




# Expression of the entomotoxic *Cocculus hirsutus* trypsin inhibitor (*ChTI*) gene in transgenic chickpea enhances its underlying resistance against the infestation of *Helicoverpa armigera* and *Spodoptera litura*

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Received: 23 December 2020 / Accepted: 17 February 2021 / Published online: 9 March 2021  
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## Abstract

A trypsin inhibitor from *Cocculus hirsutus*, commonly known as “Farid Buti” has been demonstrated to exhibit insecticidal, fungicidal, as well as nematocidal activity. The *ChTI* (*Cocculus hirsutus* Trypsin Inhibitor) gene was designed in silico and synthesized by PCR-based gene synthesis and cloned in the plant expression vector pBI121, with kanamycin, as the selectable marker. *Agrobacterium* strain LBA4404 was transformed with *pBI121:ChTI* vector for plant transformation. For developing insect-tolerant chickpea, *Agrobacterium*-mediated transformation of *ChTI* gene was performed in cultivar P-362. Twenty-day-old cotyledonary node (CN) explants were used for sonication-assisted *Agrobacterium*-mediated transformation. Three cycles of increasing concentrations of kanamycin were used for the selection of transformed shoots. In vitro grown transgenic chickpea shoots were grafted on decapitated stock of chickpea seedlings. After 45–50 days of acclimatization and hardening, pod development and its maturation occurred. After screening by PCR, seven transgenic events were confirmed to be positive by Southern blot hybridization analysis, showing 1–4 copies of the transgene. The quantitative expression of the *ChTI* gene by qRT-PCR analysis showed up to 12–17-fold change in the T<sub>1</sub> progeny. Immunoblot analysis revealed the expression of 31 kDa and 15 kDa ChTI protein in *E.coli* and transgenic plants respectively. Trypsin activity assay was performed in the T<sub>1</sub> generation and higher anti-trypsin activity was recorded. Insect tolerance against *Helicoverpa armigera* and *Spodoptera litura* were estimated by insect bioassay, wherein an overall mortality of 60–80% and weight loss (30–60% and 40–60% for *Spodoptera litura* and *Helicoverpa armigera* respectively) have been recorded in the plants of T<sub>1</sub> generation.

## Key Message

Expression of *Cocculus hirsutus* trypsin inhibitor (ChTI) gene in chickpea by *Agrobacterium*-mediated transformation, restricted the growth as well as the survival of two Lepidopteran insect pests *Helicoverpa armigera* and *Spodoptera litura*.

**Keywords** *Agrobacterium* · Chickpea · *Cocculus hirsutus* · Cotyledonary node · Insect resistance · Protease inhibitor

Communicated by Sergio J. Ochatt.

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## Introduction

Malnutrition is increasing at an appalling rate in the developing countries, due to the deficiency of proteins in the diet. Growing of pulses and their regular intake, can meet the daily requirement of proteins in the regions where animal proteins are less available and affordable. Consumption of chickpea (*Cicer arietinum* L.) a high protein crop, is effective in combating protein malnutrition and Kwashiorkor in children and adults. To attain self-sufficiency and surplus by 2050, India's total pulse production needs to reach 39 mt. In the current scenario, India holds most of the world's area under chickpea cultivation (70%) and production (67%). The requirement of chickpea is about 16–17.5 mt by 2050 from an area of about 10.5 Mha with average productivity of 15–17 q/ha. (Dixit et al. 2019). The major obstacles in pulse production are several biotic and abiotic stresses, low plant productivity, global economic crisis, and a significant degree of fluctuation in the prices of pulses (Acharjee and Sarmah 2013).

Plants face numerous abiotic and biotic stresses in nature. Noticeably, insects impose severe threat to the survival of the plant due to their diversity, adaptability and abundance. Therefore millions of years of selection pressure exerted by insect herbivores, have given rise to the evolution of a sophisticated defense mechanism in plants (Erb and Reymond 2019).

Chickpea production is severely threatened by difficulties in managing many insect pests that attack the plant parts at all stages, starting from the seedling stage to harvest and beyond. The net yield of chickpea is severely affected by Lepidopteran insects (*Helicoverpa armigera* and *Spodoptera litura*) and diseases like wilt, *Botrytis* grey mold, dry root rot, *Ascochyta* blight, etc.) throughout crop production. As a result of larval infestation on the reproductive structures such as flowers and immature pods, reduces the crop yield significantly.

*Helicoverpa armigera* Hubner (Lepidoptera:Noctuidae), the gram pod borer, inflicts up to 90% mutilation in chickpea crop from the vegetative growth to the pod formation stage. *H. armigera*, is prominently distributed in Asia, Africa, Mediterranean regions, and Oceania. The larvae of the 1st, 2nd, and 3rd instar stage, first feed on the leaves of chickpea. A shift from foliar feeders to the developing seeds and fruits has been observed in the later stages of development. Eventually, larvae eat-up the emerging seeds inside the pod. Moreover, it causes entry holes at the basal region of chickpea pods in most of the genotypes. *H. armigera* larvae have characteristic longitudinal markings on lateral sides and are often dark brown, which may vary according to the food consumed by them (Yamasaki et al. 2009). Intending to overcome this issue, the cultivators

are desirous of raising the strength of pesticides. However, the application of pesticides in an injudicious manner has resulted in the bioaccumulation, biomagnification, pesticide resistance, pest revival, and causes harm to the non-target beneficial organisms and the environment.

The following major strategies are considered effective for the management of pod borer pests: (1) development and use of resistant varieties of crops, (2) good agronomic practices, such as early sowing, optimum planting density, sufficient fertilizers, inter/trap crops (coriander, sunflower, sorghum, mustard, linseed, and marigold), (3) monitoring the pod borer through pheromone traps. Integrating these practices with biological control has shown promising outcomes for sustainable pod borer management, resulting in expected chickpea yield (Patil et al. 2017).

Tailoring crop plants for endogenous built-in resistance to insect pests through the introduction of insecticidal protein genes of *Bacillus thuringiensis* (*Bt*) and other insect-resistant proteins *via* transgenic technology and the successful commercialization of genetically modified maize, potato, tomato, cotton, canola, rice and soybean, has provided a strategy to enhance crop productivity, due to the curb in crop losses (Perlak et al. 2001; Sanahuja et al. 2011). The transgenic approach has imparted resistance to insects in different crops, e.g., Lepidopteran insects in cotton (*Gossypium hirsutum*) and Coleopteran and Lepidopteran insects in maize (*Zea mays*). Transgenic technology is superior to conventional breeding due to its advantages in widening the pool of beneficial genes, incorporating few candidate genes in a single event, thereby reducing the time required for producing a crop variety with improved agronomic traits.

Several regeneration protocols involving somatic embryogenesis and shoot organogenesis from diverse explants have been described in different cultivars of chickpea. A number of parameters like co-cultivation duration, bacterial cell density, phenolic compounds, and strain of *Agrobacterium*, play a critical role in determining the net transformation efficiency. To develop a non-chimeric transgenic plant, optimization of selection and screening parameters are the decisive steps for enhancing the transformation efficiency. However, there is a crucial requirement to increase the transformation efficiency and stability of transgenes in chickpea, for commercial release. Bt transgenic chickpea lines with resistance to *Helicoverpa armigera* for commercial release are still under development (Sanyal et al. 2005; Acharjee et al. 2010; Mehrotra et al. 2011; Asharani et al. 2011; Khatodia et al. 2014; Ganguly et al. 2014).

The higher expression of Bt toxin is responsible for reducing the growth of chickpea, which needs to be studied from a physiological point of view. However, the field-acquired resistance in *H. armigera* has reduced the efficacy of Bt crops for pest resistance (Tabashnik et al. 2013). In this regard, transgenic crops expressing pyramided Bt toxins

against similar insect pests have been extensively applied now to delay the evolution of pest resistance (Carrière et al. 2015). However, field-evolved resistance and cross-resistance in transgenic plants expressing two different types of Bt toxins have been discovered (Gassmann et al. 2014).

Another alternate choice is by the use plant protease inhibitors (PPIs) for producing transgenic pest-resistant plants. PPIs do not have a wipe-out effect similar to synthetic pesticides and do not possess strong selection pressure, which is anticipated to impressively delay the development of resistance in the pest. The cowpea trypsin inhibitor (CpTI) gene was the first PPI gene used to produce transgenic tobacco (Hilder et al. 1987), after which many transgenic plants have been developed using the PI genes (Gatehouse et al. 1993).

Plant protease inhibitors (PPIs) are natural plant defense proteins, that have a potential function in protecting plants against herbivorous insects by cleaving their digestive proteases (Zhu-Salzman and Zeng 2015). In the same context, trypsin inhibitor activity was observed in the seed flour extract of ten selected chickpea varieties. All the varieties showed inhibitory activity *in vivo* and *in vitro* against *Helicoverpa armigera* (Kansal et al. 2008). In a recent study, a trypsin inhibitor from chickpea seeds was partially characterized and tested for its insecticidal activity against *Helicoverpa armigera*. Seventeen chickpea cultivars were categorized into three groups: with high (more than 70%), intermediate (16–70%), and very low (0–15%) trypsin inhibition activity with the 20 kDa trypsin inhibitor. Results suggest that the dynamics of the stress response is triggered by trypsin inhibitor and in turn, induces field pest resistance in the plant. In the same dimension, the dose-dependent reduction of both the larval weight and the survival was validated through feeding experiments, conducted with the 5th instar larvae (Nair et al. 2013).

