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Expression of *Cocculus hirsutus* trypsin inhibitor promotes endogenous defensive response against *Helicoverpa armigera* and enhanced levels of antioxidants

Manushree V.[1,2], Devaraj V. R.[2] and Prasad D. T.[1*]

[1]Department of Plant Biotechnology, University of Agricultural Sciences, GKVK, Bangalore- 560065, India.
[2]Department of Biochemistry, Central College Campus, Bangalore University, Bangalore-560001, India.

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Proteinase inhibitors are a group of defense related proteins, natural antagonists of proteinases, induced upon herbivory, play a defensive role against polyphagous insects and phytopathogens. Serine proteinase inhibitor isolated from *Cocculus hirsutus* (L.) *Diels*, *Cocculus hirsutus* trypsin inhibtor (ChTI) was found effective as antifungal, bactericidal and nematicidal agent. Tomato plants expressing ChTI have been developed by agro-infection with almost 27% transformation efficiency. Stable integration and expression of ChTI has been established by polymerase chain reaction (PCR), inhibitory assay and western blot assay. Transgenic plants showed increased fruit yield, antioxidants, phenolics, flavonoids and titratable acidity. Protein extracts of tomato plants inhibited *Helicoverpa armigera* (gut proteinases up to 40%. Transgenic plants MT_2 and JT_2 challenged with 2nd and 4thinstar *H. armigera* (Hubner) larvae, showed delayed larval growth with 100% mortality. The results put together suggest that ChTI is a potential candidate for developing transgenic plant with multiple biotic stress tolerance.

Key words: ChTI, *Helicoverpa armigera*, insect bioassay, transgenic tomato, trypsin inhibitory assay.

INTRODUCTION

Environmental stress is one of the major challenges for plants' growth and productivity. To overcome stress spawned by herbivory, plants up-regulate defense genes encoding for proteins, secondary metabolites, toxic chemicals and repellents (Jamal et al., 2013). In plants, proteinase inhibitors are major defendants, form inhibitory complexes with specific proteinases by irreversible trapping or tight binding interactions (Clemente et al., 2019). Proteinase inhibitor genes are expressed to regulate proteinase activity within the cell (Rustgi et al., 2017), as response to insect damage, mechanical wounding (Tamayo et al., 2000; Haruta et al., 2001; Laluk

*Corresponding author. E-mail: dtprasadd@gmail.com.

and Mengiste, 2011) possibly through systemin / jasmonic acid mediated signalling cascade (Yang et al., 2015). Plant Protease inhibitors (PIs) are classified based on their specificity into 4 mechanistic classes' cysteine, serine, aspartate and metallocarboxy proteinase inhibitors and data base on plant–PIs is accessible at http://bighost.area.ba.cnr.it/PLANT-PIs (De Leo et al., 2002; Habib and Fazili, 2007) .

Most of the lepidopterans gut proteinases are trypsin/chymotrypsin like enzymes (Giri et al., 2002; Tanpure et al., 2017), hence plants accumulate SPIs in the tissues as one of the defense strategy against herbivores. These inhibitors are widely studied as defense proteins against bacteria (Kim et al., 2009; Bacha et al., 2017), fungi (Quilis et al., 2007; Bhattacharjee et al., 2010; Pariani et al., 2016), insects (Dunse et al., 2010; War et al., 2012), nematodes (Turra et al., 2009, Vieira et al., 2015; Papolu et al., 2016). Many insects combine multiple strategies to circumvent the antinutritional effect of PIs viz. (a) up-regulation of proteinases with different substrate specificity to compensate the loss (Winterer and Bergelson, 2001; Zhu-Salzman et al., 2003), (b) synthesis of proteinases degrading PIs (Wu et al., 1997) and (c) over-consumption of PI expressing tissues to minimize nutritional stress (Cloutier et al., 2000). Further, insects switch to an alternative set of proteinases that allow them to thrive on host plants. Since, insects have the ability to adapt to endogenous PIs as a result of coevolution (Wu et al. 2010, Harsulkar et al. 2002) and transgenic plants expressing these PIs. Identification of novel PIs from non-host plants and their effectiveness against insect gut proteinases and express constitutively could be a promising approach to develop plants resistant to biotic stresses (Stout et al., 1999; Tamayo et al., 2000; Srinivasan et al., 2005; Tamhane et al., 2005). Expression of the proteinase inhibitor gene has been reported to be positive and resulted in increase in seed content, growth rate and biomass (Gutiérrez-Campos et al., 2001; Schluter et al., 2010). Leaf proteome analysis indicates ectopic expression of stress related genes in leaves of transgenic plants (Munger et al., 2012). Transgenic plants with increased tolerance to abiotic stress have also been reported by many groups (Stout et al., 1999; Goulet et al., 2008). These pleiotropic effects have paved way for crop improvement.

Serine proteinase inhibitor belonging to the Kunitz inhibitor family has been characterized from *C. hirsutus* (ChTI) and found effective against mid-gut proteinases of *Helicoverpa armigera* and *Spodoptera litura*. ChTI is a monomeric protein of ~18kDa, with a narrow pH range (7-9) and higher thermo-stability (70°C). *In vitro* feeding of ChTI caused significant mortality of *H. armigera*, *S. litura* (Fabricius) larvae, and exhibited potential antifungal activity (Bhattacharjee et al., 2010). In the present study, we discuss the results of stable integration of ChTI and its constitutive expression at various stages of plant growth, effectiveness against *H. armigera* larval growth, nutritive and morphological traits during growth and fruit development in tomato.

MATERIALS AND METHODS

Plasmid constructs

pChTI (amp^r) and p*CAMBIA* (kan^r, hyg^r) were grown in LB media containing ampicillin and kanamycin at 37°C. *pChTI* was amplified with M13/ChTI specific forward and reverse primers(*ChTI*- Forward- 5' ACCTGCGCCAATCAATGAG 3'; Reverse- 5' GCAGAAGTCACGACCGAC 3' and M13 - Forward- 5' GTAAAACGACGGCAG 3'; Reverse- 5' CAGGAAACAGCTATGAC 3'). *ChTI* amplicons and p*CAMBIA* were digested with *EcoR I* and *Hind III* and separated on 1.0% agarose gel. Fragments were co-eluted by freeze thaw method, washed with phenol:chloroform:isoamylalchohol (25:24:1 v/v) followed by 70% aqueous ethanol. Air dried pellet was dissolved in 5 µl of sterilized water and ligation was carried out using T4 DNA ligase (*pCAM-ChTI*) and transformed into *Escherichia coli* DH5α and *Agrobacterium* strain LBA4404 (Sambrook et al., 1989; Jyothishwaran et al., 2007). Transformed colonies selected on LB plates containing ampicillin and kanamycin, were analyzed for the presence of *ChTI* and *hpt* (Forward- 5'-TAGAAAAAGCCTGAACTCACCG-3' and Reverse- 5'-TATTTCTTTGCCCTCGGACG-3') using gene specific primers.

Development of transgenic tomato

Seeds of cherry tomato line 252 obtained from University of Horticulture Sciences, Bagalkot were rinsed with sterile distilled water, followed by 4% (v/v) sodium hypochlorite solution for 15 min and sterile distilled water. Tomato plants were transformed with *pCAM-ChTI*, as per protocol developed in our group using cotyledons of the germinated seedling as explants (Manamohan et al., 2011; Somayaji et al., 2014). MS media composition used at different stages of transgene development are as follows: 1. MS media without any hormones; 2. MS media for co-cultivation- MS media + 0.1 mg L^{-1} IAA + 4.0 mg L^{-1} BAP + 100 µM Acetosyringone; 3. MS media for proliferation- MS media + 0.1 mg L^{-1} IAA + 4.0 mg L^{-1} BAP; 4. MS media for selection- MS media + 0.1 mg L^{-1} IAA + 4.0 mg L^{-1} BAP + 2.00 mg L^{-1} Hygromycin, and 5. MS media for rooting- MS media + 0.5 mg L^{-1} IAA + 0.5 mg L^{-1} BAP + 2.00 mg L^{-1} Hygromycin. Plantlets with well-developed roots were carefully washed and transferred to paper cups containing coco-peat and irrigated with sterile water regularly, and half strength MS media once a week, maintained at 25°C with relative humidity of 95% and light intensity 30 lux. After two weeks, plants were transplanted into large earthen pots containing farm yard manure, red soil and sand (1:2:1 w/w).

PCR analysis

Genomic DNA from a total of 56 plants was isolated from 5th leaves from the top (Doyle and Doyle, 1987) and purity of DNA was assessed by absorbance ratio at 260/280 nm. PCR was carried out using *ChTI* and *hpt* primers to confirm the recombinants: - initial denaturation at 94°C for 5 min, with 35 cycles of denaturation at 94°C for 60 s, annealing at 58°C for 90 s, extension at 72°C for 2 min and final extension of 72°C for 10 min. Positive transformants were forwarded to subsequent generations and stable integration of *ChTI* was assessed by PCR.

Trypsin inhibitory assay

Acetone powder of the leaves from individual plants was ground with extraction buffer (50 mM Tris-HCl, 50 mM EDTA, 25 mM ascorbic acid, and 10 mM β-mercaptoethanol at pH 7.4; 1:5 w/v), centrifuged at 12,000 rpm for 20 min at 4°C. Supernatant having total soluble proteins (TSPs) was incubated at 70°C for 10 min; snap chilled, centrifuged at 12,000 rpm for 20 min at 4°C (Bhattacharjee et al., 2010). Most of the plant serine proteinase inhibitors are thermostable unlike their counter acting proteinases and ensures limited contribution of endogenous proteolytic activity during the assay. Further use of acetone powder during the isolation of ChTI, also avoids the interaction of low molecular weight metabolites interfering with the inhibitory assay. Supernatant (Heat Stable Proteins -HSPs) was used for trypsin inhibitor assay using casein digestion method (Kakade et al., 1969; Bhattacharjee et al., 2010).

