Capsicum annuum proteinase inhibitor ingestion negatively impacts the growth of sorghum pest *Chilo partellus* and promotes differential protease expression

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ABSTRACT

Background: *Chilo partellus* is an important insect pest infesting sorghum and maize. The larvae internalize in the stem, rendering difficulties in pest management. We investigated the effects of *Capsicum annuum* proteinase inhibitors (CanPIs) on *C. partellus* larvae by *in-vitro* and *in-vivo* experiments.
Methods: Recombinant CanPI-7 (with four-Inhibitory Repeat Domains, IRDs), -22 (two-IRDs) and insect proteinase activities were estimated by proteinase assays, dot blot assays and *in gel* activity assays. Feeding bioassays of lab reared *C. partellus* with CanPI-7 and -22 were performed. *C. partellus* proteinase gene expression was done by RT-PCR. *In-silico* structure prediction of proteinases and CanPI IRDs was carried out, their validation and molecular docking was done for estimating the interaction strength.
Results: Larval proteinases of *C. partellus* showed higher activity at alkaline pH and expressed few proteinase isoforms. Both CanPIs showed strong inhibition of *C. partellus* larval proteinases. Feeding bioassays of *C. partellus* with CanPIs revealed a dose dependent retardation of larval growth, reduction of pupal mass and fecundity, while larval and pupal periods increased significantly. Ingestion of CanPIs resulted in differential up-regulation of *C. partellus* proteinase isoforms, which were sensitive to CanPI-7 but were insensitive to CanPI-22. *In-silico* interaction studies indicated the strong interaction of IRD-9 (of CanPI-22) with *Chilo* proteinases tested.
Conclusions: Of the two PIs tested, CanPI-7 prevents induction of inhibitor insensitive proteinases in *C. partellus* so it can be explored for developing *C. partellus* tolerance in sorghum.
General significance: Ingestion of CanPIs, effectively retards *C. partellus* growth; while differentially regulating the proteinases.

1. Introduction

Spotted stemborer, *Chilo partellus* (Swinehoe) (Lepidoptera: Crambidae) is an economically important pest of monocotyledonous crops *viz.,* maize, sorghum, pearl millet [1] as well as it feeds on wild grasses [2]. It originated in Asia and has spread to East and southern Africa, including Madagascar. It is an invasive pest capable of adjusting to warmer climates at low to high altitudes in South-East Asia and Africa. Sorghum varieties IS2205, ICSV700, ICSV93046, IS18551 show moderate levels of resistance to stem borer and shoot fly through antibiosis and antixenosis [3,4]. ICSV1 and Swarna are susceptible to

these insect pests [3]. Although insecticides are being used to control stem borer, its cryptic and nocturnal habits put a major challenge in its management, causing crop losses to the extent of 10–90% [5].

C. partellus larvae start feeding on the leaves and bore through the central whorls of young leaves and leaf bases to reach the stem region. They feed voraciously on the stem tissue and tunnel through it, resulting into death of the central leaves, which ultimately leads to 'dead heart' formation. The disruption of the nutrient supply to the upper parts leads to chaffy panicles and ultimately reduction in grain yield. Complete life cycle of *C. partellus* is around 4–5 weeks, which may extend in colder climate and shorten in warmer conditions.

Agronomic practices, natural enemies, host pant resistance and use of synthetic insecticides are the major pest control methods. A few biological control agents such as parasitoids and pathogens have been reported against *C. partellus*. *Cotesia flavipes*, a gregarious larval parasitoid is recorded as natural enemy of *C. partellus* in Asia [6]. Also the pheromone traps have been found to limit the population build-up of *C. partellus* [7]. Since *C. partellus* larvae internalizes within the plant, it's control with chemicals and natural enemies are not much effective thus emphasizing the need for strengthening plants own defense through conventional or transgenic approaches.

Plants secondary metabolites, and defensive proteins such as proteinase inhibitors (PIs) are well studied and involved in posing a direct defense against insect pests [8–11]. For example, upon ingestion, PIs affect insect growth and development by inhibiting the activities of digestive enzymes, leading to its nutrient deprivation and antibiosis [8,12]. Different types of PIs are effective against insect pests including *Spodoptera exigua* (Hub.)*, Plutella xyllostella* (L), *Helicoverpa armigera* (Hub.) and *Manduca sexta* (L.) [13–17]. Use of PIs for crop protection through various methods has been demonstrated as a sustainable pest control strategy [18–22]. In particular, non-host plants PIs have been found to be effective against many insect pests including *H. armigera* [23–28].