*Cocculus hirsutus* trypsin inhibitor (ChTI), a serine proteinase inhibitor, was isolated from the shrub *Cocculus hirsutus* (L.) belonging to the family Menispermaceae (Rakesh and Prashant 2012). It is an 18 kDa thermo-tolerant, monomeric protein present in the vegetative parts and seeds of *Cocculus hirsutus*. The leaf extracts of *Cocculus hirsutus* has been demonstrated for insecticidal activity against *Helicoverpa armigera* (fruit borer) and *Spodoptera litura*, that infest the chickpea plant during the vegetative and fruit setting stages. The 2nd and 3rd instar larvae of *H. armigera* of fed with ChTI protein (5000 TIU/ml) resulted in 84.59% and 58.71% drop in the mean larval weight respectively. Subsequently, it caused 59.53% and 74% inhibition of *Helicoverpa* gut proteases and bovine trypsin, respectively (Bhattacharjee et al. 2009). During further evaluation, *Cocculus hirsutus* PI has shown to exhibit antimicrobial, antitumor, and fungicidal activity as well (Gupta et al. 2018; Thavamani et al. 2014; Bhattacharjee et al. 2009). Thus, this trypsin inhibitor of

plant origin reflects a promising alternative for a combinatorial and synergistic attack against the field pests and fungal pathogens of chickpea.

Efficient plant regeneration systems amenable to gene transfer are a primary need for the development of transgenic plants. Due to recalcitrant response *in vitro*, and the limitation of regenerable explants for the successful delivery of T-DNA from *Agrobacterium tumefaciens*, still persists in chickpea. Excised pre-conditioned cotyledonary nodes (CNs) was preferably chosen as an explant for this study, as it was successfully attempted earlier for transformation in chickpea (Sanyal et al. 2005; Tripathi et al. 2013). Multiple parameters that influence *Agrobacterium*-mediated insertion of T-DNA were studied such as seed sterilization, age of explant, acetosyringone concentration, O.D. of *Agrobacterium* cell suspension, sonication duration, co-cultivation duration, cefotaxime concentration and kanamycin concentration, for the selection of putative transgenic plants. Transgenic chickpea plants expressing ChTI protein have been developed up to T<sub>1</sub> generation and validated for resistance against field pests like *H. armigera* and *S. litura*. Development of pest resistance in chickpea, by expressing a gene from plant origin (*ChTI*) is the first attempt to be carried out and is investigated in this paper.

## Materials and methods

### Bacterial strains and plasmids

For preparation of the construct, the 363 bp CDS of *ChTI* (GeneBank accession no. ABN04119) was used for codon usage optimization and incorporation of the stop codon (TGA). Further, in-silico designed 14 PAGE purified oligonucleotides were assembled by PCR-based gene synthesis method. Restriction sites for *KpnI* and *BamHI* sites were incorporated at 5' end and *EcoRI* and *SacI* site at 3' end of the *ChTI* gene. The synthesized gene was cloned in pBlue-script SK<sup>+</sup> cloning vector, and clones were selected through blue-white screening. Further, the synthesized *ChTI* gene was cloned and expressed in the bacterial expression vector (Champion™ pET-SUMO, Thermo Fisher Scientific, USA), for expression in *E. coli* and in the plant expression vector pBI121, for transformation of chickpea.

*Agrobacterium tumefaciens* strain LBA4404 harboring the binary vector *pBI121:ChTI* having the kanamycin resistance (*nptII*) gene for plant selection, were grown in YEB medium with appropriate antibiotics at 28 °C for co-cultivation experiments, while *E. coli* strain DH5 $\alpha$  was cultivated at 37 °C in LB medium with appropriate antibiotics (Sanyal et al. 2003). The binary vector *pBI121:ChTI* contains the *nptII* gene for kanamycin resistance driven by *nos* promoter and *nos* terminator. The *ChTI* gene is driven

by a constitutive CaMV35S promoter and *nos* terminator (Fig. 1a, b). The plant vector was mobilized into *A. tumefaciens* strain LBA4404 by electroporation (Gene Pulser, Bio-Rad, USA) and further used for genetic transformation of chickpea.

### Sequence and structural analysis of ChTI protein

A 3-D model of ChTI protein was predicted and analyzed by the open-source package of Phyre 2.0 (Protein homology/Analogy recognition engine V 2.0). I-TASSER (Iterative Threading Assembly was used for 3-D modeling, legend binding, active site and gene ontological predictions. The SMART online tool was used for functional domain prediction. The physical and chemical parameters of protein were calculated by the ProtParam online tool (ProtParam 2017).

### Plant materials and growth conditions

The mature seeds of chickpea (*Cicer arietinum* L.) variety Pusa 362 was procured from Crop Research Center, Gobind Ballabh Pant University of Agriculture and Technology, Pant Nagar, Uttarakhand, India. Firstly, the seeds were surface sterilized with 0.01% (w/v)  $\text{HgCl}_2$  for 5 min (Fig. S1a), and rinsed three times in sterile Milli-Q water followed by rinsing in 70% (v/v) ethyl alcohol for 1 min and rinsed three times in autoclaved Milli-Q water. Sterilized seeds were incubated in the dark for germination. The germinated seeds were incubated in appropriate medium, consisting of Murashige and Skoog salts, vitamins of  $\text{B}_5$ , 3% (w/v) sucrose, 0.8% (w/v) agar and 2.0 mg/l 6-Benzylaminopurine (BAP) (Fig. S1b). The culture was incubated in the culture room maintained at  $24 \pm 1$  °C under 16 h photoperiod.

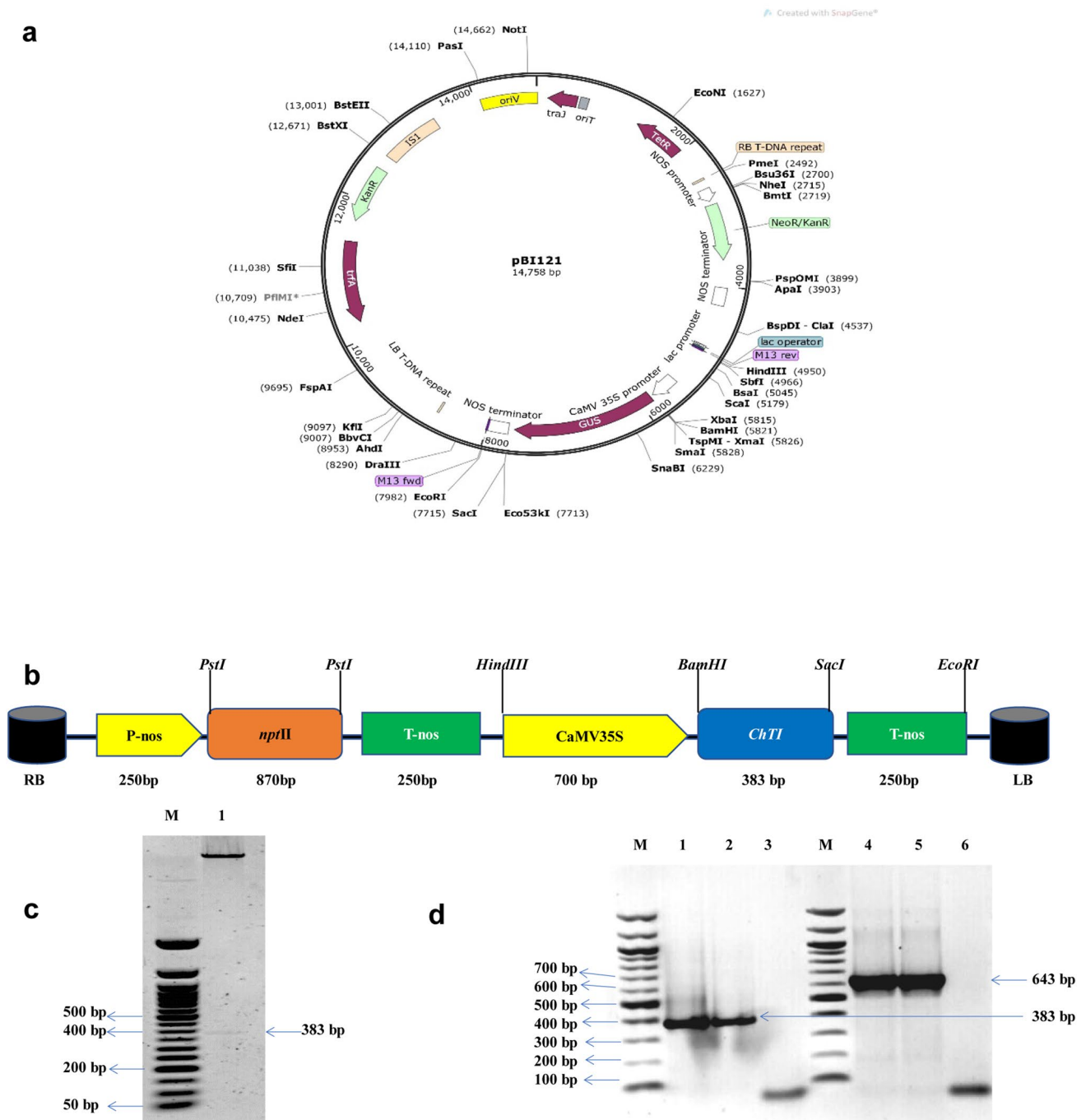
### In vitro regeneration

The cotyledonary node (CN) explants were collected from 20 days old axenically grown seedlings (Fig. S1c) by removal of the multiple adventitious shoots, main root, and developed shoot buds adjoining the axillary and epicotyl region in a maximum possible way, termed as primary excision (Fig. 2a–c). These excised explants were positioned abaxially in the Petri dish containing MS medium supplemented with L-cysteine, 30 g/l sucrose, solidified with 0.8% (w/v) agar and incubated in dark for 24 h. Further, cutting off the emerging shoot buds from the pre-existing axillary meristem of the cotyledonary region, exposes the cells of the L2 layer by transverse secondary excision of 1.5–2.0 mm thick surface layer tissues (Fig. 2d). Following the secondary excision, the excised CNs were agroinoculated according to the optimized conditions After co-cultivation with *Agrobacterium*, the explants were placed abaxially on MS medium supplemented with 2.0 mg/l BAP, 100  $\mu\text{M}$  acetosyringone,