Specific activity staining

50 µg of HSP from the leaf extract of control and transgenic plants were separated on 10% anionic PAGE (Sambrook et al., 1989) and gel was incubated with 0.1 M potassium phosphate buffer pH 7.6 for 10 min followed by incubation with trypsin solution (100 µg trypsin/ml in 0.1 M potassium phosphate buffer pH 7.6) for 30 min at 37°C. Gel was washed with distilled water couple of times, and incubated with 10 ml of substrate solution containing 2.5 mg of acetyl-DL-phenylalanine-β-napthylester and 0.55 mg/ml fast blue RR in 0.1 M potassium phosphate buffer, pH 7.6 (Filho et al., 1978; Bhattacharjee et al., 2010), till colour development. The bands containing trypsin inhibitor showed unstained clear zone against intense pink background.

Immunoblot analysis

Laying hens were injected subcutaneously with 100 µg of affinity purified ChTI (Bhattacharjee et al., 2010) in Freund's complete adjuvant followed by 3 doses in Freund's incomplete adjuvant in week interval. IgYs were purified as per method described by (Song et al. 1985). HSPs from transgenic leaf, fruit and shoot were separated on 10% SDS-PAGE and electro-blotted onto PVDF membrane (NEN Life sciences, England), ChTI specific band was detected with ChTI-IgY (1:1000 v/v) and anti-IgY-rabbit antibody conjugated with HRP (1:2000 v/v). Bands were visualised by incubating with TMB/H_2O_2 substrate solution (1:10 v/v). For *in vivo* localization of ChTI, leaf tissue imprint analysis was carried out. Fully expanded leaves were placed on PVDF membrane sandwiched between Whatman filter papers and pressed with even force. Serological reactions were carried out to detect ChTI.

Phenotyping transgenic plants

Leaf shape, plant height, number of fruits per plant, fruit weight, time taken for flowering, fruiting, and ripening were recorded in control and transgene plants. Two transgenic lines from MT_0 and JT_0 were taken for analysis. T_0 lines were forwarded for further generations up to T_2 and 5 plants from each line were used to study.

Biochemical analysis

Fruits from MT_0 and JT_0 and their subsequent generations up to T_2 from randomly selected 5 plants in each line were analyzed for nutritional traits. 10 ripened fruits were harvested and individually analysed for titratable acidity (TA), ascorbate, phenols, flavonoids, lycopene, β-carotene, total antioxidant capacity. Titratable acidity was determined by titration method using citric acid standard and expressed as % acidity (AOAC, 2000). Ascorbate was estimated by 2, 6 dichloro phenol indophenol method expressed as mg ascorbic acid equivalent per 100 g fresh weight (AOAC, 2006). Total phenolic content was measured using Lowry's method expressed as mg gallic acid equivalents per 100 g fresh weight. Total flavonoids were expressed as catechin equivalent per 100 g fresh weight (Singleton and Rossi, 1965). β-carotene and lycopene were expressed as mg of carotene equivalent /100 g fresh weight (Lichtenthaler, 1987). Total antioxidant capacity (Aoc) was measured using FRAP assay (Benzie and Strain, 1996) and expressed as mg ascorbic acid equivalent (AEAC) per 100 g of fresh weight.

Insect bioassay of transgenic plants

Detached leaf assay was carried out using fully expanded leaves of control (WT) and transgenic plants (JT_2) expressing ChTI [3100–3200 TIU/g tissue] were placed on 2% (w/v) agar plates (Giri et al., 2002). Single larva (2^{nd} instar and 4^{th} instar) per leaf was released and larval biomasses were measured at 24 h. For feeding choice assay, two larvae per plate containing control (WT) and transgenic leaves (JT_2) were used. After 24 h amount of leaf left after feeding was recorded. 5 leaves each from each transgenic plants was used per assay.

Effect of ChTI on *H.armigera* gut enzymes

Second instar *H. armigera* larvae were dissected on ice, mid gut was separated and stored at 4°C. Mid gut was suspended in 0.2 M glycine- NaOH buffer pH 10.0 (1:5 w/v), homogenized, and centrifuged for 15 min, 12000 rpm at 4°C. *H. armigera* gut proteinases (HGPs) and inhibitory activity of ChTI (2000 TIU/ml) extracted from transgenic leaves (JT_2) on gut proteinases was assayed by casein digestion method. HGP's were separated on native PAGE at 4°C and were visualized by activity staining by pre-incubation with transgenic (JT_2) and control (WT) leaf extracts.

Statistical analysis

Analysis of variance was carried out to assess the differences, some of the parameters documented in transgenic and control plants. Results were analysed by one way ANOVA program of graph pad prism 5.0. Comparisons of means were done using "Bonferroni's Multiple Comparison Test" ($p<0.05$). Correlation between inhibitory activity and phenols, flavonoids, carotenoids, ascorbate and titratable acidity was made by the Person's procedure ($p<0.001$). Intergeneration regression was carried out using Microsoft excel programme ($p<0.05$).

RESULTS

Transgenic tomato expressing ChTI

pCHTI (amp^r), p*CAMBIA1301* (kan^r) were grown in LB media with appropriate selection markers at 37°C. Since ChTI exhibited bactericidal activity when *E. coli* harbouring *pChTI* was grown for long periods, the plasmid was isolated from 3h grown culture. *pChTI* amplified with M13/ChTI specific forward and reverse

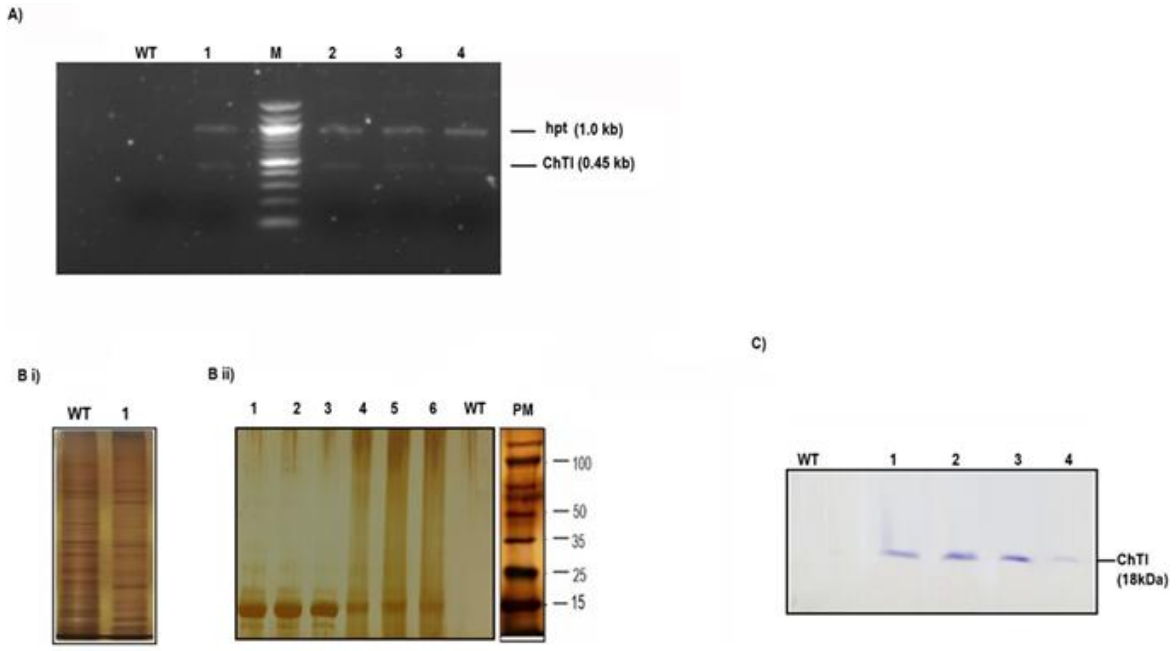

Figure 1. Screening and confirmation of ChTI in transgenic tomato plants. Plants conferring hpt resistance were screened for the presence of ChTI by PCR, and its expression by western blotting. A- PCR analysis of T0 transgenic and WT plants using *ChTI* and *hpt* specific forward and reverse primers. Lane 1-4: PCR amplicons of *ChTI* (0.45kb) and *hpt* (1kb) confirming transgene integration, M: 100bp DNA ladder, WT- non transgenic (control). B- Silver stained SDS-PAGE visualisation at different stages of extraction and purification of ChTI (18kDa) from WT and transgenic leaf extracts. B i) - lane 1- Total soluble fractions isolated from WT extract, Lane 2- TSPs from transgenic extract, B ii) Lane WT - HSPs from WT tissue , Lane 1-3- HSPs from Transgenic tissue , Lane 4-6 - Affinity purified ChTI from transgenic extracts. C -Western blot visualisation of ChTI in T0 plant tissue extracts, in the figures lanes: WT- non transgenic, expression levels of ChTI in transgenic tissue, Lane 1- root, Lane 2- shoot, Lane 3 - fruit, Lane 4- leaf.

primers, yielded 0.7 kb and 0.45 kb amplicons respectively (Supplementary Appendix Figure S1-A). M13 amplicons of ChTI and *pCAMBIA1301* were digested with *EcoRI* and *HindIII* and separated on 1.0% agarose gel. Suitable gel bits were pooled, co-eluted by freeze thaw method, and ligated using T_4 DNA ligase. Recombinant *pCAM-ChTI*, was transformed into *Agrobacterium tumefaciens* LBA4404 *(*rif^r) and selected on LB containing kanamycin. PCR amplification of *pCAM-ChTI* with *ChTI* and *hpt* gene specific primers resulting in 0.45 and 1.0 kb amplicons, confirmed the transformants selected on LB (kan^r, rif^r) media (Supplementary Appendix Figure S1-C). *Agrobacterium* mediated transformation of ChTI resulted in 25.7% efficiency and positive T_0 plants transferred to soil medium, grown under greenhouse conditions.