Sorghum bicolor transgenic plants expressing *Bacillus thuringiensis* (Bt) toxin Cry1Ac exhibited up to 40% *C. partellus* larval mortality [29]. Furthermore, jasmonic acid has been found to boost the induced defense in *S. bicolor* by genotype specific up-regulation of plant defensive enzymes and secondary metabolites [30]. *Capsicum annuum* PIs have been found effective against *H. armigera* [25–28]. The present investigation was designed to test the efficacy of non-host, multi-domain Pin-II type PIs from a dicot plant *C. annuum* against the oligophagous monocot specific pest *C. partellus*. Based on *in vitro* and *in vivo* studies, we have identified CanPI-7 protein for its potential application in spotted stem borer management.

2. Materials and methods

2.1. Insects

Chilo partellus neonates were obtained from the insect culture maintained in the insect rearing laboratory at the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Telangana, India. The insect culture was maintained on sorghum based semi-synthetic artificial diet under controlled conditions [16:8 h L: D regime at 25 ± 1 ℃ and $65 \pm 5\%$ RH] [31].

2.2. Extraction of insect enzymes

The whole larvae or the midguts were collected by dissection. The tissue was ground in liquid nitrogen and 150 mg tissue from two biological and three technical replicates comprising 6 insects per treatment was homogenized in 500 µl of 0.2 M glycine-NaOH buffer, pH 10, containing 1 mM EDTA or TrisHCl, pH 7.8 or distilled water depending on their use in enzyme assay or *in gel* assays. The pH of whole larval tissue, the midgut tissue and their extracts prepared in distilled water were determined using a pH paper. The homogenate was incubated at 4 ℃ for 2 h and centrifuged at 10, 000 rpm for 20 min at 4 ℃. Nearly 400 µl supernatant was collected and used as enzyme source for serine protease/ trypsin activity assays, proteinase inhibition assays and *in gel* assays.

2.3. Proteases and proteinase inhibitors assays

C. partellus trypsins like proteinase activity (trypsin units) and trypsin inhibitor activity (Trypsin inhibitory units, TIUs) were carried out as reported earlier using a synthetic substrate, BA*p*NA [21,32]. The protease extracts were prepared from two biological and four technical

replicates as mentioned in Section 2.2. Considering the weak trypsin like activity of *C. partellus,* its assay incubation time was increased up to 60–120 min. For characterizing pH optima of the *C. partellus* proteases, the enzyme extracts prepared in buffers with pH 10 and pH 7.8 were used in BA*p*NA assay.

For checking *C. partellus* proteinase-CanPI interaction dot blot assays were performed by placing a mixture of *C. partellus* enzyme extract (5 µl), recombinant CanPIs (5 µl, 10 µl, 15 µl) in Glycine-NaOH buffer pH 10 (5 µl) on X-ray film (gelatin coated film) [21] and incubating it for 30 min. The films were then washed in running tap water to remove the digested protein. Spot of only *C. partellus* enzyme was used as a positive control while only buffer was used as a negative control in the dot blot assay. The assay was repeated four times with two biological replicates. Only protease enzymes gave a clear zone by complete digestion of gelatin; whereas depending upon the strength/ activity of the proteinase inhibitors the gelatin digestion was reduced in the respective spots.

For *in gel* protease/PI activity assay recombinant CanPIs, synthetic serine protease inhibitor PMSF, insect enzymes and pre-incubated mixtures of both were separated on native PAGE, and the gel was further processed to visualize trypsin inhibitor (TI) activity using the gel X-ray film contact print technique (GXCT) [25] with modification in the incubation time. For visualization of *C. partellus* protease activity by GXCT the gel X-ray films were incubated together for more than 15 h. The experiment has been repeated at least five times (technical replicates) with two biological replicates comprising of 6 larvae per treatment.

2.4. Production of recombinant CanPIs

CanPIs are proteinase inhibitor genes isolated from *C. annuum* [17,33]. Previously characterized recombinant *Pichia pastoris* expressing CanPI-7 and CanPI-22 proteins [34] were used in this study. The expression of both the recombinant CanPI genes was performed as described previously [34]. The recombinant protein solution obtained after 90% ammonium sulphate precipitation and dialysis was concentrated 10 times using a lyophilizer (Christ, Osterode am Harz, Germany). This recombinant protein preparation was quantified by Bradford assay, and PI activities (TIUs) were determined as described above.