30 g/l sucrose, solidified with 0.8% (w/v) agar and incubated in dark for 48 h. Explants were transferred after 48 h into fresh MS medium supplemented with 500 mg/l cefotaxime, 30 g/l sucrose, solidified with 0.8% (w/v) agar, and incubated for a week under 16/8 light/dark cycle. Further, explants were subcultured after 15 days interval and subsequently after 45 days of culture, the individual shoots were harvested and incubated into MS regeneration medium supplemented with zeatin (0.02 mg/l) +  $\text{AgNO}_3$  (10 mM) + chlorocholine chloride (0.2 mg/l). The individual putatively transformed shoots were micrografted onto 6–7 days old rootstock of the similar genotype, prepared by decapitating and diagonally cutting the epicotyl area of the germinated seedling. Further, the scion of the putative transgenic shoot was inserted into the vertical incision already made in the stock. Firstly, the micrograft was cultured in soilrite potting mix and irrigated with half-strength MS medium in culture room for 15 days and then were transplanted into pots filled with soilrite:loam:leaf manure (1:1:1) in the greenhouse kept at 60–65% relative humidity at 22–24 °C and 14 h photoperiod (Sanyal et al. 2003).

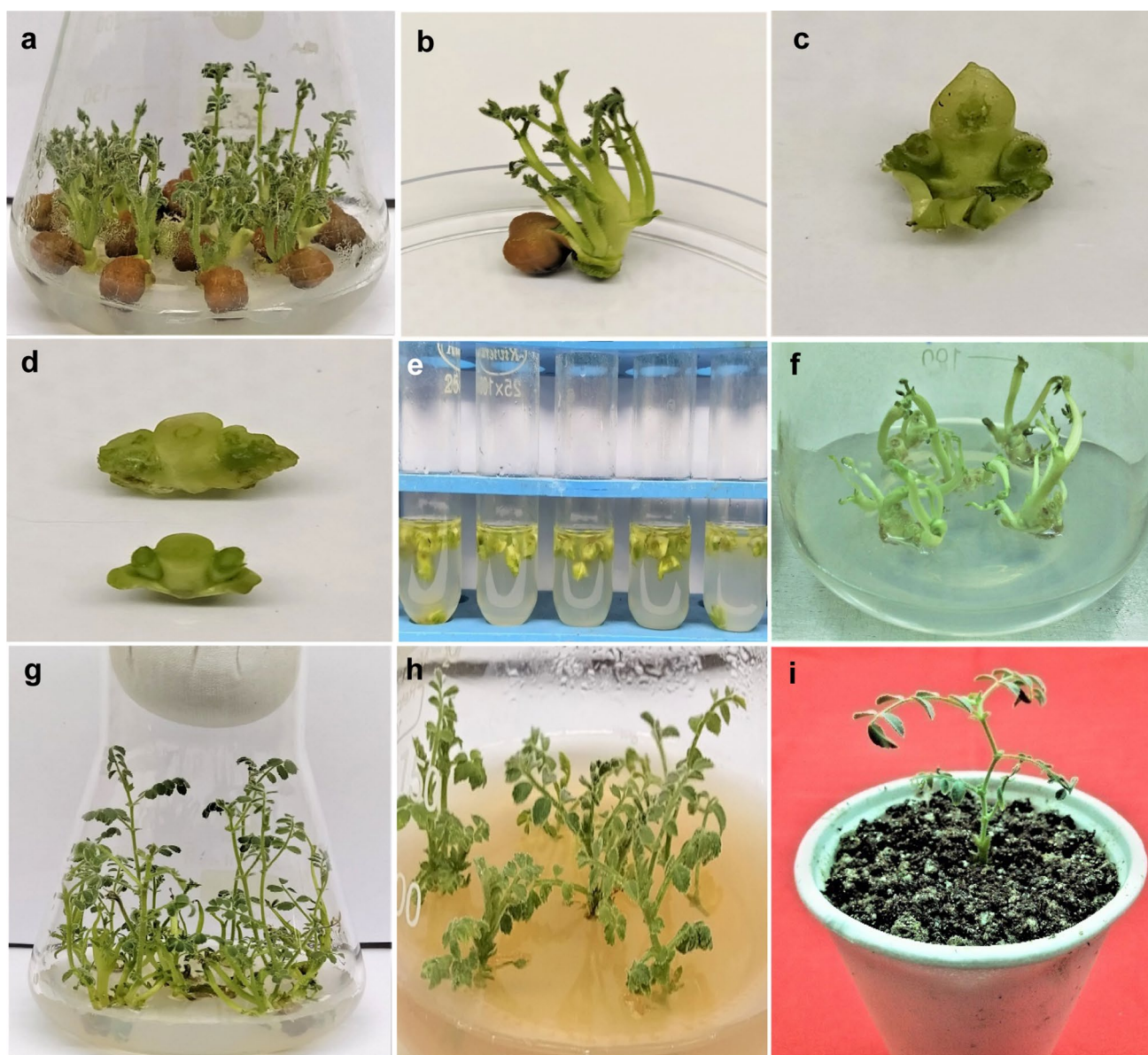
### Co-cultivation with *Agrobacterium*

The *Agrobacterium* cell density in the co-cultivation suspension culture with excised CNs suggested that the optical density of culture between 0.8 and 1.0, facilitates maximum shoot survival after the third antibiotic selection, higher or lower O.D., caused a considerable reduction in the number of putative transformants (Fig. S2a). About 10–12 excised CNs were co-cultivated with 10 ml *A. tumefaciens* suspension of  $\text{OD}_{600}$  0.8 supplemented with 100  $\mu\text{M}$  acetosyringone (Sigma-Aldrich, USA) in liquid MS medium in 30 ml borosilicate glass tubes (Fig. 2e, Fig. S2a). The increase in the acetosyringone level beyond 100  $\mu\text{M}$  significantly affected the transformation frequency in a declining manner (Fig. S2b). The expression of the *ChTI* gene in chickpea depends on the duration of sonication up to a larger extent. Sonication duration between 30 and 60 s for pre-conditioned CNs, create micro-wounding in the tissues, effective interaction of plant cells with *Agrobacterium*, and enhanced transient expression with negligible suppression of *in vitro* shoot regeneration. While sonication beyond 90 seconds resulted in 80–90% suppression of organogenesis and subsequent shoot development (Fig. S2c). These tubes were sonicated at 42 kHz for 30–60 s in a water bath sonicator (Branson 2510, USA) (Fig. S2c). After sonication-assisted *Agrobacterium*-mediated transformation (SAAT), CNs were incubated in the rotatory shaker at 75 rpm at 24 °C for 20 min. The co-cultivated CNs were blotted dry on sterile filter paper followed by their adaxial placement, on the plate of MS co-cultivation medium supplemented with 2 mg/l BAP, 100  $\mu\text{M}$  acetosyringone, and 30 g/l sucrose,



**Fig. 1** Schematic representation, characterization, and validation of the T-DNA region of the construct harboring the *ChTI* gene. **a** pBI121vector. **b** 2.7 kb fragment of *pChTI* containing the *nptII* gene driven by *nos* promoter and *nos* terminator. The position of *Bam*HI and *Sac*I on the T-DNA of the construct is used for double digestion to derive the 383 bp *ChTI* gene fragment. **c** Characterization of plasmid *pBI121::ChTI* in *Agrobacterium tumefaciens* strain LBA4404. Lane M—50 bp marker, lane one *pBI121::ChTI* digested with *Bam*HI

and *Sac*I and run in 1% agarose gel. The size of the *ChTI* gene—383 bp. **d** Characterization of plasmid *pBI121::ChTI* in *E. coli* strain DH5 $\alpha$  and *Agrobacterium tumefaciens* strain LBA4404 by PCR and run on an agarose gel. M—100 bp marker—PCR assay with internal primers and found desired band size of *ChTI*—383 bp (lane 1—DH5 $\alpha$ , lane 2—LBA4404 lane 3—Negative control), PCR assay with 35S-P-F and NOS-T-R found desired band size of *ChTI*—643 bp (lane 1—DH5 $\alpha$ , lane 2—LBA4404, lane 3—Negative control)



**Fig. 2** Explant preparation for in vitro regeneration in chickpea. **a** Seedlings of chickpea cultivar P-362 as plant material for the preparation of explants. **b** 20 day old seedlings. **c** Primary excised cotyledonary node (CN) as explant ready for preconditioning on MS medium supplemented with L-cysteine, 3% sucrose, solidified with 0.8% agar. **d** CNs excised for the second time to expose the deep-seated