Screening and expression of ChTI in tomato plants

Leaf genomic DNA amplification with *ChTI* and *hpt* primers yielded amplicons corresponding to 0.45 kb and 1 kb of *ChTI* and *hpt* in transgene plants only, confirms *ChTI* integration (Figure 1A). Further, HSPs fraction was subjected to SDS-PAGE analysis, to ascertain the presence of ChTI specific protein (Bhattacharjee et al., 2010; Figure1Bii). Western blot analysis using ChTI-IgY indicated the presence of 18 kD protein corresponding to ChTI in transgene plants (Figure 1C) and *in gel* staining activity for ChTI confirmed inhibitory activity (Figure 2B). Tissue imprinting of leaves showed the uniform distribution of ChTI (Figure 2C). HSPs exhibited high inhibitory activity ranging from 3000-3500 TIU/ g in aerial tissues (leaf, shoot and fruits) and 2000-2800 TIU/g in roots. However, there was no ChTI activity in the WT (control) plant tissues (Figure 2A). Leaf TSP's, HSPs, and trypsin affinity purified inhibitor (4.5, 2.1 and 0.89 mg/ml) showed 1310, 3100 and 5940 TIU/g tissue, respectively.

Morphological attributes

Transgenic plants expressing high levels of ChTI (3100-3300 TIU/g tissue, Table 2) were found taller than controls with increased internode distance and stem diameter (Table 1 and Figure 3A). Difference in number of leaves at inflorescence was not significant. Transgenic plants had larger leaf area, smoother edges with more

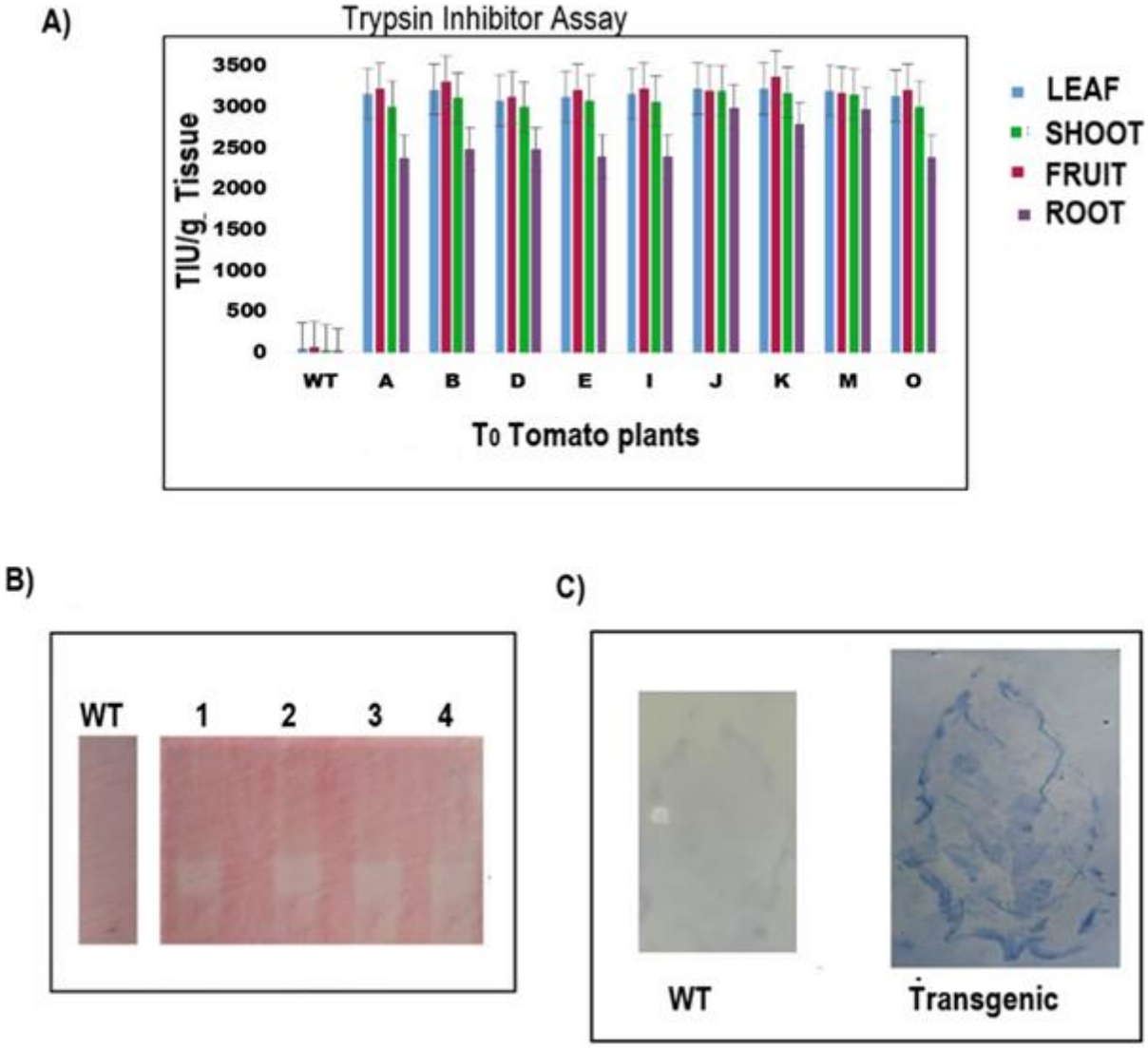

Figure 2. Trypsin inhibitory activity of Transgene Tomato plants expressing ChTI. Positively screened transgenic plant tissues were assayed for inhibitory activity. A- Trypsin inhibitory activity from tissues (leaf, shoot, fruit and root) from control and ChTI expressing T_0 plants. Stable inhibitory activity observed in M and J plant tissue extracts. B- In gel activity of ChTI. HSP of MT_0 and JT_0 leaf extracts were separated on 10% SDS gels and bovine trypsin inhibitory activity was visualised using acetyl-DL-phenylalanine-□-napthylester as substrate. Lane WT- control, Lane 1&2- transgenic leaf extracts from MT0, Lane 3&4- transgenic leaf extracts from JT_0 and C- Tissue print immune-localization of ChTI showing uniform distribution of transgene in transgenic leaf.

heft compared to small, serrated and narrow leaves in WT (Figure 3A). Although, flowering occurred 10 ± 2 days earlier than WT plants, there was hardly any difference in flower morphology (Table 1). WT plants produced more flowers than transgenic plants. Fifteen days' delay in fruit ripening was noticed in transgenic plants (Table 1). Total number of fruits (MT_0-18 ± 2; JT_0-16 ± 2) per transgenic plants was less than WT (30 ± 3), and individual fruit weight in transgenic plants (MT_0-36.46 ± 1.67; JT_0-34.86 ± 2.23) was 10 fold higher than WT (3.85 ± 0.70, Table 1, Figure 3C), resulting in improved net yield.

Biochemical analysis

Titratable acidity (TA) in transgenic fruit (MT_0-0.31 ± 0.07, JT_0-0.34 ±0. 01) was 25% higher than WT fruits (0.27 ± 0.01). Further transgenic fruits had almost 35% higher ascorbic acid (MT_0-16.07 ± 1.06 and JT_0-14.32 ± 0.21 mg per /100 g respectively) than WT (10.53 ± 0.24 mg/100 g, Table 2), resulting in a positive correlation between TA and ascorbate (Table 3). Carotenoids, lycopene and β-carotene were high in transgenic fruits (Lycopene: MT_0-2.65 ± 0.06 and JT_0- 2.24 ± 0.04 mg/100 g fw; β-

Table 1. Variation in the vegetative and reproductive parameters of WT and ChTI expressing tomato plants at T0 generation.

T_0	Internode length (cm)	Stem diameter (cm)	No. of Leaves at first inflorescence	Plant height at first inflorescence (cm)	Onset of flowering[@] (days)	Blossom set to mature green[#] (days)	Mature green to red[$] (days)	Fruit weight (g/ fruit)	No. of fruits / plant
WT	1.49 ± 0.04	0.52 ± 0.02	12.00 ± 1.00	33.00 ± 0.11	33.00 ± 0.11	21.5 ± 0.70	26.50 ± 0.70	3.85 ± 0.70	30.00 ± 3.00
M	1.90 ± 0.24	0.80 ± 0.03	8.00 ± 1.00	42.88 ± 2.60	26.55 ± 1.06	30.0 ± 1.56	40.22 ± 2.61	36.46 ± 1.67	18.00 ± 2.00
J	1.94 ± 0.36	0.81 ± 0.04	7.50 ± 2.00	40.37 ± 2.97	24.25 ± 1.03	32.0 ± 2.61	38.75 ± 1.66	34.86 ± 2.23	16.00 ± 2.00

[@]Onset of flowering corresponds to first 5 flowers appearing, [#]First flower opening up to first fruit breaker, [$]First fruit breaker maturing to red. Data represented as mean ± SD (p values were significant from each other at p value <0.05) corresponds to 2 T_0 generation lines M, J (Transgenic) are compared with WT plant (non transgenic). 10 samplings are taken for each observation.

