Improved tolerance against *Helicoverpa armigera* in transgenic tomato over-expressing multi-domain proteinase inhibitor gene from *Capsicum annuum*

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Received: 14 March 2017 / Revised: 27 May 2017 / Accepted: 1 June 2017 / Published online: 26 June 2017
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Abstract Plant proteinase inhibitors (PIs) are plant defense proteins and considered as potential candidates for engineering plant resistances against herbivores. *Capsicum annuum* proteinase inhibitor (CanPI7) is a multi-domain potato type II inhibitor (Pin-II) containing four inhibitory repeat domains (IRD), which target major classes of digestive enzymes in the gut of *Helicoverpa armigera* larvae. Stable integration and expression of the transgene in T1 transgenic generation, were confirmed by established molecular techniques. Protein extract of transgenic tomato lines showed increased inhibitory activity against *H. armigera* gut proteinases, supporting those domains of CanPI7 protein to be effective and active. When T1 generation plants were analyzed, they exhibited antibiosis effect against first instar larvae of *H. armigera*. Further, larvae fed on transgenic tomato leaves showed delayed growth relative to larvae fed on control plants, but did not change mortality rates significantly. Thus, better crop protection can be achieved in transgenic tomato by over-expression of multi-domain proteinase inhibitor *CanPI7* gene against *H. armigera* larvae.

Keywords *C. annuum* · *CanPI7* · Transgenic tomato · Proteinase inhibitor · *H. armigera*

Introduction

Helicoverpa armigera is an important lepidopteran pest responsible for severe yield losses in tomato and other many important crop plants. However, as efficacy of current pest controlling strategies such as insecticides, transgenic crops with *Bacillus thuringiensis* (Cry) gene (Tabashnik et al. 2013) and resistant crop varieties is threatened by the emergence of resistance, new approaches to control this pest are urgently needed.

Since the key discovery by Green and Ryan's (1972) that wound-inducible proteinase inhibitors inhibit digestive herbivore gut proteases, numerous plant proteinase inhibitors (PIs) have been characterized for their potential to control herbivorous insects (Hilder et al. 1987; Tamhane et al. 2005; Telang et al. 2009; Chen et al. 2014). In several plants, PIs are generally present in reproductive tissues such as flowers, tubers and seeds where they are produced and stored (Damle et al. 2005; Ryan 1990; Koiwa et al. 1997). PIs showing large structural and functional diversity are induced in vegetative tissues by insects wounding. Various PIs have been used for development of insect-resistant transgenic crops (Gatehouse 2011). Expression of potato inhibitor-II (Pin-II) gene in tobacco plants reduced the larval growth of lepidopteran pest *Manduca sexta* (Johnson et al. 1989). Also, transgenic rice plants expressing potato Pin-II gene has conferred protection against pink borer (Duan et al. 1996). In a reciprocal experiment, Ortego et al. (2001) showed that silencing of PI gene expression in transgenic potato plants increased larval weights of *Leptinotarsa decemlineata* and

Electronic supplementary material The online version of this article (doi:10.1007/s12298-017-0456-5) contains supplementary material, which is available to authorized users.

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Spodoptera exigua. However, the success of PI based approach to control insects is relied on the availability of novel PI forms effective against different insect pests. For instance, PIs obtained from non-host plants to which insect had no or minimal earlier exposure may prove extremely useful for increasing insect resistance in transgenic plants (Harsulkar et al. 1999; Dawkar et al. 2011). Pin-II family of serine PIs, predominantly found in Solanaceae is extensively studied in relation to plant defense system (Green and Ryan 1972; Kim et al. 2001). Precursor PIs of Pin-II type inhibitors in different plants contains 1 to 8 inhibitory repeat domains (IRD) of about 55 amino acids (AA) linked by proteinase sensitive linker regions of 5 AA, which upon cleavage by endogenous proteases release single inhibitor protein effective against one or several proteinases (Horn et al. 2005; Heath et al. 1995).