$L_2$  layers. **e** Preparation of CNs for sonication at 42 kHz for 0–60 s. **f** shoot buds emerging in selection medium, supplemented with 250 mg/l cefotaxime and 100 mg/l kanamycin. **g** shoots subjected to the third cycle of antibiotic selection. **h** shoots incubated on regeneration medium. **i** shoots of putative transformants micro-grafted on 7 days old rootstock

solidified with 0.8% (w/v) agar. Co-cultivation was conducted for 48 h, at 24 °C under a 16 h photoperiod. Further, explants were plated on MS medium fortified with 500 mg/l cefotaxime concentration as optimized for one week (Fig. S2d, e). The selection of putative transformants was performed on a selective medium similar to MS culture medium supplementing 100 mg/l kanamycin as optimized (Sigma-Aldrich, USA), 500 mg/l cefotaxime and 0.02 g/l  $AgNO_3$  for the shoot proliferation (Fig. S2f). Thereafter, the 15 days

old explants were transferred on to fresh selective media containing 100 mg/l kanamycin and subjected to regeneration for 30 days with sub-culturing after 15 days (Fig. 2f). Following the selection, individual putative transgenic shoots was transferred on regeneration medium supplemented with 0.02 mg/l zeatin + 10 mM  $AgNO_3$  + 0.2 mg/l chlorocholine chloride for another 15 days (Fig. 2g, h). The emerging putative transgenic shoots were micro-grafted on the pre-grown rootstock of the control non-transgenic plant

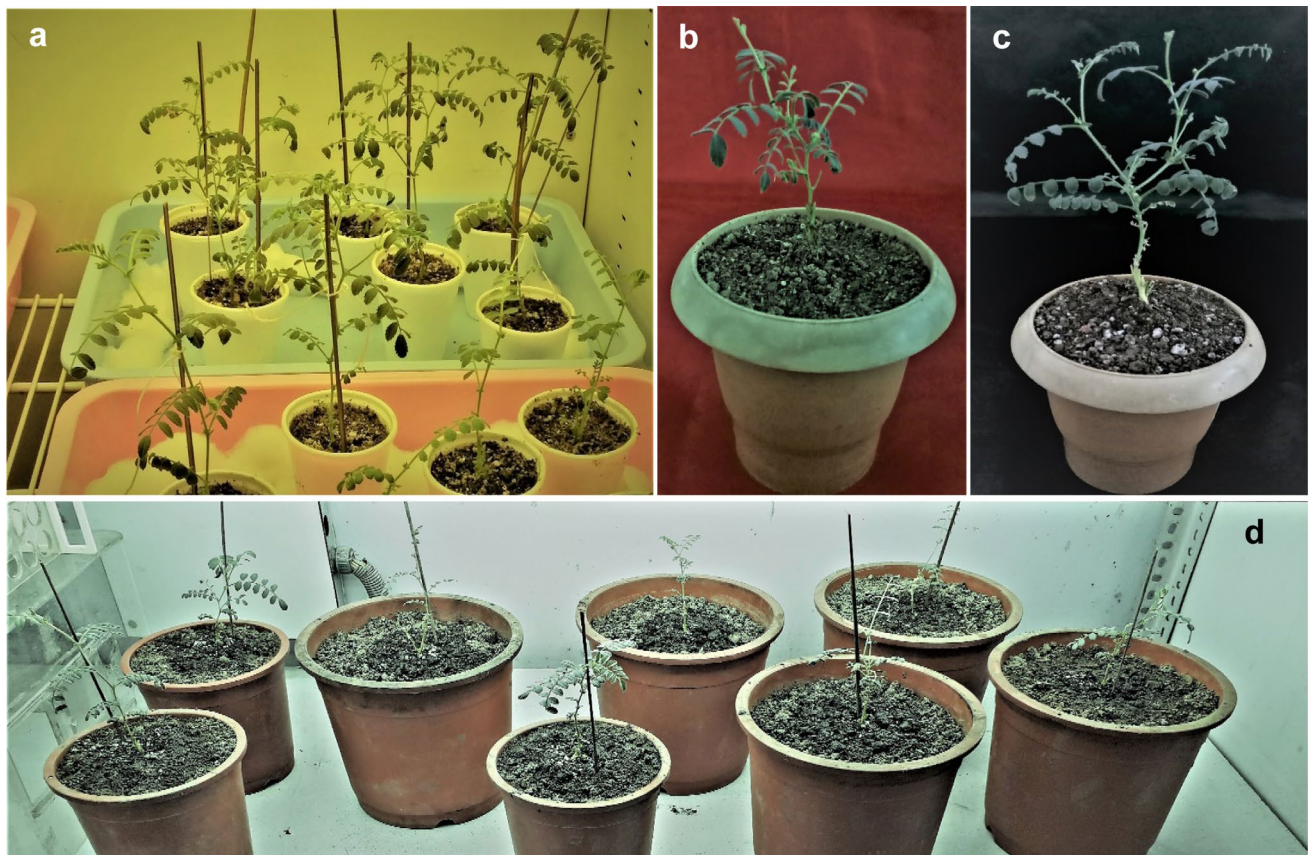
and grown in a culture room for 15–45 days, until plantlets were hardened enough for ready to survive in the greenhouse (Figs. 2i, 3a–d). Explants pre-conditioned for 24 h before co-cultivation for 48 h gives more transformation events, rather than CNs pre-conditioned beyond 24 h (Fig. S3a). Another important factor for increasing the transformation efficiency is the duration of co-cultivation with *Agrobacterium*. In this regard, CN explants co-cultivated for 48 h were found to be more efficient for gene transfer, than those co-cultivated for 24 and 60 h (Fig. S3b).

### PCR and Southern blot hybridization analysis

Chickpea genomic DNA was isolated by the method described by (Sanyal et al. 2005). Characterization of plasmid pBI121:*ChTI* in *Agrobacterium tumefaciens* strain LBA4404 was carried out through digestion with *Bam*HI and *Sac*I and run in 1% agarose gel (Fig. 1c). Characterization of the plasmid pBI121:*ChTI* in *E. coli* strain DH5 $\alpha$  and in *Agrobacterium tumefaciens* strain LBA4404 was done by PCR using internal primers and the desired band size for

*ChTI*—383 bp was seen and with 35S-P-F and NOS-T-R primers the desired band size of *ChTI*—643 bp was observed (Fig. 1d). Seventeen regenerated transformed shoots were screened by PCR analysis for actual transgenic events through internal (*ChTI* forward—5'-GGTACCGGATCC ATGAGTGATCTAAC-3' and *ChTI* reverse—5'-GCGAAC GCTATGTCCTGATA-3') primers and with a set of flanking primers (CaMV35S forward—5'-TATTCGGCTATGACT TGGGC-3' and *ChTI* reverse primer). The amplicon size of 383 bp and 483 bp were obtained with the internal and flanking primers, respectively. PCR was performed for 35 cycles, each cycle of 1 min denaturation at 95 °C, annealing at 58 °C for 30 sec, and extension at 68 °C for 30 sec followed by a final extension at 68 °C for 5 min. Amplicons were electrophoresed on 1.0% (w/v) agarose gel and documented on gel-doc (Bio-Rad, USA).

Southern blot hybridization was performed to confirm T-DNA integration in the putative transformants following the established procedures (Sambrook et al. 1989). About 10  $\mu$ g of chickpea genomic DNA was digested with 50 units of *Hind*III (New England Biolabs, USA) for 18–24 h and



**Fig. 3** Hardening and acclimatization of putative transformants micro-grafted on seedling stocks for the development of the T<sub>0</sub> plants. **a** Pre-acclimatization of micro-grafted shoots in a growth chamber at 22–24 °C at 60–65% relative humidity and 16 h photoperiod for

15 days. **b–d** T<sub>0</sub> plants were shifted in culture room at 22–24 °C, 60–65% relative humidity and 16 h photoperiod for 30 days for the development of seeds

electrophoresed on 0.8% (w/v) agarose at 15V and transferred on to Zeta-Probe GT nylon membrane (Bio-Rad, USA) by vacuum transfer mechanism as per manufacturer's instructions (Bio-Rad, USA). The blots were hybridized with the 383 bp *ChTI* gene fragment as a probe radiolabeled with  $\alpha^{32}\text{P}$ -dCTP (BRIT Mumbai, India). *Bam*HI-*Sac*I digested fragment from p*ChTI* vector was used as positive control. The putative transgenic events with confirmed gene integration, and validated by Southern blot hybridization, were selected to progress in the next-generation ( $T_1$ ) for further analysis.

### Gene expression analysis of *ChTI* by qRT-PCR

To evaluate the expression level of *ChTI* gene in the transgenic events, total RNA was isolated from the leaves of the selected plants (control and transgenics) in the  $T_1$  generation by using the Spectrum plant total RNA kit (Sigma-Aldrich, USA). cDNA was synthesized using the Enhanced Avian HS RT-PCR kit (Sigma-Aldrich, USA). Further, cDNA was used as a template for qRT-PCR to assess the total transcript level by using SYBR Green PCR Master Mix (Applied Biosystems, USA) and was performed in StepOne real-time PCR system (Applied Biosystems, USA). Chickpea *beta-actin* gene was taken as an endogenous control. The real-time expression of the genes was estimated by using the  $2^{-\Delta\Delta C_T}$  method (Livak and Schmittgen 2001). The primer sequences designed for analysing the *ChTI* gene and chickpea *beta-actin* are shown in Table S1.

### Trypsin inhibition activity assay and Western blot analysis

100 mg leaf tissue from the individual  $T_1$  plant was harvested and chopped into small pieces followed by washing in cold PBS. Further, 4X-6X volumes (500–1000  $\mu\text{l}$ ) of trypsin activity assay buffer was added and were incubated on ice followed by homogenization in ice, and immediately centrifuged at 4 °C, for 2–5 min at maximum speed. The supernatant having total soluble protein (TSP) were collected in a fresh tube. The supernatant was further used for trypsin inhibition activity assay, as described in trypsin activity assay kit (colorimetric) (Abcam ab102531, USA) (Robles et al. 2015).