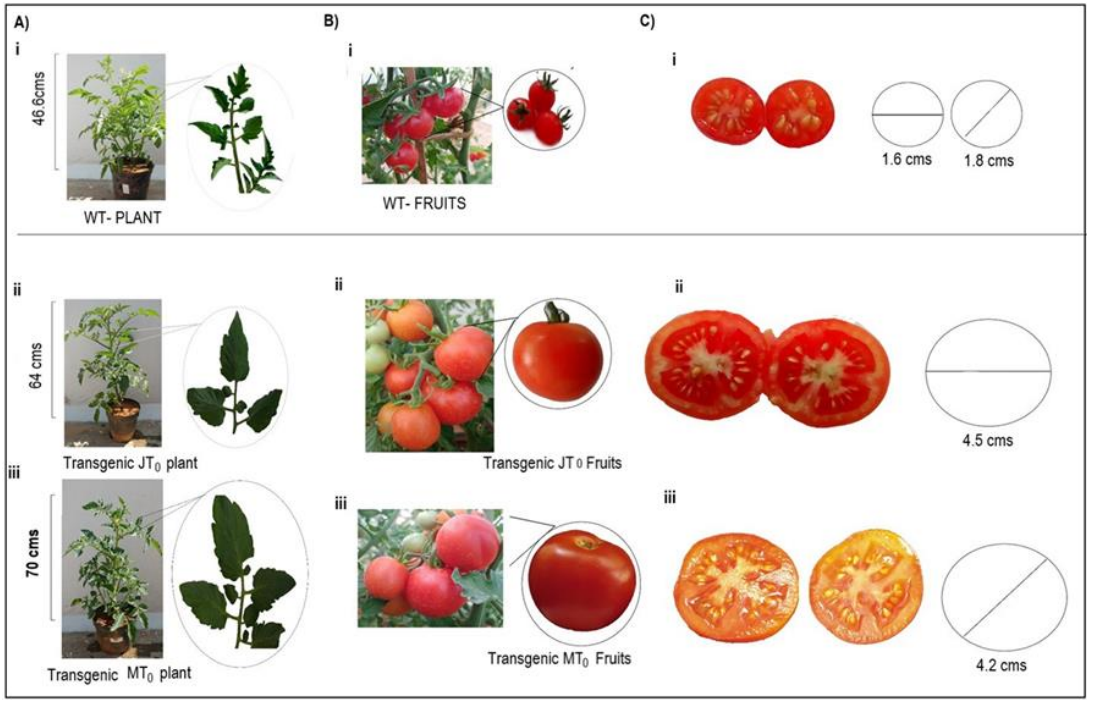

Figure 3. Phenotypes of transgenic tomato plants expressing ChTI. A- Plant height of WT and Transgenic plants (MT_0 and JT_0) at flowering stage, transgenic leaves with larger area, smoother edges and small, serrated and narrow WT leaves. B- WT and Transgenic fruits at harvesting stage. C- Cross section of WT and transgenic fruits. Note: WT - non transgenic plants considered as control. M and J - T_0 generation transgenic plants.

Table 2. Trypsin inhibitory activity and variation in nutrition and morphological parameters among T0 - control and transgenic plants.

T_0	WT	M	J
		Inhibitory activity@	
Leaf	0.00 0.00	3220.0– 3224.0 3221.00 ± 1.51	3197.0 – 3198.0 3198.00 ± 0.63
Fruit	0.00 0.00	3187.0 – 3199.0 3195.00 ± 3.96	3159.0 – 3166.0 3161.00 ± 3.55
Shoot	0.00 0.00	3196.0– 3199.0 3197.00 ± 1.41	3154.0 – 3157.0 3155.00 ± 1.47
Root	0.00 0.00	2975.0 – 2978.0 2976.00 ± 1.16	2972.0 – 2978.0 2976.00 ± 1.16
		Biochemical parameters	
Ascorbate[ß]	10.21 - 11.09 10.53 ± 0.24	15.32 - 17.88 16.07 ± 1.06	13.98 - 14.47 14.32 ± 0.21
Titratable acidity[ơ]	0.23 - 0.28 0.26 ± 0.08	0.34 - 0.36 0.31 ± 0.07	0.31 - 0.32 0.34 ± 0.01
Phenols[đ]	20.11 - 21.99 20.52 ± 0.13	49.76 - 51.14 50.45 ± 0.55	46.07 - 49.47 47.04 ± 1.20
Flavonoids[ε]	9.87 - 10.25 10.22 ± 0.02	15.01 - 15.79 15.22 ± 0.28	14.12 - 15.05 15.58 ± 0.40
Lycopene[ŧ]	1.62 - 01.84 1.82 ± 0.04	2.55 - 2.72 2.65 ± 0.06	2.11 - 2.56 2.24 ± 0.04
β -Carotene[ŧ]	2.11 - 2.13 2.12 ± 0.02	4.24 - 4.31 4.25 ± 0.12	3.25 - 3.89 3.60 ± 0.26
AoC[Ӣ]	7.10 - 7.15 7.14 ± 0.01	24.02 - 25.11 24.84 ± 0.84	22.00 - 24.12 22.67 ± 0.86
		Morphological attributes	
Onset of flowering in days	29.00 - 36.00 33.00 ± 2.00	28.00 - 31.00 29.00 ± 1.00	23.00 - 25.00 24 .50 ± 1.00
Mature green to red in days	21.00 - 22.00 23.66 ± 00.57	32.00 - 38.00 35.00 ± 3.00	37.00 - 39.00 37.80 ± 2.01
Fruit weight[Ï]	3.80 - 04.00 3.90 ± 0.10	25.60 - 32.55 31.05 ± 3.04	29.45 - 34.56 28.99 ± 3.63
Fruits / plant [Ţ]	30.00 - 40.00 35.33 ± 5.03	16.05 - 17.03 16.58 ± 0.76	17.40 - 21.44 19.69 ± 2.20

Data represented as mean ± SD. ($p < 0.05$) of Transgenic lines M T_0 and J T_0 and compared with non-transgenic plants – WT. 10 samplings from each line are taken for each observation. @- Inhibitory activity expressed as TIU/g tissue. Various Biochemical parameters of T_0 fruit values expressed as ß- mg ascorbic acid equivalent /100 g fw, ơ –% acidity, đ –mg gallic acid equivalents / 100 g fw, Ɛ – Flavonoids content expressed as mg catechin equivalents / 100 g fw, ŧ – carotene equivalents/ 100 g fw, Ӣ – AEAC/100 g fw, Ï – weight in grams/fruit , Ţ- fruits/ plant in number.

Table 3. Relationship between inhibitory activity and biochemical constituents of T0 transgenic fruits.

Variable	Inhibitory activity	Phenols	Flavonoids	βCarotene	Lycopene	Titratable acidity	Acsorbate	Total antioxidant capacity
Inhibitory activity	0.00	0.85***	0.54*	0.75**	0.87***	0.75***	0.70***	0.90***
Phenols	0.85***	0.00	0.75**	0.63**	0.82***	0.68**	0.77***	0.93***
Flavonoids	0.54*	0.75**	0.00	0.59*	0.54*	0.74***	0.82***	0.59*
β-Carotene	0.75**	0.77**	0.59*	0.00	0.72**	0.61*	0.83***	0.64*
Lycopene	0.87***	0.74**	0.54*	0.72***	0.00	0.77**	0.69**	0.96***
Titratable acidity	0.75**	0.86***	0.74**	0.61**	0.77**	0.00	0.51*	0.47*
Acsorbate	0.70**	0.82**	0.54*	0.82***	0.69**	0.51*	0.00	0.65**
Total antioxidant capacity	0.90***	0.93***	0.59*	0.64**	0.96***	0.47*	0.65**	0.00

Transgenic plants - T_0 M, T_0 J were considered. n*** , n**, n* indicates values were significant at p-value < 0.001 , < 0.05 ,< 0.01 respectively, n# indicates non-significant.

Table 4. ChTI activity of HSPs from the leaf extracts of tomato expressing ChTI against H.armigera gut protease.

Protein	Trypsin like activity of proteinases (TU/mg)	Trypsin like activity of proteinases incubated with ChTI (TU)	% Inhibition	IC_{50} of ChTI required to inhibit proteinases
H.armigera gut proteinase (HGPs)	144.00 ± 2.87	93.67 ± 0.94	34.5	700 TIU/g tissue

Leaf extracts from transgenic lines - M T2, J T2 were taken for the assay. The data obtained are the means ±SD (P<0.001). TU/mg – Trypsin unit , TIU/g- Trypsin inhibitory units.

carotene: MT_0-4.25 ± 0.12 and JT_0-3.60 ± 0.26 mg/100 gfw, respectively) which was about 30% higher than WT (lycopene: 1.82 ± 0.04 and β-carotene: 2.12 ± 0.02 mg/100 g fw; Table 2). Phenolic content in transgenic fruits was 60% higher than WT. Transgenic fruits had the highest phenol content (MT_0- 50.45 ± 0.55 mg, JT_0-47.04 ± 1.20 mg /100 g fw, respectively) over the WT (20.52 ± 0.13 mg /100 g fw). Flavonoid content was 40% more in transgenics, (MT_0-15.22 ± 0.28, JT_0-15.58 ± 0.40 mg /100 g fw, respectively), relative to WT (10.22 ± 0.02 mg/100 g fw; Table 2). Total Aoc activity in transgenic fruits (MT_0-24.84 ± 0.84 mg, JT_0-22.67 ± 0.86 AEAC/100 g fw, respectively) was higher compared to WT (7.14 ± 0.01mg AEAC/100g fw; Table 2). Positive correlation was observed in transgenic fruits at T_0 generation between biochemical traits and ChTI activity (Tables 3 and 4).

Inheritance ChTI in T_1 and T_2 generations

Based on the performance of T_0 plants and keeping inhibitory activity as the main criteria for selection, plants were forwarded to next generation. Plants from MT_1 and JT_1) were subjected multiplex PCR and inhibitory assay. Twelve plants, confirmed for the presence of *ChTI* and *hpt* by multiplex PCR (Supplementary Appendix Figure S2-A) and those with higher inhibitory activity were forwarded (Supplementary Appendix Figure S3) to next generation. Western blot analysis of plants expressing stable inhibitory activity showed similar immunobloting pattern (Supplementary Appendix Figure S2-C). Parent progeny regression analysis showed positive correlation with respect to their inhibitory activity and morphological/ nutritive traits (Supplementary Appendix Tables S1, S2, S3). Based on the morphological, biochemical, PCR analysis and Trypsin inhibitory activity (TIA), plants were further forwarded to T_2 generation. Random analysis of 10 plants from each, confirmed the presence of ChTI gene (Supplementary Appendix Figure S2-

