activity of ChTI against *E. coli*: Serial dilution assay was carried out to test the bactericidal activity of ChTI. a and b) Different concentrations of ChTI 100 - 1500 TIU was incubated with *E. coli* cells (10⁻¹ - 10⁻³). Water and non-transgenic plant extracts were used as control. c) The above mixture was incubated with Evan's blue dye for 2h resulting in the correlation of results of serially dilution assay and Evan's blue assay. MIC was recorded at 100 TIU with 34% mortality rate.

Membrane protein binding assay

Membrane proteins bound to ChTI - sepharose column was eluted with 2% SDS and was separated on SDS-PAGE gels (Fig-

ure 2B). Protein bands of about 50 kDa were visualised from Coomassie brilliant blue staining. Western blot analysis with ChTI-IgY showed reactivity with eluted cell membrane protein, suggest the possible interaction of cell membrane proteins with ChTI (Figure 2c and 2d).

Bioevaluation of transgenic tomato plants against *R. solanacearum*

Transgenic tomato plants were used to evaluate whether ChTI would confer resistance to *R. solanacearum*. The wilting symptoms was measured every 5 days for 30 days. In non-transgenic plants, symptoms of the disease were observed after 11 days of infection (Figure 2e-4) and subsequently plant showed complete mortality. Transgenic plants were healthy with hardly any symptoms of wilt caused by *R. solanacearum* (Figure 2e-3).

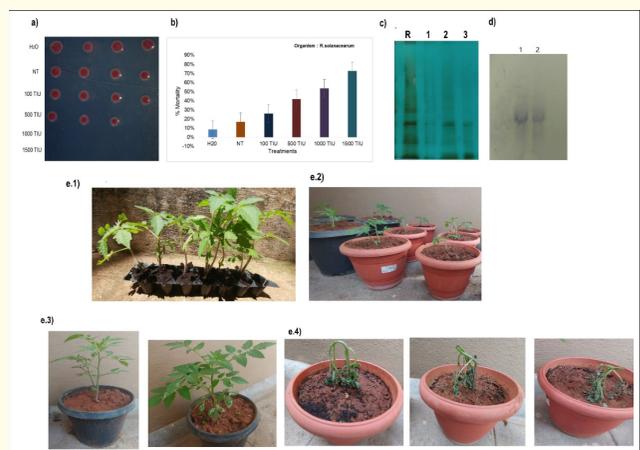


Figure 2: Evaluation of ChTI against *R. solanacearum*. *In vitro* as well as *in vivo* evaluation against *R. solanacearum* was carried out. *In vitro* evaluation was done by serial dilution assay, Different concentrations of ChTI 100 - 1500 TIU was incubated with serially diluted bacterial cells (10⁻¹ - 10⁻³). MIC is observed to be 100TIU. Water and Non transgenic plant extracts were used as control. b) The above mixture was incubated with Evans blue dye for 2 h resulting in the correlation of results of serially dilution assay and Evan's blue assay. MIC was recorded at 100TIU with 24% mortality rate. c) SDS -PAGE visualization of membrane proteins eluted from ChTI-sepharose affinity column. Lane R: membrane proteins before elution used as control, lane 1: Unbound eluted fraction, lane 2 and 3: ChTI bound eluted using 2% SDS. d) Western blot analysis of ChTI bound eluted fractions with ChTI-IgY. e) Transgenic plants were challenged with *R. solanacearum* 1: Day 1 before inoculation, Non transgenic plants were taken as control. 2: day 3 post inoculation, 3: Transgenic plants at day 15 were plants showed no symptoms of wilt, 4: Non transgenic plants at day 11 were plant almost succumbed to infection.

ChTI-PLP in-silico docking

In silico docking analysis was used to study the possible interaction between PLP (chain A) - ChTI. The overall structure consists of 300 amino acid residues. ChTI consists of 5 helices with 4 loop structure. Interaction patterns regarding the conserved residues of domain mlaC (Figure 4) with ChTI was carried out. 8 hydrogen bonds, Lys 124 (A) - Glu 96 and Ser 100; Arg 127 (A) - Arg 19, Arg 48, Glu 42; Asp 131(A) - Arg 12; Asp 151 (A) - Cys 103 at bond distance ranging between 2.12 and 3.22 was observed in this interaction studies (Figure 3).