Total soluble proteins (TSPs) were incubated at 70 °C for 10 min, chilled on ice, and centrifuged at 12,000 rpm for 20 min at 4 °C (Bhattacharjee et al. 2009). Almost all the plant serine proteinase inhibitors are known to thermostable, apart from their counteracting proteinases and have negligible endogenous proteolytic activity throughout the assay. The supernatant (Heat Stable Proteins-HSPs) was used for Western blot assay. HSPs from transgenic leaves were separated on 15% SDS-PAGE. Further, the gel containing separated proteins was transferred on a PVDF membrane using a Trans-blot Semi-Dry Transfer Cell (Bio-Rad, USA),

at 15V for one hour. Immunoblot was performed for the ChTI protein expressed in the  $T_1$  transgenic chickpea plants ( $T_{11}$  and  $T_{15}$ ), and ChTI expressed in *E. coli* (BI21) by using ChTI-IgG antibodies (Chromous Biotech, Bangalore, India).

### Insect bioassay

Insecticidal activity of the toxin ChTI in  $T_1$  chickpea plants was assayed by conducting a no-choice detached leaf-feeding bioassay, using the second instar neonatal larvae of *H. armigera* and *S. litura*. Larvae of *H. armigera* were initially reared on an artificial diet at  $26 \pm 2$  °C, 70% relative humidity under 14 h light, and 10 h dark light regime and thereafter by feeding on castor leaves to complete their life cycle. In this experiment, about 200–250 mg of fresh leaves from transgenic and control chickpea plants, were placed in a 50 ml capped plastic bottle, and 10 neonate larvae were infested and allowed to feed on the leaves. The bottles were sealed with Parafilm and placed in the insect rearing room at 70% relative humidity,  $26 \pm 2$  °C, and a 16 h photoperiod, to prevent desiccation. Under the same conditions, feeding was allowed for 4 days, with the supply of fresh plant leaves on alternate days and the data was documented on larval mortality and larval weight. Feed choice assay was carried out to determine the preferential choice of leaves, when kept in the same vicinity for the larvae.

### Statistical analysis

Values in the results reported are mean S.D. of three replicates, and a minimum of 50 explants was used in each experiment. The data were analyzed by using Microsoft excel (MS-Excel) and SPSS statistical package. A significant difference analysis within the means ( $p < 0.05$ ) was performed with the help of DMRT (Duncan's Multiple Range Test), and compared each parameter independently. Bars that contain the same letter(s) do not differ significantly.

## Results

### Sequence and structural analysis of ChTI protein

Retrieval of *Cocculus hirsutus* trypsin inhibitor sequence in FASTA format has been carried out from UniProt Primary accession number: A2TJS6 (Fig. S4). A 3-D model of protein predicted by the open-source package of Phyre 2.0 (protein homology/Analogy recognition engine V 2.0) (Fig. S5a). Secondary structure with percent disorders was evaluated and summarised (Fig. S5b). 15 Protein sequences are showing variable homology as their domain alignment indicated (Fig. S5c). Out of 15 homologues, four (c2mw7A,



c6f0fB, c6f0gC, c6f0gD) have > 70% similarity as shown by their alignment (Fig. S5d) (Kelley et al. 2015).

Although I-TASSER (Iterative Threading Assembly Refinement) is a hierarchical approach to predict protein function and structure, instead of Phyre 2.0 it provides comprehensive output for a 3-D model by using meta-threading approaches in addition to ligand binding site, active site, and gene ontological predictions (Figs. S6, S7, S8) (Zhang 2009; Roy et al. 2012; Yang and Zhang 2015).

PSI search online tool has been used for functional domain analysis for homologue sequences in the database, against different profiles such as SMART, Pfam, etc. (Madeira et al. 2019) (Fig. S9). Two functional domains were annotated by the SMART online tool: the ZnF\_TAZ domain and IKK beta NEMO binding domain. TAZ (Transcription Adaptor putative Zinc finger) domains are zinc-containing domains found in the homologous transcriptional co-activators CREB-binding protein (CBP) and the P300, positions predicted between 40 and 112 in the sequence, CBP and P300 are histone acetyltransferase, which catalyzes the reversible acetylation of all 4 histones in nucleosomes, eventually, regulate transcription through chromatin remodeling. However, another domain is the IKK-betaNEMO bind domain; positions are predicted between 64 to 98 in the sequence, and these proteins have a key role in inflammatory reactions. Further, this domain was validated as the binding site of IKK-beta for NEMO (Letunic and Bork 2017).

The physical and chemical parameters of the protein is calculated by the ProtParam online tool (ProtParam 2017). The estimated half-life of ChTI protein is 30 h (mammalian reticulocytes, in vitro), > 20 h (yeast, in vivo), and > 10 h (*E. coli*, in vivo). However, the instability index is computed to be 86.18. This classifies the protein as unstable. The grand average of hydropathicity (GRAVY) is calculated as -0.984, which indicates the hydrophilic nature of the ChTI protein.

### In vitro regeneration from cotyledonary nodes explants of chickpea

To complement higher susceptibility and efficiency for *Agrobacterium*-mediated transformation, CNs having uncovered cells of L2 layers were used for in vitro regeneration. The CNs from 20 days old germinated seedlings of chickpea, showed the optimal response for the development and induction of adventitious shoots (Fig. S1a). After 2 weeks of culture, the development of shoot buds was initiated, and the formation of meristematic domes on the surface of excised CN was observed. These primordial structures further elongated into shoots. Micrografting of putative transgenic shoots on 6–7 days old rootstock of a similar genotype,

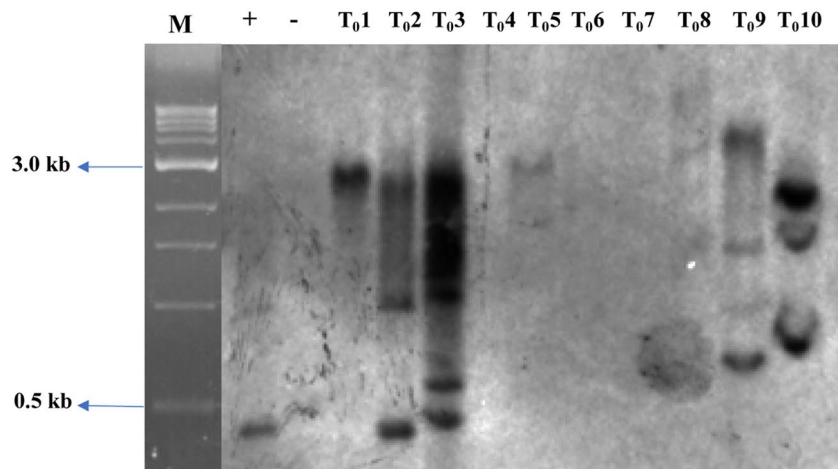
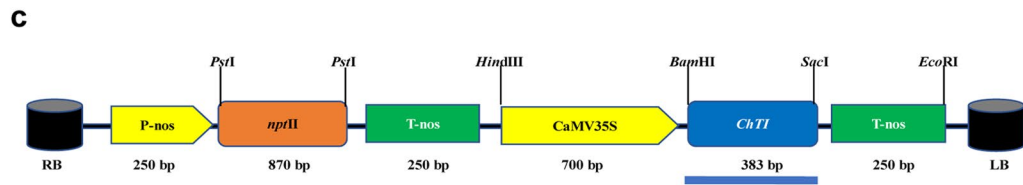
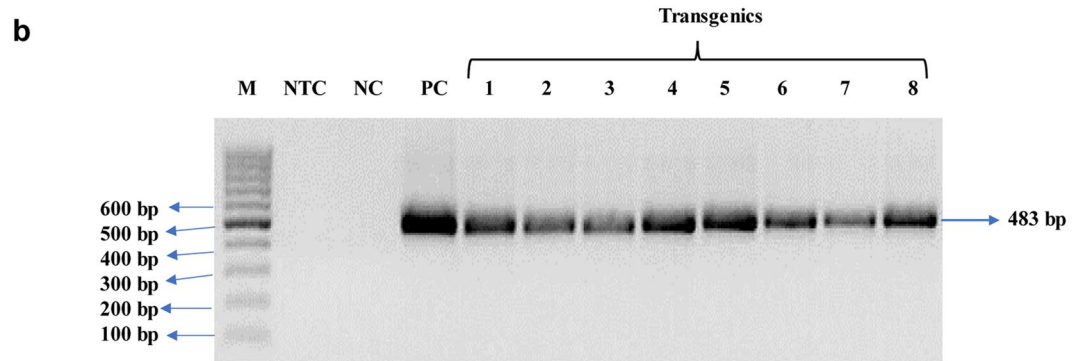
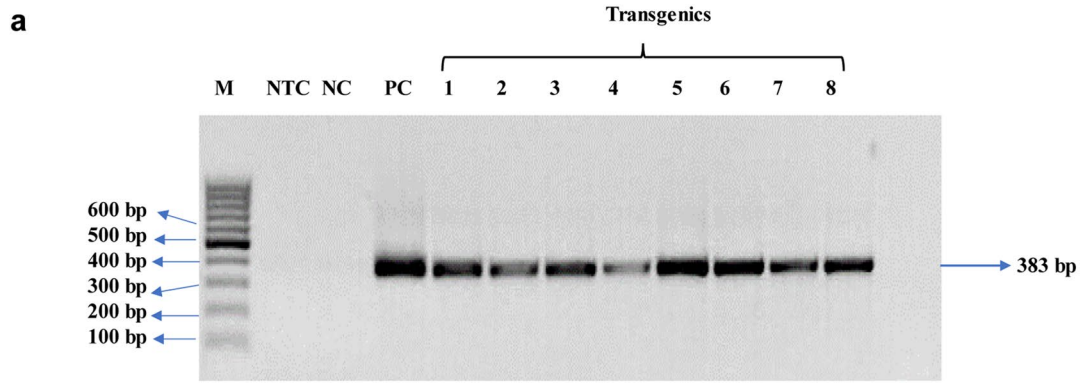
reflects 82–85% endurance and complete plant development under the optimized glasshouse conditions (Sanyal et al. 2003).