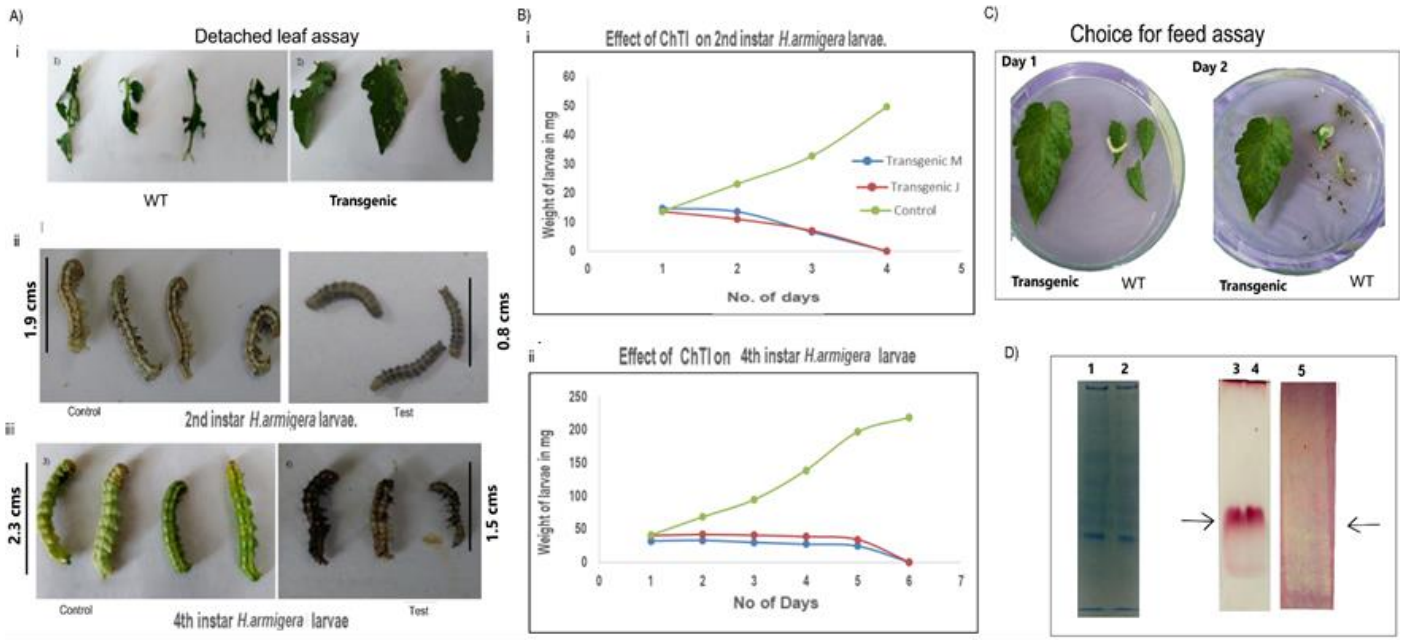

Figure 4. Effect of feeding on transgenic tomato plants expressing ChTI on H. armigera larval growth. A- Detached leaf feeding assay using WT and transgenic leaf damage with 2nd instar larvae. ii, iii) Retardation in growth of 2nd and 4th instar larvae fed on transgenic and WT leaves. B- Rate of H.armigera larval growth of 2nd and 4th instar feeding on WT and transgenic tomato leaves, C- Feeding choice assay using 2nd instar larvae. Larvae feed on WT leaves preferentially over transgenic leaves. D- In-gel assay visualize the effect of ChTI on HGPs. Lane 1, 2: HGPs visualized on SDS- PAGE. Lane 3: HGPs incubated with phosphate buffer, lane 4: HGPs incubated with WT leaf extract showing no inhibition in activity. Lane 5: HGPs incubated with transgenic leaf extract showing inhibition in activity. Note: Leaves and leaf extracts from two individual transgenic plants (MT2 and JT2) and non-transgenic (WT) were taken for the assay. The data obtained are the means ± SD ($p<0.001$) from 15 larvae performed in duplicates.

D) as well its expression (Supplementary Appendix Figure S2-F). Inter-generation regression and correlation analysis (Supplementary Appendix Table S4) showed strong association between individual mean of traits in T_1 and T_2 generation plants. These results put together indicated the inheritance of traits analysed from T_0 to T_2 generation.

Bioassay against *H. armigera*

T_2 generation plants were used to evaluate the effect of ChTI on growth of 2nd and 4th instar *H.armigera* larvae. Larval feeding assay showed significant reduction in mean larval weight and increase in mortality compared to WT (control). LT_{50} for 2nd instar larvae ranged between 3.5 to 4 days, and 4-6 days was for 4th instar larvae (Figure 4B) followed by 100% mortality in both cases at later stages of growth (Figure 4A ii; iii). Leaves of WT plants were severely damaged compared to transgenic plants (Figure 4i). Feeding choice assay showed that *H. armigera* larvae preferred WT over transgenic leaves. Transgenic leaves remained untouched whereas complete feeding on WT leaves was observed (Figure 4C). *In gel* assay showed that ChTI inhibits some of the major HGPs (Figure 4D). HSPs (700 TIU/g) from transgenic leaf extracts inhibited gut caseinolytic activity up to 34.5% (Table 4). These results together suggest that the amount of ChTI constitutively in transgenic plants (~2000 to 3500 TIU per g. of fresh weight), is more than enough to retard the growth and development of *H. armigera* larvae.

DISCUSSION

C. hirsutus possesses serine proteinase inhibitor (ChTI) which has potential insecticidal and antifungal activity (Bhattacharjee et al., 2010). *H. armigera* is a true generalist and agricultural pest that feeds on at least 161 plant species in 49 plant families (Wang et al., 2017). Since *C. hirsutus* is a non-host plant of *H. armigera,* we presumed that the insects are less likely to develop resistance against ChTI compared to similar type of inhibitors from host plants. The study was focussed on the development of transgenic tomato plants expressing ChTI and its *in vivo* evaluation against *H. armigera*. *Agrobacterium* mediated transformation (Manamohan et al., 2011; Somayaji et al., 2014) allowed us to achieve transformation efficiency up to 25.7% in tomato. Selection of plants were made based on the high expression of ChTI in vegetative tissues and fruits, and forwarded to

subsequent generations. SDS-PAGE followed by western blot analysis / *in gel* activity assay suggests that ChTI is 18 kDa protein, expressed constitutively in all these tissues. High level ChTI was expressed in leaves followed by fruits, shoot and roots. Earlier study report that proteinase inhibitor gene expressed under 35S promoter showed more accumulation of inhibitor in mature leaves than in flowers (Thomas et al., 1994). SDS-PAGE / western blot analysis using ChTI-IgYs showed the presence of ~18 kDa protein in partially purified tissue extracts corresponding to ChTI in T_0-T_2 plants. Leaf tissue imprinting analysis showed uniform distribution of ChTI. In earlier study, immuno-analysis in *L. peruvianum* has revealed the presence proteinase inhibitor throughout fruit development (Wingate et al. 2008). Plants expressing ChTI showed 40-45% increased plant height, early flowering by 10 days and increased fruit size. Although, there was delay in fruit ripening, an increase in nutrient and antioxidant levels was observed. Transgenic tobacco plants over-expressing OCI, showed increased plant height, biomass, earlier flowering and decreased life cycle (Gutierrez-Campos et al., 2001). Serine proteinases are likely to be associated in regulating programmed cell death, or associated processes such as senescence and cellular metabolic processes at every stage of plant growth and development (Fluhr et al., 2012; Santamaria et al., 2014; Ghorbani et al., 2016). High level constitutive expression of ChTI in tissues seem to have interfered in signalling mechanisms associated with physiological processes related to plant growth and fruit development.

Digestive process in lepidopteron gut mainly depends upon amylases, proteinases and lipases. Trypsin and chymotrypsin like serine proteinases play major role in providing amino acids pool for the growth and development of insects/pests through hydrolysis of ingested proteins. Large amount of the larval gut proteolytic enzymes are serine proteinases (Johnston et al., 1991). Use of proteinase inhibitors targeting these enzymes is one among the accomplishable crop management strategies against insect/pest control. However, insects under selective pressures have developed multiple mechanisms of adaptation to overcome plant's defense, especially, to proteinase inhibitors of domesticated crops by modifying their digestive physiology (Gatehouse, 2011). Up-regulation of chymotrypsin and other diverged serine proteinases and down regulation of trypsin like enzymes in gut has been reported in *H. armigera* larvae fed with artificial diet containing SkTI (Kuwar et al., 2015). Studies also suggest up-regulation of inhibitor insensitive proteinases in chickpea, pigeon pea, and cotton resulting in 35-55% larval growth. Non host plant PIs from *Pongamia pinnata, Mucuna pruriens*, *Capsicum annuum*, *Nigela sativa* and wild relatives of Chickpea (*Cicer arietinum)* showed maximum inhibitory potential towards HGPs *in vivo*, also exhibited moderate level of inhibition of pro-proteinases, *H. armigera* gut pro-proteinases (HGPPs) (Parde et al., 2010; Golla et al., 2018). Plant proteinase inhibitors from groundnut, potato, winged bean caused 80-100% larval mortality (Harsulkar et al., 2002). Affinity purified ChTI caused significant reduction in 2^{nd} and 3^{rd} instar larval growth (up to 84%), and resulted in 100% mortality in *in vitro* assay (Bhattacharjee et al., 2010). Earlier studies have shown that pro-proteinase levels increased during larval growth, and maximum HGPPs activity was observed in the fifth-instar. Larvae fed on diets with non host plant PIs showed greater inhibition of HGPPs as compared to HGPs. *In vitro* studies on HGPs treated with gut extract of larvae fed on *D. alba* inhibitor showed that out of 10 proteinase isoforms, two were activators of pro-proteinases. Larval growth and development were significantly reduced in the larvae fed on non-host plant PIs, resulting in stunted growth of *H.armigera* larvae. *In vivo* studies indicated that non-host plant PIs were good candidates as inhibitors of the HGPs as well as HGPPs (Parde et al., 2010). Considering the importance of crop protection / improvement, transgenic tomato plants over-expressing ChTI were developed. Leaf feeding bioassay using second and fourth instar *H.armigera* on transgenic tomato plants expressing ChTI showed larval mortality within 2-6 days. Several groups have reported plant protection with development of transgenic plants expressing non host PIs. Transgenic cotton over expressing PI-I and PI-II have reported LT_{50} of 11 days (War et al., 2012). Transgenic tomato over expressing CanPI-17 proved effective against *H. armigera* larvae with $LT_{50}$of 7days (Giri et al., 2010). It is observed 40% larval mortality in *H. armigera* larvae fed with cowpea trypsin inhibitor and 33% mortality of *H. armigera* larvae was observed upon feeding artificial diet impregnated with mung bean (Kansal et al., 2009). The results of our study indicate that 1000 TIU of transgenic leaf extract bring in almost 40% inhibition of HGPs. Constitutive levels of ChTI in leaves is almost 3000TIU, is more toxic leading to severe larval mortality. The reasons for decreased larval mass fed with inhibitors or feeding on transgenic leaves expressing sufficient amount of PIs, could be ascribed to amino acid starvation. Accumulation of proteins and nutrients is very crucial for larvae to progressively switch from one instar to another, disruption of which results in growth retardation, finally resulting in mortality. Feeding choice assay shows preference for WT leaves, but not ChTI expressing leaves, suggest that change in olfactory network. Avoidance of transgenic leaves might be due to release of anti-agents, volatiles gases, which may have mimicked the non host plant volatiles (Wang et al., 2017; Anderson and Anton, 2014). Previous work on *H. armigera* feeding choice assay has indicated that neurons in the medial sensillastyloconica on the maxillary glea contribute to the gustatory discrimination between cotton and pepper leaf saps (Tang et al., 2006). Further, isoforms of gut trypsin like enzymes are expressed in different developmental