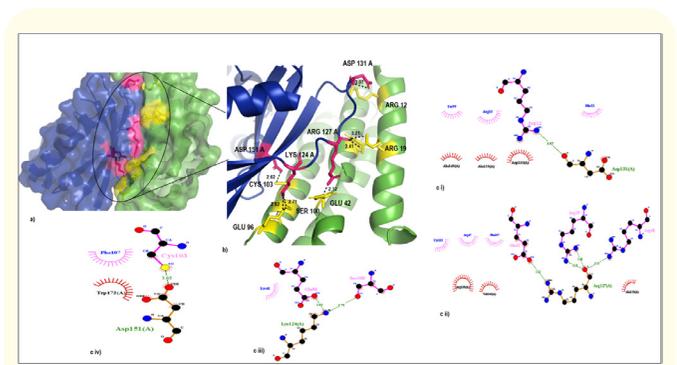


Figure 3: Interaction of ChTI on *R. solanacearum* membrane proteins. a) Surface view of the interaction between ChTI (Green) and PLP (Blue). b) 3-D visualisation of interaction residues of ChTI (yellow-sticks) and PLP (magenta-sticks). c) 2-D representation of interaction between ChTI and PLP (chain A).

consensus	1	HPKKSLLTLLALVIAFAS...LAAAPAPVSLVGEADKLSLKNQ...KIQDQVLRIVQQLPVDF	72
15892847	35	DYHQIITLGLWITFYAY...SHEVPAQLNSVYVNLNEASSLDS...KLSRVKIKARLHNSQLDF	102
16127923	7	THRIYITTRRSHVIALFFBGLSAPALQGGIDPAREAPVYQMGVILASISPHSEKQVYFQWQKLDV	86
15792695	1	--HKKFLITLFFNAFA...LQ-LDEISSTKKNIDASKLLQDN...KEEKIQANGIFKLFDSIDY	61
15793487	1	--HKKSSPISAIGIGLISG...HAFAPADAVWTRQNAQVLSLKS...DANTARQKAEAVAYVDF	64
15677793	1	--HKKSSPISAIGIGLISG...HAFAPADAVWTRQNAQVLSLKS...DANTARQKAEAVAYVDF	64
15599649	1	--MLTLRGLVLAAP...LLSVAFTQVQVQVDELSDSKANK...AYKADPKLYATLRLIGPVDA	69
15689972	1	--HQIITLGLWITFYAY...SHEVPAQLNSVYVNLNEASSLDS...KLSRVKIKARLHNSQLDF	66
17547677	3	KVLLGLLHLELFPANAV...HAKQEQDHTVYASDQVLTYSQ...LSPGKQFVLYQVQVWADP	74
15837020	9	HFAVALSTAPPMSHLQASS...LAEVQDQSKMVDSSARVLSLEQ...DFRQDQALRQPHTEFTIFOR	80
73	KYAAKLVIG-KYKYSAPQEQAF...FARFVLEQYVQALQVLDKLVKPSRPLDSE-DVIVRVEIDP-QQKPVKL	149	
15892847	105	DHMAKVTLGHQIKTLSGQVQEF...VSKVYVSYDLDKDKGQKLVQVPLSST--DFVAVNIISHEEQDPTK	180
16127923	87	PISTVPLVGLKAVRTFEGBARF...VAVHVSRSDDREQLVTSVVRKPS--DVEVNTIADQLQGVPI	163
15792695	62	KLMKLSLS-KVNSKLTKEQKFT...FATLSKSKFDKLSLVKQVQLVKNELNKE--VFLTSSVVDG-EKNIIF	138
15793487	65	QHITLAVG-NPVRTASDAQALAE...FTQLLIRYTSQTLKLNANWVNDP...TVNKGKKEIIVRAVGVV-QQVPM	142
15677793	65	QHITLAVG-NPVRTASDAQALAE...FTQLLIRYTSQTLKLNANWVNDP...TVNKGKKEIIVRAVGVV-QQVPM	142
15599649	70	EQIAKSVTV-KVYSRQPEQLR...FEVFKVLSLQVFNALVQNDIVL...PSSAPSD--RASVNEIRD--KGVY	145
15689972	67	DHMAKVTLGHQIKTLSGQVQEF...VSKVYVSYDLDKDKGQKLVQVPLSST--DFVAVNIISHEEQDPTK	144
17547677	75	KRTTQZAG-KFSGDTEEQDQ...QDQFLLVYVAGLAVNINWVY...VPLQAGMD--DVRVSTVNIH--DQVAV	150
15837020	83	DVAARLVIG-KYARNASDQV...LFDALITENLQVFNALVQNDIVL...PSSAPSD--RASVNEIRD--KGVY	145
15837020	81	DVAARLVIG-KYARNASDQV...LFDALITENLQVFNALVQNDIVL...PSSAPSD--RASVNEIRD--KGVY	145
consensus	158	DFLWKNQGTGK--MKVVDVIEGVSLVTTVQVQDGLRKK...IDGLQLESIN	202
15892847	181	EYLVREIKGKQVDFVSDIIT...EVSILGQDQEFDTLKNQ...FRLIQLESRS	235
16127923	164	SHRLLNSG--GK--MKVVDVIEGVSLVTTVQVQDGLRKK...IDGLQLESIN	202
15792695	139	KFYDNN--MLLYDQVLSVQVTSVQVSGDGLAQ...FDALQLESIV	187
15793487	143	DFTTYQ--GK--VRYVWVAGSALVTVYRQVBEIIRK...VGLIQLKANN	193
15677793	143	DFTTYQ--GK--VRYVWVAGSALVTVYRQVBEIIRK...VGLIQLKANN	193
15599649	146	PVSVYTNLAGE--MKVVDVIEGVSLVTTVQVQDGLRKK...IDGLQLESIN	202
15689972	145	EYLVREIKGKQVDFVSDIIT...EVSILGQDQEFDTLKNQ...FRLIQLESRS	199
17547677	154	QVWIKSAG--GK--MKVVDVIEGVSLVTTVQVQDGLRKK...IDGLQLESIN	202
15837020	159	DVLLRNN--GQ--MKVVDVIEGVSLVTTVQVQDGLRKK...IDGLQLESIN	209