We previously reported that non-preferred host plant *Capsicum annuum* L. of *H. armigera*, possess a wide range of wound inducible Pin-II type genes (*CanPIs*) with difference in the number of IRDs, isoforms among individual IRD (1- to 4-IRD) and signal sequences joined by linker sequences (Tamhane et al. 2007, 2009; Mishra et al. 2010, 2012). Among the various isolated *CanPI* genes, multi-domain PI gene (*CanPI7*) with four IRD showed strong inhibition of *H. armigera* proteases and adversely affected the growth and development of *H. armigera* through artificial diet (Tamhane et al. 2007; Mahajan et al. 2013). In order to assess the potential of *CanPI7* gene for protection of tomato plants against *H. armigera*, *CanPI7* gene was introduced into the tomato. We report that transgenic tomato plant overexpressing *CanPI7* gene showed reduced fitness in the first instar *H. armigera* larvae.

Materials and methods

Bioinformatics analysis and construction of plant expression vector

Multiple sequence alignment of deduced AA sequences among all individual IRDs of *CanPI7* gene was performed using ClustalW program. Furthermore, three dimensional structure for these individual IRDs was predicted using homology modeling. A docking study was performed to determine binding energy and interaction of inhibitory domains with *H. armigera* proteinases. Detail methodology for interaction analysis is provided in Joshi et al. (2014). For expression and transformation *CanPI7* gene ORF (Accession No.: DQ005913.1) was amplified with gene specific primers with BamHI and SacI restriction sites (Supplementary Table 1). The amplicon was digested with BamHI and SacI restriction enzymes and cloned into pRI101-AN vector (Takara, Shiga, Japan).

Generation of transgenic tomato plants

Transgenic tomato plants were produced using protocol described by Sharma et al. (2009). Incubation conditions for in vitro culture, unless stated otherwise, were $25 \pm 1^\circ\text{C}$ and 16 h photoperiod of light intensity 2500–3000 lux. Initial T0 rooted kanamycin resistant plantlets were confirmed by PCR and transferred to a greenhouse for fruiting. The seeds collected from positive transgenic plants were selected on MS plates containing 200 mg/L kanamycin for segregation and selection of transgenic progeny. Finally, germinated T1 plants were transferred to greenhouse for further investigation. Putative T1 transgenic plants were examined by genomic PCR, Southern blot and reverse transcriptase PCR (RT-PCR) analysis. PCR amplification of genomic DNA for presence of *CanPI7* gene was performed using genomic DNA as template and gene-specific primers (Supplementary Table 1). For Southern blotting, DNA (10 µg) was digested by restriction enzymes (BamHI and SacI) (Promega, Madison, WI, USA), resolved on 0.9% agarose gel and transferred onto nylon membrane (Hybond-N+, Roche, Switzerland) using standard protocol according to Sambrook et al. (1989). The gene-specific probe (789 bp) was used to hybridize blotted membranes. Digoxigenin-labeled gene-specific probe generation, hybridization and detection was performed with DIG High Prime DNA Labeling and Detection Starter Kit I according to the manufacturers' instructions (Roche, Switzerland).

Total RNA was isolated from wild type (WT) and transgenic tomato plants under normal growth condition using Plant Spectrum RNA kit (Sigma-Aldrich Chemical Co., St. Louis, MO, USA). cDNA was synthesized using a reverse transcriptase (RT) kit with ~2 µg purified total RNA, according to the manufacturer's protocol (Applied Biosystems, Foster, CA, USA). PCR amplification of cDNA was performed using specific primers of *CanPI7* gene, with tomato PIN-II and elongation factor 1 α (EF) gene as an internal control (Supplementary Table 1).

Proteinase inhibitor activity assays

Total soluble protein extracted from leaves from 2 month old WT and T1 transgenic plants was assayed for proteinase inhibitory activity. Leaf tissues (200 mg) were grind to a fine powder and extracted in 500 µL of Tris–HCl buffer (0.1 M; pH 7.8) at 4 °C for period of 2 days. The extract was then centrifuged at 12,000 g (4 °C; 20 min) and clear supernatant was used for enzymatic assays. Total protein of clear supernatant was quantified following method described by Bradford (1976). For the enzyme inhibitor assay, a suitable volume of enzyme equivalent to 0.4 U *H. armigera* gut proteinases (HGP), was mixed with

leaf crude protein (50 µg), incubated at 37 °C for 10 min and residual proteinase activity was estimated using enzyme specific chromogenic substrates, Benzoyl-DL-Arginyl-*p*-Nitroanilide (BApNA) and N-succinyl-L-ala-L-ala-L-pro-L-leu-*p*-nitroanilide (SAApLNA) (Sigma, St. Louis, MO, USA), as reported earlier (Damle et al. 2005; Tamhane et al. 2005). One proteinase unit was defined as the amount of enzyme that increased the absorbance by 1.0 OD under the given assay conditions.