stages and also on the basis of diets they were fed with. During the feeding experiments, it has been observed the proteolytic enzymes produced in early instars get inhibited and inhibitor resistant enzymes get expressed. Serine like proteinases expressed in early stages of larval growth is predominantly sensitive to PIs than the once expressed in the later stages of growth. Therefore, the larval growth and mortality rates depend on the stage of the larval growth and the effectiveness of the PIs resulting in delay of mortality rate of *H. armigera* (Bhattacharjee et al., 2010; Lomate et al., 2018; Chikate et al., 2013). These findings indicated that ingestion of transgenic leaves expressing ChTI at early stages of larval growth could control the lepidopteran population effectively.

Significant increase in the fruit size was observed in T_0-T_2 tomato plants expressing ChTI. Earlier studies in breeding crops for higher yield has been invariably associated with compromise on nutritional traits. Micronutrient malnutrition is a major threat in the present scenario, related to quantity and quality of food produced using modern agricultural technologies. Antioxidants are more stable in acidic pH. Increase in TA (68%) with increase in fruit size shows positive correlation of ascorbate (R^2= 0.511). Tittonell et al. (2001) report that higher TA (lower pH) provides stability of ascorbate and related antioxidants. Besides, intensity of light and amount of foliage is of particular interest contributing to the level of ascorbate in fruits (Ntagkas et al., 2019). Transgenic tomato plants expressing ChTI are taller, with low foliage levels compared to shorter control plants with heavy foliage. Burge et al. (1975) report that the higher fruit ascorbate levels in plants with less foliage (23 mg /100 g fw) and reduced level (18 mg/100 g fw) in plants with heavy foliage. Development of horticulture crops with increased Aoc is becoming increasingly relevant in accomplishing nutritional security in addition to increased production. Ascorbic acid, lycopene, flavonoids and phenols contribute to Aoc of the fruits (Toor and Savage, 2005). High Aoc activity in ChTI expressing tomato fruits showed strong positive correlation with TA, ascorbate, lycopene, phenols and carotenoids. Regression and correlation analysis between T_0-T_2 showed improvement in almost every trait documented in our study, thus suggesting successful inheritance of the traits along with ChTI expression for resistance to *H. armigera*.

Conclusion

The results of our study suggest that tomato plants incorporated with ChTI shows very high constitutive expression in all the vegetative tissues and fruits. ChTI effectively inhibits HGPs and larval growth of *H. armigera* effectively. The data reveals no compromise on the phytonutrients content viz. titratable acidity, antioxidant content, phenolics, flavonoids, ascorbate and lycopene, which was significantly higher in transgene fruits. PIs from the non-host plants have the potential to be expressed in genetically engineered plants to confer resistance to *H. armigera*. However, insect herbivores develop multiple mechanism of adaptation to overcome the defensive effects due to selection pressure. Future prospects for using proteinase inhibitor genes to enhance insect resistance in transgenic crops will require assessment of their mechanisms of action like their role in cell signalling, PCD other metabolic processes at various stages of plant growth and development.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ABBREVIATIONS

Aoc, Antioxidant capacity; **ChTI,** *C. hirsutus* Trypsin Inhibitor; **EDTA,** Ethylene di-amine tetra-acetic acid; **HGP,** *H. armigera* gut proteinase; **HGPP,** *H. armigera* gut pro-proteinase; **Hpt,** Hygromycin phosphotransferase; **HRP,** Horse radish peroxidase; **HSPs,** Heat stable proteins; **LD,** Lethal dose; **MS,** Murashige and Skoog; **PCD,**Programmed cell death; **PIs,** Proteinase Inhibitors; **SPIs,** Serine proteinase inhibitors; **TA,** Titratable acidity; **TIA,** Trypsin Inhibitory activity; **TIU,** Trypsin inhibitory unit; **TMP,** 3,3',5,5'-Tetramethylbenzidine; **TSPs,** Total soluble proteins.

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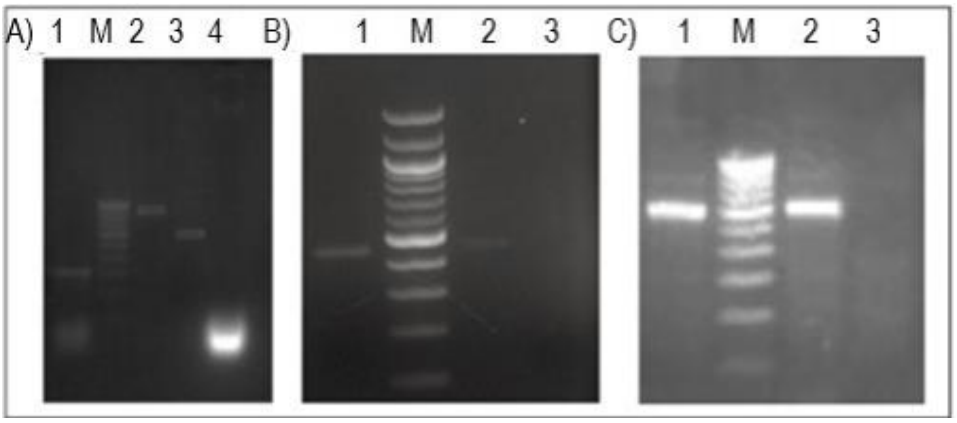
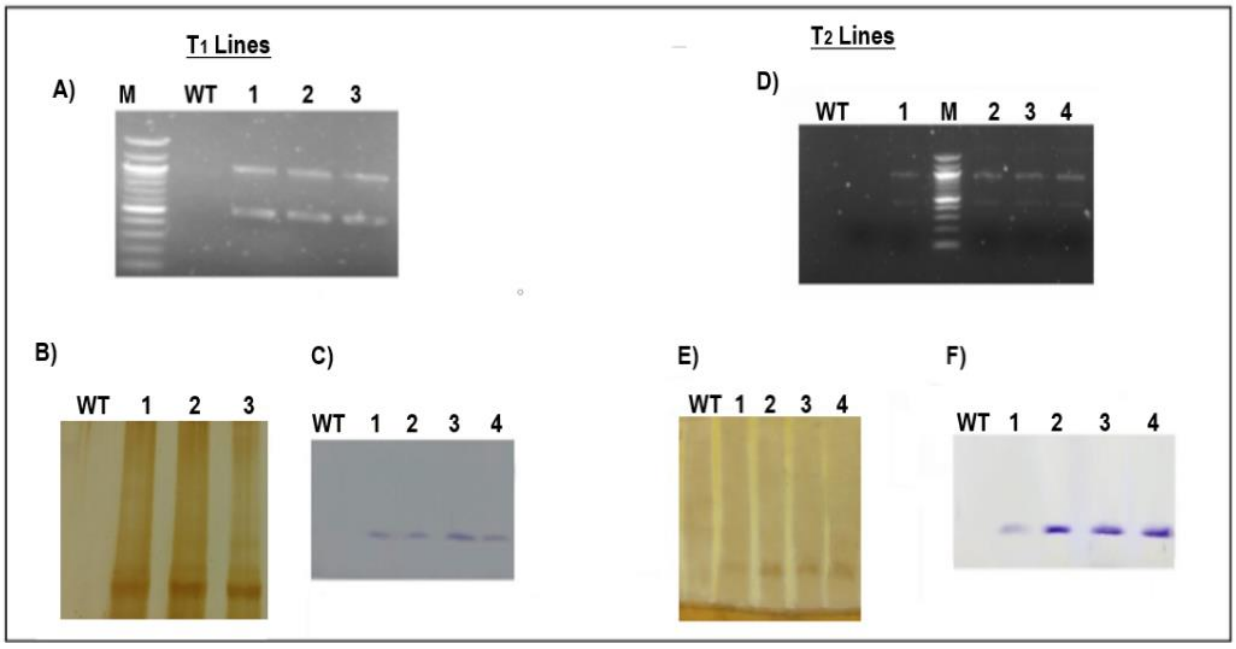
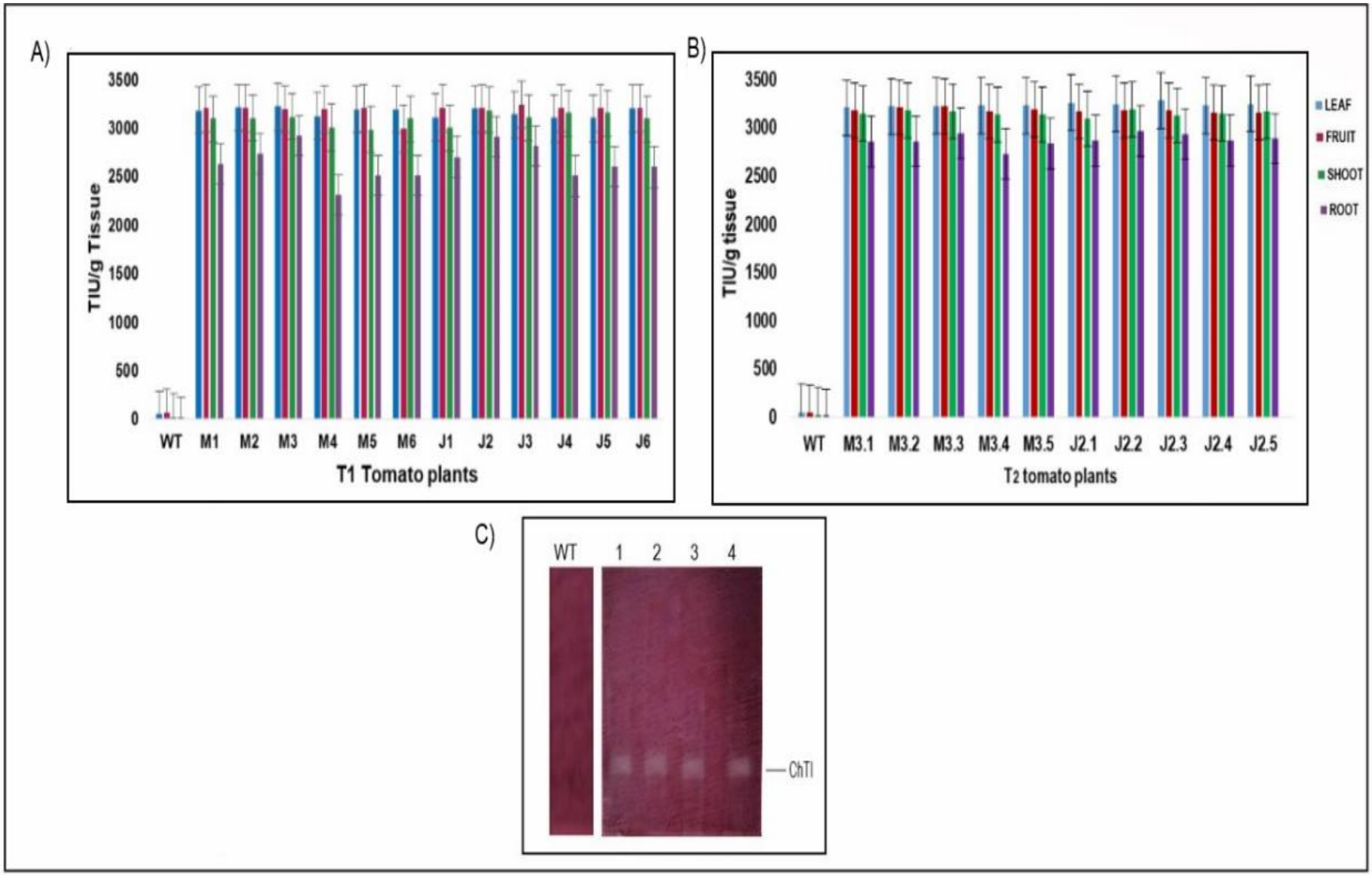
SUPPLEMENTARY MATERIAL