Figure 4: Conserved amino acid residues of mlaC domain. The highlighted residues are predicted to be interacting with ChTI.

ChTI-phospholipid interaction

The possible interaction of ChTI with Di- Palmitoy-3-Sn-Phosphatidylethanolamine was also carried out through the mechanism of ChTI binding to membrane proteins. ChTI amino acid residues: Arg 12, Glu 96, Ser 100, Cys 103 interacted with Asp 131 A, Lys 124 A, Asp

151 A, respectively and Arg 19, Glu 42, Arg 48 with Arg 127A, were involved in the interaction via hydrogen bonding (Figure 5).

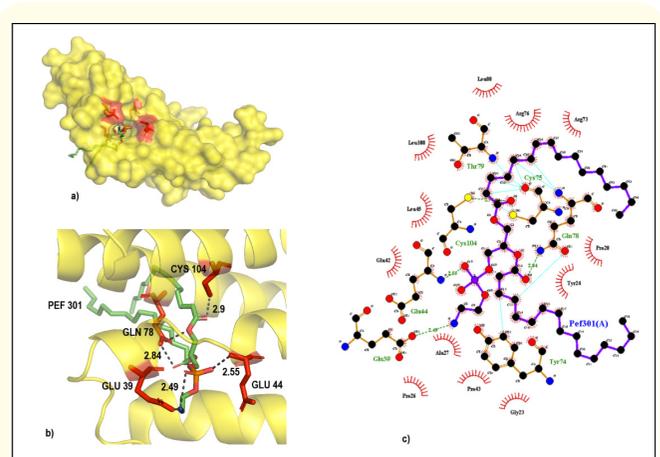


Figure 5: Possible mechanism of action of ChTI and phospholipid. a) Surface view of the interaction between ChTI (Yellow) and PEF (green). b) 3-D visualisation of interaction residues of ChTI (red-sticks) and PLP (green-sticks). c) 2-D representation of interaction between ChTI and PEF (chain A).