Insect bioassay of transgenic plants

Insect resistance of T1 plants was assayed by using detached leaves of WT and transgenic tomato plants using first instar larvae of *H. armigera* following the method of Senthilkumar et al. (2010). For leaf bioassay, fully expanded leaves of similar size and weight of approximately 2 month old greenhouse grown T1 tomato plants were placed in 9 cm Petri dishes containing 2% (w/v) agar and covered with filter paper to retain proper moisture. To carry out this assay, 15 leaves from WT and 15 leaves from each of three transgenic tomato lines were taken and one leaf per larva of *H. armigera* (60/treatment) was placed in Petri dish. Fresh leaves were supplied after every 48 h. Individual larval body mass was measured after every 24 h period. Also mortality of *H. armigera* larvae was recorded for a period of 7 days. The experiment was repeated three times.

Statistical analysis

Data was analyzed by one-way analysis of variance (ANOVA) with Tukey–Kramer multiple comparisons test. Data points were considered significant at $p \leq 0.01$, $p \leq 0.001$ and $p \leq 0.0001$.

Results

Bioinformatics analysis

Multiple sequence alignment of AA sequences of IRD variants of *CanPI7* gene viz. Domain 1 and 3 were typical trypsin inhibitory domain (TI) while Domain 2 and 4 represented a chymotrypsin inhibitory domain (CI) (Fig. 1a). These IRDs showed variation in active site residues. Trypsin and chymotrypsin inhibitory sites had Lys (K) or Arg (R) and Leu (L), respectively at P1 site of reactive core. Docking studies revealed significant differences in binding energies suggesting that inhibitory domains had variable interaction with *H. armigera* serine proteinase(s). Among these domains, 1 and 3 showed strong interaction with the lowest binding energy with

various *H. armigera* chymotrypsins (Fig. 1b). Domain 2 and 4 showed interaction with most of the serine proteinases, but they exhibited remarkable binding with *H. armigera* trypsins. Interaction pattern for domain 1 and 3 with most of the serine proteinases was found to be similar. Domain 1 and 3 are clustered together for all the analyzed serine proteinases, similarly domain 2 and 4 are clustered in separate clan. This suggests difference in overall binding pattern with most of gut serine proteases for domain 1 and 3 with respect to domain 2 and 4.

Characterization of transgenic tomato plants confirms integration of *CanPI7* gene

To study *CanPI7* gene function in insect resistance, *Agrobacterium tumefaciens* strain GV3101 having plasmid pRI101-*CanPI7* was used to generate transgenic tomato plants (Fig. 2a). Tomato transformation efficiency of 7.5% was achieved. Tomato transformation events gave rise to 9 kanamycin resistant T0 plants, which were confirmed as transgenic origin by genomic PCR analysis. Germinated kanamycin resistant T1 transgenic plants are shown in (Fig. 2b). Further, these transgenic tomato plants were examined by genomic PCR, Southern blot and RT-PCR (Fig. 3). Three T1 independent transgenic lines were selected and analyzed for inhibition activity assay and insect bioassay.

Increased qualitative and quantitative proteinase inhibitor activity in tomato plants expressing *CanPI7*

The inhibitory activity of protein extracts of transgenic and WT tomato leaf tissue was assayed against HGP. Transgenic plants exhibited significantly higher HGP inhibition as compared to WT (Fig. 4a). HGP inhibition was observed highest in transgenic line1 as compared with WT. Qualitative analysis of WT and transgenic leaves revealed that inhibition of trypsin-like activity was higher than inhibition of chymotrypsin-like activity of HGPs. However, quantitatively the amount of HGPIs was about 4–6 times higher in case of trypsin like activity while 4–7 times higher for chymotrypsin like activity in transgenic leaves as compared to those in WT leaves.