Supplementary Figure 1 (S1). Construction of recombinant vector pCAM-ChTI. ChTI amplicons amplified with M13 forward and reverse primers as well as pCAMBIA 1301 were digested with EcoR I and Hind III to provide cohesive termini for ligation. Fragments separated on 2% agarose gel were co-eluted and ligated. Construction of pCAM-ChTI was confirmed by PCR analysis. a) M-100 bp DNA ladder, lane2 - ChTI amplicons amplified with M13 forward and reverse primers, lane3 - ChTI amplicons amplified with M13 forward and reverse primers digested with EcoR I and HindIII, and lane4 - pCAMBIA digested with EcoR I and HindIII; b) M-100 bp DNA ladder, lane 1 and 2 - pCAM-ChTI amplified with ChTI specific primers, showing 450bp amplicons of ChTI, lane3 - pCAMBIA 1301 amplified with ChTI specific forward and reverse primers, showing the absence of 450bp amplicons of ChTI; c) Confirmation of transformation in pCAM-ChTI in Agrobacterium strain LB4404 by PCR using ChTI specific primers. Lane1 and 2 - pCAM-ChTI isolated from Agrobacterium strain LB4404, lane3 - pCAMBIA 1301 isolated from Agrobacterium strain LB4404.



Supplementary Figure 2 (S2). Confirmation of ChTI stability in transgenic tomato plants. Plants exhibiting trypsin inhibitory activity were screened for the stability of ChTI in T1 and T2generation. Plants conferring hpt resistance were screened for the presence of ChTI by PCR, and its expression by western blotting. A, D- PCR analysis of T1 and T2 plants using ChTI and hpt specific primers. Lane 1-3: PCR amplicons of ChTI (0.45kb) and hpt (1kb), M- 100bp DNA ladder, WT- non transgenic (control). B, E- Silver stained SDS-PAGE showing ChTI (18kDa) purified via Trypsin affinity column from WT, T1 and T2 plants, in the figures lane WT: control tissue extract, lane 1-4: transgenic tissue extract. C, F- Western blot visualisation of ChTI in T1 and T2 plant tissue extracts, in the figures lanes: WT- non transgenic, expression levels of ChTI from lane 1- root, lane 2- shoot, lane 3 - fruit, lane 4- leaf.



Supplementary Figure 3 (S3). Trypsin inhibitory activity of Transgene expressing T1 and T2 - tomato plants. Positively screened transgenic plant tissues were assayed for inhibitory activity. A, B - Trypsin inhibitory activity from tissues (leaf, shoot, fruit and root) from WT and ChTI expressingT1 and T2 tomato plants. C - In gel activity of ChTI. HSP fraction of leaf extracts were separated on 10% SDS gels and bovine trypsin inhibitory activity was visualised using acetyl-DL-phenylalanine-□-napthyl ester as substrate. Lane WT: control, lane 1-4: transgenic leaf extracts from two independent T1 and T2 plants.

Supplementary Table 1 (S1). Variation in the vegetative and reproductive parameters of WT and ChTI expressing tomato plants at T1 and T2 generation.

T_1	Internode length (cm)	Stem diameter (cm)	No. of leaves at first inflorescence	Plant height at first inflorescence (cm)	Onset of flowering[@] (days)	Blossom set to mature green[#] (days)	Mature green to red[$] (days)	Fruit weight (g/ fruit)	No. of fruits / plant
WT	1.45 ± 0.06	0.56 ± 0.08	12.00 ± 1.00	38.00 ± 0.28	31.00 ± 0.05	21.0 ± 0.56	24.50 ± 0.68	3.72 ± 0.81	32.00 ± 2.00
M	2.10 ± 0.15	0.78 ± 0.06	8.00 ± 2.00	48. 39 ± 1.80	23.95 ± 1.01	28.0 ± 1.90	39.18 ± 3.80	36.00 ± 1.55	14.00 ± 3.00
J	1.90 ± 0.26	0.80 ± 0.03	7.00 ± 1.00	46.41 ± 2.00	23.21 ± 1.09	28.0 ± 1.91	38.61 ± 1.66	36.06 ± 1.03	19.00 ± 1.00
T_2									
WT	1.39 ± 0.10	0.58 ± 0.13	11.00 ± 2.00	37.00 ± 0.10	30.00 ± 0.10	22.0 ± 0.60	27.50 ± 0.11	3.90 ± 0.11	25.00 ± 5.00
M	2.08 ± 0.12	0.81 ± 0.10	9.00 ± 1.00	49. 19 ± 2.10	24.81 ± 2.00	29.0 ± 1.00	39.10± 2.00	34.00 ± 1.00	15.00 ± 3.00
J	2.00 ± 0.11	0.71 ± 0.30	7.00 ± 1.00	49.70 ± 1.92	22.05 ± 1.00	30.0 ± 1.00	40.9 ± 0.05	36.13 ± 0.09	18.00 ± 2.00

[@]Onset of flowering corresponds to first 5 flowers appearing, [#]First flower opening up to first fruit breaker, [$]First fruit breaker maturing to red. Data represented as mean ± SD (p values were significant from each other at p value < 0.05) corresponds to 2 independent T_1 and T_2 generation lines - M, J (Transgenic), and 5 progeny plants from each lines (T_1 M and T_1J) are compared with WT plants. (non transgenic). 10 samplings are taken for each observation.

Supplementary Table 2 (S2). Trypsin inhibitory activity and variation in nutrition and morphological parameters among T1, T2- control and transgenic plants.