2.5. Effect of CanPIs on C. partellus

The effect of CanPIs on growth and development of *C. partellus* was studied by rearing the larvae on CanPI incorporated artificial diets. For preparing the diets, specific amount of CanPI was mixed with 135 ml of artificial diet. 0.25–0.025 TIU of recombinantly expressed CanPI-7 and CanPI-22 were incorporated in the artificial diets and the resulting four test diets were named as CanPI-7 I, CanPI-7 II, CanPI-22 I and CanPI-22 II. Each diet was poured in the plastic jars and 120 newly hatched larvae were released in each jar. Two replications were maintained for each treatment. In the control, larvae were released on the artificial diet without any recombinant protein added. After 12 and 20 days of treatment, larval weights were recorded using a digital balance (Mettler-Toledo AB304-S, Leicester, United Kingdom). Data were also recorded on larval period, pupal mass, pupal period. Ten moths per treatment (5 males and 5 females) were kept in a mating chamber and number of egg masses laid by *C. partellus* females reared on standard artificial diet and PI treated diet were recorded.

2.6. Proteinase structure prediction and docking with inhibitors

Trypsins and chymotrypsins from *C. suppressalis* (CsuChy002, CsuTry005, CsuTry007, CsuTry009 and CsuChy012) were selected on the basis of their differential expression profiles in mid-gut and haemolymph [35]. Homology modelling was done for predicting the

structure of these proteinases; 3D structures of trypsin and chymotrypsin IRDs from CanPI-7 and CanPI-22 (IRD4 CI, IRD5 CI, IRD9 TI, IRD10 TI and IRD14 TI) were also modelled. ModWeb (https://modbase.compbio.ucsf.edu/modweb/), an online server that works on Modeller algorithm was used to generate the 3D structures of both, proteinases and IRDs. Model validation of the predicted structures was done using Verify 3D (http://services.mbi.ucla.edu/Verify_3D/), RAMPAGE: Assessment of the Ramachandran plot (http://mordred.bioc.cam.ac.uk/~rapper/rampage.php) and ProSA (https://prosa.services.came.sbg.ac.at/prosa.php). Structures of different substrate/inhibitors (PMSF, TLCK and TPCK) were downloaded from PubChem (https://pubchem.ncbi.nlm.nih.gov/). Validated structures of proteinases and IRDs were used for molecular docking study using PatchDock online server (http://bioinfo3d.cs.tau.ac.il/PatchDock/) to analyze the strength of interaction between proteinase-PI complex. Binding energy of each proteinase-IRD complex having the highest docking score revealed by PatchDock was used to construct a heat map using MS-excel. Interaction strength was shown by gradient ruler, which indicated a range from −582.63 (red, strong) to 258.97 (green, weak).

2.7. RNA isolation, quantification, RT PCR and cDNA profiles of C. partellus

Total RNA of *C. partellus* larvae raised on untreated control diet was isolated using RNA isolation kit (NucleoSpin RNA XS) (MACHEREY-NAGEL, Düren, Germany) as per the manufacturer's protocol. Isolated RNA was quantified by spectrophotometer (SpectraMax M5, California, USA) and its quality was checked by agarose gel electrophoresis. cDNA was prepared using reverse transcription kit (Ambion reverse transcription kit, Thermo Fisher Scientific, Waltham, MA, USA). RT-PCR was carried out using primers for *C. suppressalis* trypsin and chymotrypsin genes [35]. For each 20 µl of reaction, 1 µl of cDNA template, 2 µl of buffer (10x), 0.4 µl of dNTP's (10 mM), 1 µl of forward and reverse primer each (10 µM), 0.2 µl of *Taq* polymerase and 14.4 µl of ddH$_2$O were used. This reaction mixture was set to denature at 95 °C, followed by 32 cycles at 94 °C for 30 s, then 50 °C for 45 s and 72 °C for 90 s; and extension at 72 °C for 10 min. PCR product was analyzed on 0.8% agarose gel.

2.8. Statistical analysis

The data on characterization of C. partellus proteinases and insect growth and development were subjected to analysis of variance (ANOVA) performed by Generalized Linear Model (GLM) procedure using Statistical Analysis System (SAS 9.3; SAS Institute Inc., Cary, NC, USA). Means were separated by Tukey's honest significant difference (HSD) (p≤0.05), to find if treatment effects were statistically significant.