Transgenic tomato plants expressing *CanPI7* exhibit insect tolerance

After feeding for 7 days, average mass of larvae fed on transgenic leaves depending on line ranged from 4.4 to 5.8 mg per larva while on WT leaves was 11 mg per larva (Fig. 4c). Larvae feeding on leaves from *CanPI7* transformed plants had significantly reduced mean larval mass

Fig. 1 **a** Diagrammatic representation highlighting various regions of the *CanPI7* gene indicating the locations of the signal peptide sequence (SP), linker region and domains. Multiple sequence alignment of AA sequences of IRD variants (Domains) of *CanPI7* gene. **b** Interaction energy of binding of all four IRDs with *H. armigera* proteases is compared by using Heatmap analysis. Heatmap with hierarchical clustering of relative free binding energy (obtained from docking study) normalized by mean values of cumulative free

energy obtained from *H. armigera* trypsins, chymotrypsin, cathepsins and other serine proteases binding with IRDs of *CanPI7*. The gradient ruler from -5 (blue) to 5 (red) is an indicator of interaction strength, where blue colour indicate weak binding and red indicate strong binding. Accession number of each protease sequence (Uniprot IDs) is given in the bracket. The scale values (-0.37 to 1.0) on the cluster branches indicates the degree of correlation in different interaction and hence further used for the clustering (colour figure online)

at 2, 3, 4 and 6 days as compared to WT. Based on mass of larvae recovered in this insect bioassay experiment, larval population was distributed into various groups: 3.1–4.9, 5.0–9.9 and 10.0–20.0 mg. Larvae from transgenic lines

remained in lower larval mass range (3.1–9.9 mg). While, most of larvae recovered from WT leaves reached to higher larval mass (>10 mg). Highest reduction in mass of larvae was up to 60% in transgenic line 3 as compared to larvae

Fig. 2 **a** Schematic map of T-DNA regions of the expression construct pRI101-*CanPI7* used for tomato transformation. **b** Various stages of *Solanum lycopersicum* var. Pusa Ruby transformation **A** Cotyledonary explants on selection medium after co-cultivation with *Agrobacterium* strain GV3101 **B** Callus with regenerating shoot

buds growing on selection medium **C** Shoot elongation **D** Root formation **E** Mature T0 transgenic tomato plant in greenhouse **F** Transgenic fruits **G** Germination of T1 seeds on selection media **H** T1 transgenic plants in greenhouse

Fig. 3 **a** Molecular analysis of T1 transgenic plants **a** PCR amplification of the 342 and 173 bp fragment of the tomato PINII gene and *CanPI7* gene, respectively. Lane M: marker; Lane 1–2 DNA from WT; Lanes 3–8 DNA from transformed plants; Lane 9 negative template control, **b** RT-PCR analysis of *CanPI7* expression from transgenic tomato and WT. Lane 1–2 cDNA from WT; Lanes 3–8

cDNA from transformed plants, tomato Elongation factor-1 α (EF) and Pin-II gene was analysed as internal control; Lane 9 negative template control, **c** Southern blot hybridization analysis of six transgenic PCR positive T1 lines. The genomic DNA (digested with BamHI and SacI) hybridized with *CanPI7* probe. Lane 1 genomic DNA from WT; Lanes 2–7 genomic DNA from six T1 lines

Fig. 4 **a** HGP inhibitory activity of WT and transgenic leaves. Graph indicates the concentration in terms of HGP inhibitory units (HGPIUs) in WT and transgenic leaves, **b** Representative *H. armigera* first instar larvae fed on WT and transgenic tomato plant leaves for 7 days. Three independent plants were used per assay and data were obtained from the average weight ($\pm$ SE) of 60 larvae per treatment from independent experiments performed in triplicate. Representative

WT and transgenic leaves showing damage after infestation by *H. armigera* larvae, **c** Larval mass of first instar *H. armigera* larvae fed on transgenic lines and WT plant leaves. Larval mass was recorded after every 24 h for 7 days. Standard mean errors are indicated. a***, b** and c* indicate that values are significantly different from each other at $p < 0.0001$, $p < 0.001$ and $p < 0.01$ respectively, 'ns' denotes non-significant

fed on WT leaves on 7th day. Representative image of larvae recovered at the end of 7th day experimental period is shown in (Fig. 4b). Insects made severe damage to WT leaves compared to transgenic plants (Fig. 4b). No significant differences in larval mortality rate on transgenic and WT leaves were noted. Together, feeding experiment established that larvae fed on tomato plants expressing a *CanPI7* gene under a constitute promoter showed a significant reduction in their mass when compared with that of larvae fed on WT leaves.