Discussion

PIs act as therapeutic intervention of certain microbial infection. These are known as defense molecules in plants and protect them from phytophagous insects and microbes [25-27]. ChTI, 18kDa thermostable protein inhibits the growth of *E. coli* with an MIC of 0.1 mg/ml and IC₅₀ of 0.8 mg/ml. Studies on bactericidal activity of *Coccinia grandis* protease inhibitor on *Staphylococcus aureus*, *Bacillus subtilis*, *E. coli* showed MIC of 1 mg/ml [10]. Thermostable chymotrypsin inhibitor, Potide G inhibited the growth of *S. aureus*, *E. coli* and *Clavibacter michiganense* [28]. Trypsin inhibitor isolated from *Brassica chinensis* seeds showed bactericidal activity against *B. subtilis* and *B. cereus* with an IC₅₀ of 236 μM and 222 μM, respectively [29]. Effect of ChTI on *R. solanacearum* showed complete mortality of bacterial cells with IC₅₀ of 0.5 mg/ml and MIC of 0.1 mg/ml. Transgenic plants tested against *R. solanacearum* performed better than the control plants with 80% survival rate. Dual expression of proteinase inhibitor genes, Sporamin (trypsin inhibitor) and CeCPI (Phytocystatin) in transgenic tobacco plants have conferred resistance to bacterial pathogens *Erwinia carotovora* and *Pythium aphanidermatum* [30]. PIs have been conferred with insecticidal and fungicidal activity since their identification and characterization, but not as bactericidal agents. Many AMPs have been reported to act on membrane surface resulting in pore formation followed by leakage of cell contents, resulting in the variation in

membrane polarization, finally leading to cell mortality [31]. Many AMPs are reported to interact with membrane lipids. Amphiphatic nature and positive charge at physiological pH make PIs adhere to membrane lipids and the mode of action is yet to be understood.

Binding of membrane proteins to ChTI affinity sepharose column suggest that ChTI interacts with *R. solanacearum* cell membrane (Figure 2c). Phospholipid binding protein from *R. solanacearum* was docked with ChTI. The results predicted that ChTI residues bound to conserved residues of domain mlaC, part of mla operon aiding in shuttling lipid substrates between inner membrane and outer membrane [32]. Screening *Acinetobacter baumannii* ABC transporter system homologs to the *Mla* system in *E. coli* showed that the pathogen uses this complex for phospholipid transport from inner membrane to outer membrane [33]. We predict that ChTI binds to this domain, and cause hindrance in biogenesis of lipid membrane resulting in loss of structural integrity of the cell wall leading to cell mortality. Further interaction of ChTI with phospholipid via hydrogen bonding, especially ChTI residue Glu 44 to phosphate group moiety along with other stabilizing interactions with membrane lipid also predicts the masking of the lipid moiety, thus making the substrates unrecognizable to lipid binding proteins.

Conclusion

The present study led to the identification of proteinase inhibitor gene, ChTI exhibiting bactericidal activity. Transgenic plants expressing these conferred resistance to *R. solanacearum*, one of most devastating pathogens infecting tomato. Affinity column as well as *in silico* analysis revealed the probable mode of action of ChTI is by binding to bacterial phospholipid binding protein or by making the host membrane phospholipids unrecognizable to these proteins.

Author Contributions

Dr. Devaraj V.R. , Dr. Theertha Prasad. D conceived and designed the experiments; Ms. Manushree. V, performed the experiments. Manushree V wrote the manuscript. All authors reviewed the manuscript.

Declaration of Interest

This manuscript has not been submitted to, nor is under review at, another journal or other publishing venue.

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