### Co-cultivation parameters for improved transformation efficiency

The transformation efficiency of *Agrobacterium*-mediated delivery of T-DNA into plant cells is determined by several physiological and physicochemical parameters. Alteration in parameters is taken into the account to optimize *Agrobacterium*-mediated gene delivery and collection of transformants using CN explants. Considering the four *Agrobacterium* strains (GV2260, GV3850, LBA4404, and EHA105), strain LBA4404 was found statistically significant compared to GV2260, GV3850 and EHA105 as reported in earlier studies (Sanyal et al. 2005). Based on the optimized transformation and tissue culture conditions, *Agrobacterium* co-cultivation was carried out with 24 h pre-conditioned CNs explants in the presence of 100  $\mu$ M acetosyringone with 0.8 OD<sub>600</sub> of *Agrobacterium* cell density, sonication for the 30 s followed by 48 h co-cultivation into MS medium supplemented with 2.0 mg/l BAP, 100  $\mu$ M acetosyringone, 3% sucrose and 0.8% agar. Meanwhile, transformed shoots were selected with 100 mg/l kanamycin in the first and second round, followed by 150–200 mg/l kanamycin in the third round. Following the selection cycles, 17 putative transgenic shoots were recovered and reflects a transformation frequency of 2.36% with the p*ChTI* construct (Table S2).

### Molecular analysis of T<sub>0</sub> putative transgenic plants

Genomic DNA was isolated from all the 17 primary transformants (T<sub>0</sub>) and subsequently used for PCR screening using the internal primers and flanking primers *ChTI* gene, for the amplification of DNA fragments of 383 and 483 bp, respectively. The results summarized in (Fig. 4a, b) which suggested that 8 plants were PCR positive for both *ChTI* internal and flanking primers out of the 17 T<sub>0</sub> putative transgenic plants evaluated, while 9 putative transformed plants did not show amplification with either set of primers. Furthermore, T<sub>0</sub> chickpea plants were checked for gene integration as well as copy number by Southern blot hybridization analysis. Southern blot analysis with specific radiolabelled *ChTI* probe was performed with genomic DNA isolated from the chickpea plants (T<sub>0</sub>) showed stable integration of *ChTI* in 7 events (T<sub>0</sub>1, T<sub>0</sub>2, T<sub>0</sub>3, T<sub>0</sub>5, T<sub>0</sub>8, T<sub>0</sub>9 and T<sub>0</sub>10) out of the 10 events tested (Fig. 4c). Events T<sub>0</sub>1, T<sub>0</sub>5 and T<sub>0</sub>8 was found with single-copy insertion of the *ChTI* gene, T<sub>0</sub>2 and T<sub>0</sub>10 showed three copies and events T<sub>0</sub>3 and T<sub>0</sub>9 have a copy number of 4. These events, validated by



◀ **Fig. 4** Molecular analysis of transgenic events in chickpea. **a** using the internal primers of *ChTI* and the desired band size of 383 bp (M—100 bp ladder, NTC—non-template control, NC—negative control, PC—positive control, lane 1–8 are putative  $T_0$  transgenic events. **b** with flanking primers of 35S-P-F and *ChTI*-R, and desired band size as 483 bp (M—100 bp, NTC—non-template control, NC—negative control, PC—positive control, lane 1–8 are putative  $T_0$  transgenic events. **c** Southern blot hybridization with the radiolabelled probe of *ChTI* gene (+) positive control (*Bam*HI-*Sac*I digested fragment from p*ChTI* vector), (–) negative control (non-transformed chickpea plants), lines  $T_01$ ,  $T_02$ ,  $T_03$ ,  $T_05$ ,  $T_08$ ,  $T_09$  and  $T_010$  are the putative transgenic events hybridizing with the probe and lane  $T_04$ ,  $T_06$ , and  $T_07$  are not, indicating the integration of *ChTI* gene). **d** qRT-PCR analysis—the relative expression of the transgene with gene-specific primers of *ChTI*, and the *actin* gene of chickpea as an endogenous control. A significant difference analysis within the means ( $p < 0.05$ ) was performed with the help of DMRT (Duncan's multiple range test), and compared each parameter independently. Bars that contain the same letter(s) do not differ significantly. All the given values are means of a minimum of three replicates  $\pm$  SD

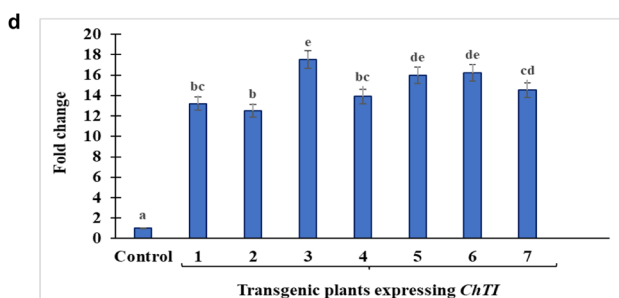
Southern blot hybridization were allowed to proceed to the next-generation ( $T_1$ ).

#### qRT-PCR assay

The relative expression of the *ChTI* was evaluated by qRT-PCR using *ChTI* gene-specific primers and actin gene of chickpea was utilized as an endogenous control. The fragments of 137 and 134 bp were amplified through the set of primers for *ChTI* and chickpea actin genes, respectively. The transcript levels in the 7 selected transgenic  $T_1$  lines expressing *ChTI* gene was analyzed along with the endogenous control, in triplicates. The result indicated a higher transcript level of the *ChTI* gene ranging from 12 to 17-fold change (Fig. 4d).

#### Trypsin inhibition activity assay and Western blot analysis

Total soluble protein (TSPs) was used for trypsin inhibition activity assay as described by trypsin activity assay kit (colorimetric, abcam ab102531).  $T_1$  transgenic lines expressing the *ChTI* gene have exhibited a higher trypsin inhibitory



**Fig. 4** (continued)

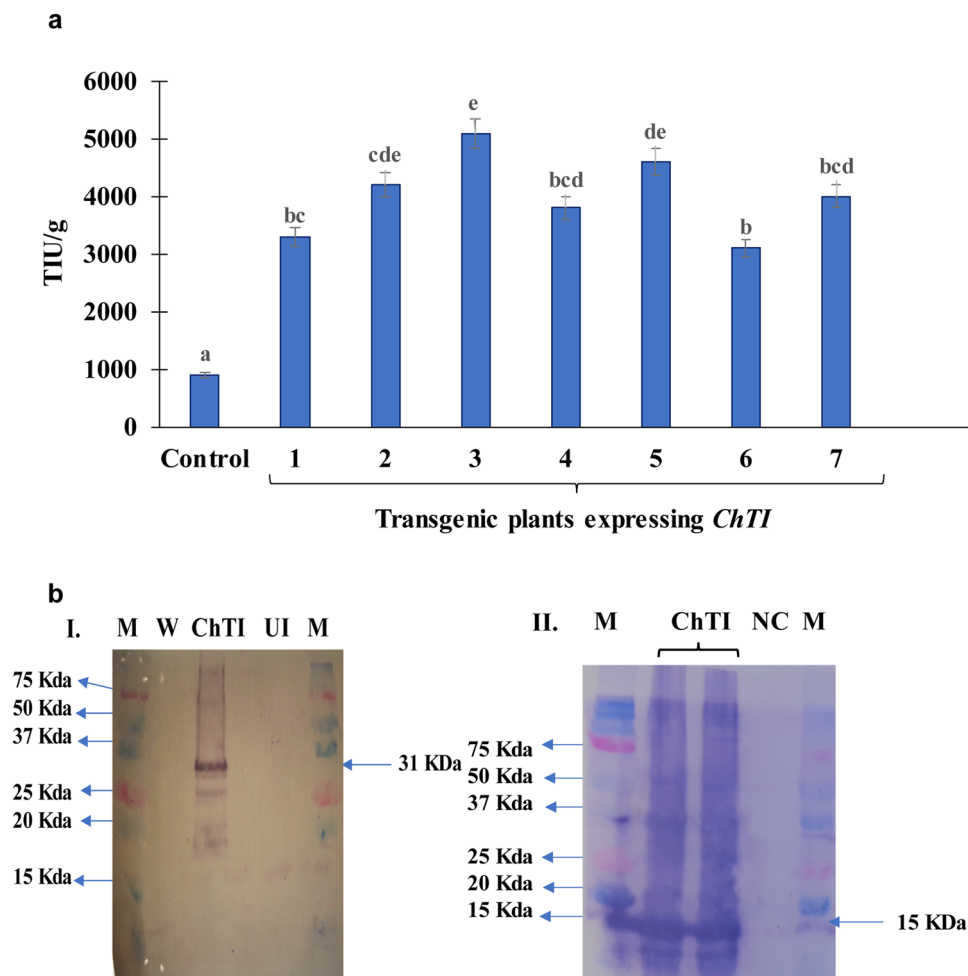
activity, ranging from 3100 to 5100 TIU/g (trypsin inhibition unit/gram) in comparison to control, which has very low antitrypsin activity, 900 TIU/g, due to the endogenous trypsin inhibitors (Fig. 5a). HSPs were used for Western blot analysis. HSPs isolated from the transgenic leaves ( $T_11$  and  $T_15$ ) and control were separated on 15% SDS-PAGE. 31 kDa ChTI protein was expressed, induced, and purified, and immunoblotted from bacteria (*E.coli*. BL121 cells) (Fig. 5b (I), Fig. S 10). Immunoblot analysis using ChTI-IgG confirmed the presence of 15 kDa protein corresponding to ChTI in chickpea transgenic plants ( $T_11$  and  $T_15$ ) (Fig. 5b (II)).