Discussion

Heterologous PI genes expressed in crop plants has led increased resistance to a varied range of insect pests (Abdeen et al. 2005; Quilis et al. 2014; Schneider et al. 2017). The effectiveness of PIs against other pathogens such as bacteria, fungi and nematodes is also reported. Transgenic tobacco plants expressing the pyramided inhibitor genes

from potato and taro exhibited broad-spectrum resistance to *H. armigera*, *Erwinia carotovora* and *Pythium aphanidermatum* (Senthilkumar et al. 2010). In transgenic tomato over-expression of cysteine proteinase inhibitor from rice has conferred protection against white potato cyst nematode (Urwin et al. 1995). However, insects have also been shown to be adequately adaptable to the existence of either endogenous PIs of their host plants or transgenic PIs. Insect pests adapt to PI ingestion by over-producing PI sensitive proteinases, and/or producing proteinases that have either the ability to degrade inhibitors (Jongsma et al. 1995, 1996; Giri et al. 1998) or are insensitive to them. However discovery and characterization of novel PIs to address induced and inherent complexity of insect gut proteinases is obligatory. Keeping this aspect in view, PIs that derived from non-host plants containing repeated domains of inhibitors could be active against variants of proteinases. This could be a more effective alternative approach to enhance plant resistance in opposition to insect pests. In this work, we have shown that transgenic tomato expressing of multi-

domain proteinase inhibitor gene from *C. annuum* targeting different proteinases delays growth and development of *H. armigera* larvae.

In earlier reports from our lab we have shown that neonates had more prominent retardation upon exposure to recombinant CanPI7 as compared to third instar larvae (Mahajan et al. 2013). Thus to study the effect of transgenic plant with CanPI7 on growth of first instar larvae would be very crucial and gives insight into antibiosis effect of CanPI7 during initial stage of insect development from crop protection point of view. Insect feeding experiments revealed that mass of *H. armigera* larvae fed on *CanPI7* containing transgenic tomato plants was decreased as compared to larvae which were fed on WT plants. Based on the previous reports of inhibitory properties of CanPI7, a broad spectrum of insect digestive proteinases (serine proteinases) might be affected by activity of inhibitory domains. The possible reasons for delayed growth of *H. armigera* larvae could be AA starvation due to inhibition of insect gut proteinases and increased stress on gut proteinase expression system to express more and higher amounts of proteinases (Broadway 1995). Protein extracts from *CanPI7* plants exhibited inhibitory activity against HGP, which is in agreement with earlier reports of inhibition assays for recombinant CanPI7 protein (Tamhane et al. 2007). Binding energy comparison and hierarchical clustering analysis provides overview of specific interaction of inhibitor with various proteases. Furthermore, it will also give speculation about mode of action and effect of various inhibitory domains on *H. armigera* digestive proteases. Due to presence of multi inhibitory domains in capsicum serine proteinase inhibitor it would be more difficult for insect pest to raise expression of different insensitive proteases against this inhibitor. Moreover, novel inhibitors containing artificial multi-domain inhibitors with more potent inhibitor potential than its natural counterparts can be designed (Outchkourov et al. 2004). It has also been shown that by stacking two insecticidal genes with different mechanisms of action or by using combinations of PI genes, the possibility of evolution of adaptation or resistance in the larvae can be significantly reduced (Dunse et al. 2010; Chen et al. 2014). Together, the results presented here suggest that *CanPI7* gene in combination with other PI or resistance transgenes may provide a valuable strategy for the protection of economically important crop species against *H. armigera* pest. However, the further evaluation in field is needed for insect resistance of transgenic plants. A further challenge would be to find the right balance between efficacy, durability and specificity requirements for controlling *H. armigera* pest.

Acknowledgements RT thanks to CSIR for Research Fellowship and VVD is thankful to the Science and Engineering Research Board,

Department of Science & Technology, Government of India, New Delhi for funding under Young Scientist Scheme (SB/YS/LS-260/2013). This work is supported by CSIR under XII five year plan program (BSC0107) to CSIR-National Chemical Laboratory.

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