	T_1			T_2		
	WT	**M**	**J**	**WT**	**M**	**J**
			Inhibitory activity[@]			
Leaf	0.00	3200.0 – 3216.0	3209.0 – 3248.0	0.00	3021.0– 3232.0	3169.0– 3189.0
	0.00	3208.00 ± 6.34	3216.00 ± 15.38	0.00	3190.00 ± 83.45	3176.00 ± 11.76
Fruit	0.00	3125.0 – 3224.0	3108.0 – 3201.0	0.00	3170.0 – 3221.0	3175.0 – 3189.0
	0.00	3190.00 ± 35.19	3148.00 ± 46.31	0.00	3195.00 ± 19.75	3174 ± 11.86
Shoot	0.00	3154.0 – 3157.0	3005.0 – 3190.0	0.00	3140.0– 3185.0	3100.0 – 3198.0
	0.00	3155.00 ± 01.41	3120.00 ± 65.91	0.00	3156.00 ± 18.53	3143.00 ± 37.36
Root	0.00	2712.0– 2931.0	2510.0 – 2917.0	0.00	2512.0– 2931.0	2873.0– 2970.00
	0.00	2823.00 ± 89.16	2727.0 ± 217.10	0.00	2606.00± 213.89	2904.00 ± 38.96
Biochemical parameters						
Ascorbate[ß]	9.57 - 11.89	14.51 - 17.35	13.66 - 17.03	10.97 - 11.08	15.14 - 18.13	14.35 - 17.85
	11.32 ± 1.53	16.28 ± 1.28	13.88 ± 1.59	10.83 ± 0.40	16.98 ± 1.10	16.01 ± 1.23
Titratable acidity[ơ]	0.20 - 0.22	0.38 - 0.43	0.31 - 0.40	0.20 - 0.21	0.35 - 0.43	0.31 - 0.36
	0.21 ± 0.01	0.39 ± 0.02	0.33 ± 0.04	0.20 ± 0.05	0.40 ± 0.04	0.33 ± 0.01
	21.10 - 22.44	49.98 - 60. 81	45.19 - 56.99	20.01 - 21.06	46.19 - 60.13	44.57 - 56.13
Phenolsđ	22.11 ± 0.86	55.36 ± 4.28	51.19 ± 5.82	20.78 ± 0.51	54.31 ± 5.46	48.81 ± 4.22

Supplementary Table 2 (S2). Contd

Flavanoidsε	8.34 - 10.23 9.43 ± 0.84	14.83 - 16.33 15.92 ± 0.64	14.69 - 16.35 15.32 ± 0.69	10.29 - 10.03 10.09 ± 0.13	15.68 - 17.54 16.17 ± 0.93	14.55 - 15.56 15.08 ± 0.37
Lycopenef	1.02 - 1.13 1.08 ± 0.06	3.68 - 4.12 3.96 ± 0.15	3.14 - 4.02 3.57 ± 0.46	1.09 - 2.07 1.80 ± 0.47	2.34 - 3.53 3.26 ± 0.47	2.98 - 3.44 2.83 ± 0.87
β-Carotenef	1.96 - 2.03 2.00 ± 0.03	3.47 - 4.23 3.94 ± 0.35	3.14 - 4.02 3.50 ± 0.46	2.08 - 02.28 2.13 ± 0.09	3.27 - 4.23 3.94 ± 0.95	3.04 - 3.91 3.50 ± 0.37
AoCИ	10.23 - 12.93 10.87 ± 0.96	22.88 - 27.65 25.28 ± 1.70	22.06 - 24.11 23.12 ± 0.89	10.23 - 11.08 10.83 ± 0.40	22.88 - 26.19 25.28 ± 1.70	23.14 - 24.11 23.12 ± 0.89
Morphological attributes						
Onset of flowering in days	30.00 -31.00 31.00 ± 1.00	27.00 - 30.00 28.00 ± 2.00	24.00 - 28.00 25.00 ± 3.00	29.00 - 33 .00 31.00 ± 2.00	24.00 - 26.00 25.00 ± 1.00	25.00 - 28.00 27.00 ± 1.00
Mature green to red in days	21.00 - 23.00 22.00 ± 1.00	34.00 - 37.00 35.00 ± 1.00	31.00 - 40.00 35.00 ± 5.00	21.00 -23.00 22.00 ± 01.00	34.00 - 42.00 39.00 ± 2.00	36.00 - 42.00 39.00 ± 2.00
Fruit weightΪ	3.20 - 3.80 3.40 ± 0.32	31.00 - 38.00 33.80 ± 2.50	34.00 - 37.00 35.25 ± 1.50	3.50 - 4.20 3.90 ± 0.11	28.86 - 34.93 32.17 ± 2.50	27.89 - 35.90 31.44 ± 3.38
Fruits / plantΓ	30.00 - 36.00 33.00 ± 3.00	15.00 - 17.00 16.20 ± 0.83	14.00 - 16.00 15.05 ± 1.29	30.00 - 40.00 36.00 ± 03.00	16.04 - 20.01 17.95 ± 01.45	17.50 - 23.00 20.45 ± 02.00

Data represented as mean ± SD. ($p< 0.05$) of M T_1 and J T_1, M T_2 and J T_2 - Transgenic plants, WT- Non transgenic plants. 5 plants from each T_1 ,T_2 was taken for study. With 10 samplings per plant. The results are compared with non-transgenic plant (WT). Note : @- Inhibitory activity expressed as TIU/g tissue. Various Biochemical parameters of T_0 fruit values expressed as ß- mg ascorbic acid equivalent /100g fw, ơ –% acidity, đ –mg gallic acid equivalents / 100 g fw, ε – Flavanoids content expressed as mg catechin equivalents / 100g fw, f – carotene equivalents/ 100g fw, И – AEAC/100g fw, Ï – weight in grams, Γ- fruits/ plant in number.

Supplementary Table 3 (S3). Relationship between inhibitory activity and biochemical constituents of T1, T2 transgenic fruits.

	Inhibitory activity		Phenols		Flavanoids		βCarotene		Lycopene		Titratable acidity		Ascorbate		Total antioxidant capacity	
	T_1	T_2	T_1	T_2	T_1	T_2	T_1	T_2	T_1	T_2	T_1	T_2	T_1	T_2	T_1	T_2
Inhibitory activity	0.00	0.00	0.9^{***}	0.9^{***}	0.93^{***}	0.93^{***}	0.88^{***}	0.81^{***}	0.8^{***}	0.83^{***}	0.77^{***}	0.77^{***}	0.67^{**}	0.40^{*}	0.72^{**}	0.81^{***}
Phenols	0.9^{***}	0.9^{***}	0.00	0.00	0.73^{**}	0.76^{**}	0.63^{**}	0.77^{**}	0.68^{**}	0.78^{***}	0.64^{**}	0.61^{**}	0.76^{***}	0.72^{***}	0.69^{*}	0.89^{***}
Flavanoids	0.93^{***}	0.93^{***}	0.73^{**}	0.76^{**}	0.00	0.00	0.62^{**}	0.70^{**}	0.78^{***}	0.59^{**}	0.76^{***}	0.8^{***}	0.89^{***}	0.63^{*}	0.87^{***}	0.87^{***}
β-Carotene	0.88^{***}	0.81^{*}	0.63^{*}	0.77^{**}	0.62^{**}	0.7^{***}	0.00	0.00	0.79^{**}	0.89^{***}	0.57^{*}	0.45^{*}	0.67^{*}	0.80^{***}	0.56^{*}	0.78^{**}
Lycopene	0.8^{***}	0.33^{*}	0.68^{*}	0.78^{***}	0.78^{***}	0.59^{***}	0.79^{**}	0.89^{***}	0.00	0.00	0.67^{*}	0.31^{*}	0.75^{**}	0.77^{**}	0.57^{**}	0.74^{**}
Titratable acidity	0.77^{**}	0.77^{**}	0.79^{*}	0.61^{**}	0.76^{***}	0.8^{***}	0.57^{*}	0.45^{*}	0.67^{**}	0.31^{*}	0.00	0.00	0.55^{*}	0.42^{*}	0.63^{**}	0.72^{**}
Acsorbate	0.67^{*}	0.40^{*}	0.76^{**}	0.72^{**}	0.89^{***}	0.63^{*}	0.67^{***}	0.80^{***}	0.75^{***}	0.77^{***}	0.55^{*}	0.42^{*}	0.00	0.00	0.75^{**}	0.51^{**}
Total antioxidant capacity	0.72^{**}	0.81^{**}	0.69^{**}	0.89^{***}	0.87^{***}	0.87^{***}	0.56^{*}	0.78^{*}	0.57^{**}	0.74^{**}	0.75^{*}	0.72^{**}	0.75^{**}	0.51^{**}	0.00	0.00

Five $T_1$and T_2- Transgenic plants M and J with 10 samplings each were considered. n*** , n**, n* indicates values were significant at p-value <0.001 , < 0.05 ,< 0.01 respectively, $n^{\#}$ indicates non-significant.

Supplementary Table 4 (S4). Intergeneration correlation and regression analysis from T1-T2 generation.

Characters	$T_1 - T_0$				$T_2 - T_1$			
	M		J		M		J	
	Correlation	Regression	Correlation	Regression	Correlation	Regression	Correlation	Regression
Inhibitory activity								
Leaf	0.81**	0.65**	0.78**	0.61*	0.98***	0.97***	0.93***	0.86**
Shoot	0.93**	0.86**	0.84***	0.71**	0.76**	0.57*	0.95***	0.9***
Fruit	0.93***	0.87***	0.88**	0.78**	0.93***	0.87**	0.9***	0.81**
Root	0.65*	0.42*	0.79**	0.63**	0.94***	0.89**	0.88***	0.78**
Biochemical attributes								
Phenols	0.85**	0.72**	0.85**	0.73**	0.9***	0.82**	0.94***	0.89***
Flavanoids	0.8**	0.64**	0.87**	0.75**	0.95***	0.9***	0.94***	0.89***
Lycopene	0.89**	0.8**	0.75**	0.56*	0.83***	0.69*	0.76**	0.59*
β- Carotene	0.96***	0.92***	0.92***	0.84**	0.98***	0.97***	0.93***	0.87**
Ascorbate	0.93***	0.86**	0.83**	0.7**	0.94***	0.89**	0.78**	0.61*
Acidity	0.85**	0.73**	0.84**	0.7**	0.86***	0.74**	0.78**	0.61*
Total antioxidant capacity	0.78**	0.62*	0.86**	0.72**	0.84**	0.72**	0.87**	0.75**
Morphological attributes								
Onset of flowering	0.92***	0.85**	0.63*	0.39#	0.8**	0.65*	0.86**	0.74**

Supplementary Table 4 (S4). Contd

Blossom set to mature green	0.81**	0.66**	0.63*	0.4 #	0.88***	0.77**	0.92***	0.85**
Mature green to red	0.72**	0.52*	0.59*	0.35 #	0.93***	0.86**	0.71**	0.5*
Fruit weight	0.93***	0.86***	0.9***	0.81**	0.78**	0.61**	0.9***	0.81***
Fruits / plant	0.78**	0.61*	0.7**	0.41#	0.83**	0.69**	0.87**	0.65**

T_0, T_1, T_2 - Transgenic plants M and J were considered. n*** , n**, n* indicates values were significant at p-value < 0.001 , < 0.05 ,< 0.01 respectively, $n^{\#}$ indicates non-significant.