#### Assessment of insecticidal activity

The insecticidal activity of  $T_1$  plants expressing *ChTI* protein was evaluated using the leaves of individual transgenic and control chickpea plants for feeding assays, using the second instar neonate larvae of *S. litura* and *H. armigera* (Fig. 6a). Data revealed that most larvae ceased feeding after 2 days on leaves of  $T_1$  plants (Fig. 6b, d).  $T_1$  chickpea plants expressing a large amount of ChTI protein, which triggers a significant reduction in the weight of larvae from 30 to 60% and 40 to 60% for *S. litura* to *H. armigera*, respectively (Fig. 6e). Whereas, larvae challenged on the leaves of individual plants of  $T_1$  generation, reflect moderate to high (60% to 80%) mortality due to stunted growth and impaired life cycle, with early pupation of *S. litura* and *H. armigera* (Fig. 6f). Feed choice assay for determination of the larvae's preferential choice indicated that second instar larvae of *S. litura* to *H. armigera*, preferred control plants as feed choice compared to transgenic lines expressing the ChTI entomocidal protein (Fig. 6c).

#### Assessment of plants in $T_1$ generation

Total of seven numbers of  $T_0$  transgenic events and four untransformed tissue culture grown control plants of chickpea were moved to the glasshouse for seed setting and for the analysis of inheritance of the transgene (*ChTI*) in the next generation. All the seven  $T_0$  plants were fertile and produced seeds, although the number of pods in transgenic plants was surprisingly low (4–6 per plant) in comparison to (8–10) pods in the control plants. However, the viability of  $T_1$  seeds from plants grown through tissue culture remained low, compared to seeds developed from the field-grown plants. PCR analysis of  $T_1$  transgenic plants has been carried out with internal primers and flanking primers (Fig. 7). The pattern of kanamycin resistance in  $T_1$  seeds on antibiotic-supplemented growth medium, exhibited the typical 3:1 Mendelian ratio as anticipated for single dominant gene inheritance, apart from one transgenic line, where more susceptible seeds were



**Fig. 5** Characterization of ChTI protein. **a** trypsin inhibitory activity of transgenic plants expressing ChTI protein. Positively screened transgenic plant tissues ( $T_1$ ) were assayed for inhibitory activity of trypsin. Trypsin inhibitory activity from total soluble protein (TSP) from the leaves of control and  $T_1$  plants expressing ChTI protein was calculated as trypsin inhibitory unit/gram of tissue (TIU/g): control—900 TIU/g, 1—3300 TIU/g, 2—4200 TIU/g, 3—5100 TIU/g, 4—3800 TIU/g, 5—4600 TIU/g, 6—3100 TIU/g, 7—4400 TIU/g. **(b)** Immunoblot of ChTI (I) purified protein fraction from *E.coli* (BI21) with Penta-His antibody lane 1—protein marker (M), lane 2—wash

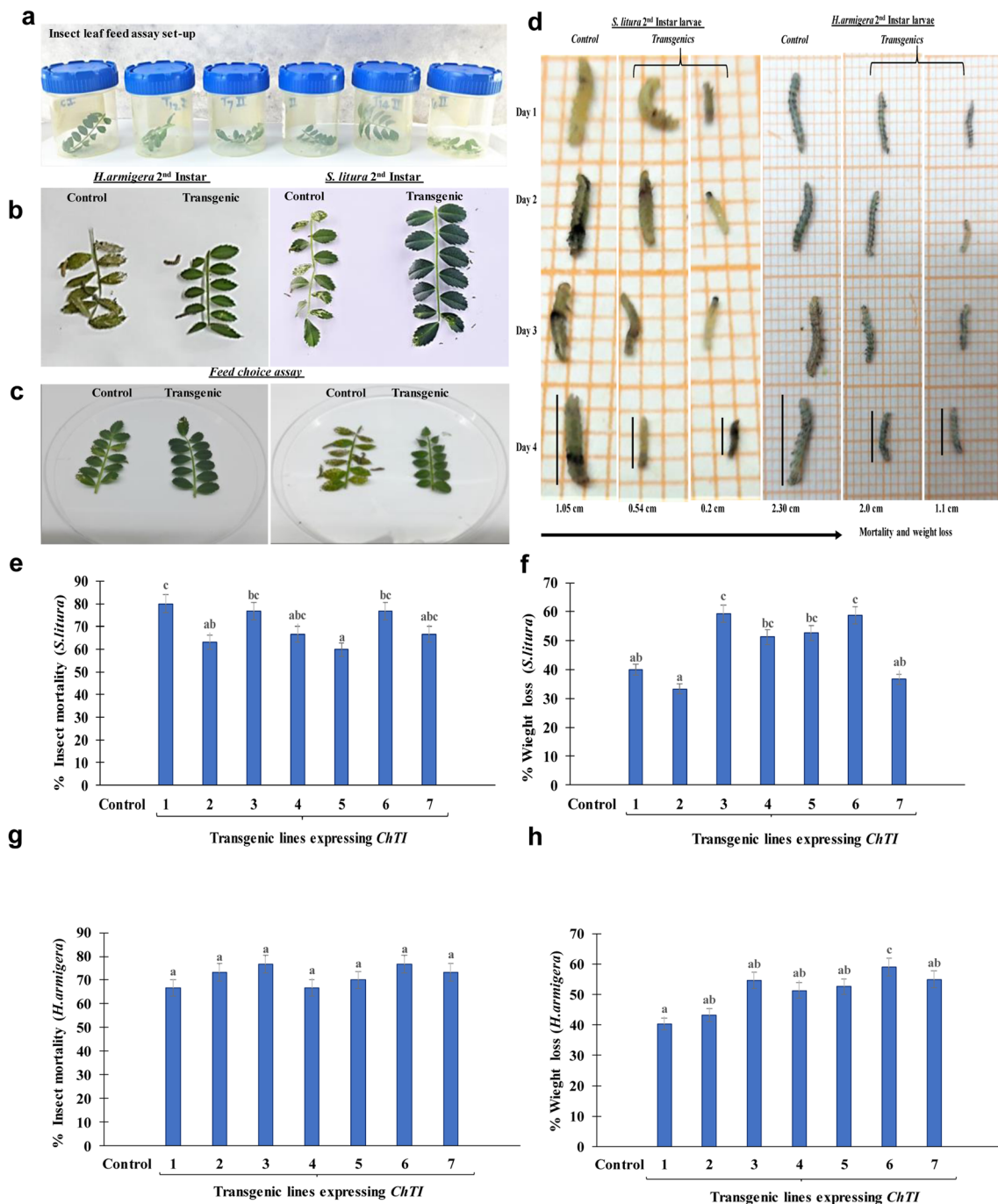
protein (W) from purification column, lane 3—purified ChTI protein lane 4—uninduced (UI) culture protein, lane 5—protein marker (II) Expression of ChTI in chickpea, lane 1—protein marker, lane 2 and 3—ChTI from plant protein fraction ( $T_{1,1}$  and  $T_{1,5}$ ), lane 4—negative control, lane 5—protein marker. A significant difference analysis within the means ( $p < 0.05$ ) was performed with the help of DMRT (Duncan's multiple range test), and compared each parameter independently. Bars that contain the same letter(s) do not differ significantly. All the given values are means of a minimum of three replicates  $\pm$  SD

found, than the anticipated Mendelian ratio. The segregation pattern of expression of *ChTI* gene in the progenies of the promising  $T_0$  lines is shown in Table S3.

## Discussion

Previous studies revealed that the ChTI protein has an important role in plant defense against biotic stresses, such as antimicrobial and insect resistance (Bhattacharjee et al. 2009). However, the utilization of ChTI to develop insect-resistance in an economically valuable crop is entirely unexplored. In a recent study, the expression of *Coccilus hirsutus* trypsin inhibitor (ChTI) in transgenic tomato and

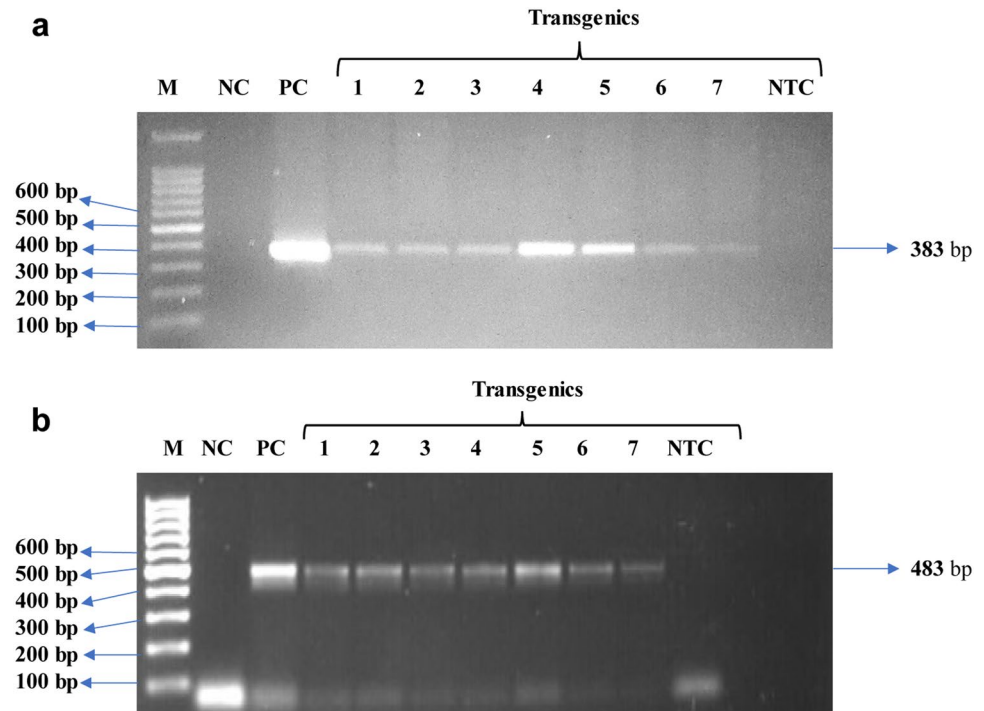
the subsequent upregulation of the endogenous plant defense response, as well as the level of antioxidants against *H. armigera* has been reported. The tomato plant expressing *ChTI* gene showed early flowering in the transgenic plants by 10 days, approximately 45% increase in the plant height, increased diameter of fruits, and delay in fruit ripening. Besides increased nutrient and antioxidant levels, 100% mortality of 2nd and 4th instar larvae of *H. armigera*, fed on the transgenic tomato leaves was documented (Manushree et al. 2020). This study demonstrates the first report on the expression of ChTI protein in any plant belonging to the legume family. In comparison to tomato, the regenerative potential is limited in chickpea, besides this 60–80%



**Fig. 6** Leaf feed assay for toxicity assessment on 2nd instar *H. armigera* and *S. litura* larvae. **a** Insect leaf feed assay was set up in 50 ml tube, with control and transgenic leaves (T<sub>1</sub>) in triplicates. **b** In leaf feed assay, the maximum damage is observed in control leaves, and larvae fed on transgenic leaves showed minimum damage against *H. armigera* and *S. litura*. **c** Feed choice assay was demonstrated for validation of preferential feeding of leaves by larvae of *H. armigera* and *S. litura*. **d** Insect mortality and weight loss assessment of 2nd instar *S. litura* and *H. armigera* larvae fed on leaves. 60–80% mortality and 30–60% weight loss were observed in 2nd instar lar-

vae of *S. litura* and *H. armigera* fed on transgenic leaves, in comparison to negligible loss in larvae fed on control leaves. **e, f** insect weight loss and survival assessment of *S. litura*. **g, h** insect weight loss and survival assessment of *H. armigera*. A significant difference analysis within the means ( $p < 0.05$ ) was performed with the help of DMRT (Duncan’s multiple range test), and compared each parameter independently. Bars that contain the same letter(s) do not differ significantly. All the given values are means of a minimum of three replicates  $\pm$  SD

**Fig. 7** Assessment of the transgenic events of  $T_1$  generation by PCR. **a** Using internal primers of *ChTI* gene and found the desired band size of 383 bp (*M* 100 bp marker, *NC* negative control, *PC* positive control, lane 1–7 are putative transgenic events, lane 8: *NTC* non-template control). **b** using flanking primers with 35S-P-F and *ChTI*-R and found desired band size of 483 bp (*M* 100 bp marker, *NC* negative control, *PC* positive control, lane 1–7 are putative transgenic events, lane 8: *NTC* non-template control)



mortality and 30–60% weight loss were recorded from transgenic chickpea plants against the Lepidopteran insects.

Earlier, several attempts were carried out to express plant protease inhibitors in different crops and notable mortality/weight loss or both, were reported against the field pests. Transgenic tobacco plants were developed, expressing cowpea trypsin inhibitor (CpTI) and 35–40% mortality was validated against *S. litura* (Sane et al. 1997). Altpeter et al. (1999) developed transgenic wheat by incorporating the barley trypsin inhibitor CMe (BTI-CMe), showed resistance with 29% mortality against *Sitotroga cerealella*. In a different study, *Solanum americanum* proteinase inhibitor II (PIN2) was used to develop resistance in tobacco with 50–56% mortality against *H. armigera* (Luo et al. 2012).

Despite gene alone strategies, few gene pyramiding approaches were demonstrated such as *Nicotiana glauca* proteinase inhibitor (NaPI) and *Solanum tuberosum* potato type I inhibitor (StPin1A) were co-integrated in the cotton genome and 90% weight loss was documented against *Helicoverpa punctigera* (Dunse et al. 2010). Furthermore in another report, 40% mortality was observed against *Tuta absoluta* by co-expressing the serine proteinase inhibitor (BTI-CMe) and cysteine proteinase inhibitor (Hv-CPI2) in tomato (Hamza et al. 2018).

Although several attempts have been carried out to develop insect resistance in chickpea (Sanyal et al. 2005; Ignacimuthu and Prakash 2006; Gowri Neelima et al. 2008; Chakraborti et al. 2009; Acharjee et al. 2010; Asharani et al. 2011; Ganguly et al. 2014; Khatodia et al. 2014; Khatodia

2017), minimal success was observed particularly in case of *S. litura* and *H. armigera*. In this study, we have focused on using a transgene from plant origin itself, i.e., *ChTI*, and the development of transgenic chickpea expressing *ChTI* for insect resistance.

This study is focused on developing transgenic chickpea plants harboring the *ChTI* gene and its assessment against *S. litura* and the chickpea pod borer *H. armigera*. Although widely regarded as a highly recalcitrant crop, chickpea transformation and regeneration has shown minimal success to date (Sanyal and Amla 2008). *Agrobacterium*-mediated transformation and the optimized tissue culture procedures have directed this particular study to achieve a transformation efficiency up to 2.36% by using cotyledonary node as an explant. The selection of plants was made based on the kanamycin as a selection marker, utilizing an iterative procedure. After the final round of selection, 17 putative transgenic shoots were collected and subsequently micrografted for hardening and seed setting. Furthermore, the putative transgenic lines were screened through PCR, by using internal as well as flanking markers. Finally, seven PCR positive events were successfully validated through Southern blot hybridization, except one escape, possibly due to the chimeric nature of the transformant.

Real-time qPCR analysis in  $T_1$  plants revealed 12–17-fold expression upregulation of *ChTI* gene, which is quite impressive to confirm the potential of *ChTI* for the development of insect resistance in chickpea. Moreover, leaf feed assay was carried out to assess the insecticidal potential of ChTI, against two very challenging pests of this economically

important legume crop, *S. litura* and *H. armigera*. T<sub>1</sub> chickpea plants showed a significant weight loss of 30–60% and 40–60% for *S. litura* to *H. armigera*, respectively. Whereas, the 2nd instar larvae challenged on leaves of individual plants from T<sub>1</sub> generation, have shown moderate to high (60% to 80%) mortality of *S. litura* and *H. armigera*. The immunoblot analysis and trypsin activity assay (3100–5100 TIU/g) were performed, and protein expression was validated in *E. coli* and in T<sub>1</sub> generation of chickpea plants.

Albeit, transgenic chickpea plants expressing ChTI alone wouldn't be ideally forwarded to offer 100% mortality against pod borer and leaf caterpillar. To attain 100 percent mortality and optimal weight loss, gene pyramiding approach alongwith protein engineering of ChTI may prove beneficial to achieve further improvement in context to field pest resistance. Protein engineering through the strategies such as molecular simulation and specific localization, to enhance the functional properties of ChTI such as specific activity, stability (thermal stability, tolerance to salts and organic solvents, pH tolerance and solubility), inhibition by reaction products, and specificity against non-natural substrates.

In the future, we need to do a controlled assessment of responses by these plants for field pest tolerance. Identifying and screening of new endogenous proteases in chickpea and whether these endogenous chickpea protease inhibitors can specifically modify the resistance potential of plants against field pests, will be futuristic. It will be very interesting and crucial to identify, characterize, and validate the antimicrobial and pharmaceutical (anti-neoplastic, anti-inflammatory, anti-oxidative, anti-diabetic) potential of the ChTI protein.

## Conclusions

This particular study demonstrated the role of the *ChTI* gene against biotic stress in an economically important leguminous crop (*Cicer arietinum* L.), which has not been investigated earlier in detail. This study reveals that transgenic chickpea expressing the *ChTI* gene can overcome the biotic stress imposed due to insects, by augmenting the plants' defense mechanism, such as anti-trypsin activity. The reduction in larval weight, mortality, and preferential feeding choice boosts the survivability in transgenic plants of chickpea against herbivory. The obtained results from the insect resistance evaluation demonstrates that the expression of *ChTI* offers better resistance in transgenic plants, in contrast to control plants against stress generated by insects. It was validated that the *ChTI* gene expression can be used to develop insect-resistant cultivars to improve crop productivity.

**Supplementary Information** The online version contains supplementary material available at <https://doi.org/10.1007/s11240-021-02041-2>.

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