3. Results

3.1. Characterization of C. partellus proteinases and their inhibition by recombinant CanPIs

The pH of whole larval tissue, the midgut tissue and their extracts prepared in distilled water was found to be alkaline (pH 8). The *C. partellus* trypsin-like activity was evaluated at pH 7.8 and 10, and it was noted to be high at pH 10.0 (Fig. 1A). Visualization of *C. partellus* proteinases on the gel revealed three proteinase isoforms, which were sensitive to inhibition by a serine protease inhibitor, PMSF (Fig. 1B).

Two recombinant CanPIs namely, CanPI-7 and CanPI-22 (Fig. 1C), which differ in the number and type of inhibitory repeat domains (IRDs) were selected for inhibition studies [34,36]. The recombinant

CanPIs were, visualized on native PAGE gel for activity profiles (Fig. 1D). Both CanPIs (0.28 µg) inhibited the *C. partellus* larvae proteinase activity (Fig. 1E).

3.2. Effects of CanPIs on C. partellus growth and development

Ingestion of two concentrations of CanPI-7 and -22 (0.25 and 0.025 TIUs/ml diet) incorporated in artificial diet, showed dose dependent effect on the *C. partellus* larval body mass (Fig. 2A). Low and high dose of CanPI-7 and CanPI-22 showed 2 and 2.6-fold decrease in larval mass, respectively as compared to larvae raised on diet without CanPIs at 20 days of feeding. Further, CanPI ingestion by *C. partellus* larvae resulted in 25 to 30% reduction of pupal mass. However, the difference in pupal mass across the CanPI treatments was not significant (Fig. 2B). Feeding on CanPI prolonged the larval period of *C. partellus* by 5–8 days (Fig. 3A). The pupal periods of *C. partellus* larvae reared on CanPI diets were prolonged by 1–5 days (Fig. 3B). The fecundity of *C. partellus* was also hampered by 50–80% when reared on the CanPI diets (Fig. 3C)...

3.3. CanPI induced C. partellus larval proteinases are sensitive to CanPI-7 but not to CanPI-22

Total proteinase activity of *C. partellus* larvae raised on CanPI diet exhibited differential profiles. Each CanPI uniquely induced some protease isoforms in *C. partellus* larvae, which were different than the larvae reared on inhibitor free control diet (as marked in Fig. 4A). *C. partellus* control diet raised larval proteinase isoform 1, was not inhibited by either CanPI-7 or CanPI-22. Isoform 2 was sensitive to CanPI-7 but not CanPI-22, while isoform-3 could be inhibited by both CanPIs (Fig. 4B). Unique protease isoforms induced in *C. partellus* larvae fed on CanPI-7 and CanPI-22 diets were completely inhibited by CanPI-7 (6 µg); while CanPI-22 (6 µg) was ineffective in inhibiting the same (Fig. 4B)..

3.4. Chilo proteinase display structural diversity and varied interactions with CanPI IRDs

Due to the lack of *C. partellus* nucleotide sequence information in the database, the available *C. suppressalis* proteinase sequences [35] were used to study the proteinase gene expression patterns in *C. partellus* (Table 1). *C. partellus* transcripts for trypsin and chymotrypsin genes amplified using CsuChy002, CsuTry005, CsuTry009, CsuChy012 specific primers expressed strongly, while those with CsuTry007 primers showed low expression (Supplementary Fig 1). Three-dimensional structures of trypsins (CsuTry005, CsuTry007 and CsuTry009), chymotrypsins (CsuChy002 and CsuChy012) and CanPI-IRDs were predicted *in silico* and validated by Verify3D, RAMPAGE and ProSA (Supplementary Table 1). Studies were carried out to provide insights into the interaction between *C. partellus* proteinases and the CanPIs. For this the validated, predicted structures of enzymes and inhibitors were used. Proteinases (trypsins and chymotrypsin) and IRDs/substrate complexes revealed variation in binding energies (Fig. 5A). IRD9 displayed the strongest binding with CsuChy002, CsuTry009 and CsuTry005. On the other hand IRD10 and IRD4 showed strong binding with CsuTry005 and CsuChy002. Interestingly, IRD5, which is chymotrypsin inhibitor, exhibited strong binding with CsuTry007 and CsuChy002 while IRD14 showed weak binding with all the trypsins and chymotrypsins except CsuTry007. Amongst different synthetic inhibitors (PMSF, TLCK, TPCK) TLCK displayed strong binding with trypsins and chymotrypsins, and moderate to weak binding was noted with TPCK and PMSF (Fig. 5A). CsuChy002 was inhibited by IRDs, except IRD14 TI, whereas CsuChy012 and CsuTry009 were least inhibited by IRDs..
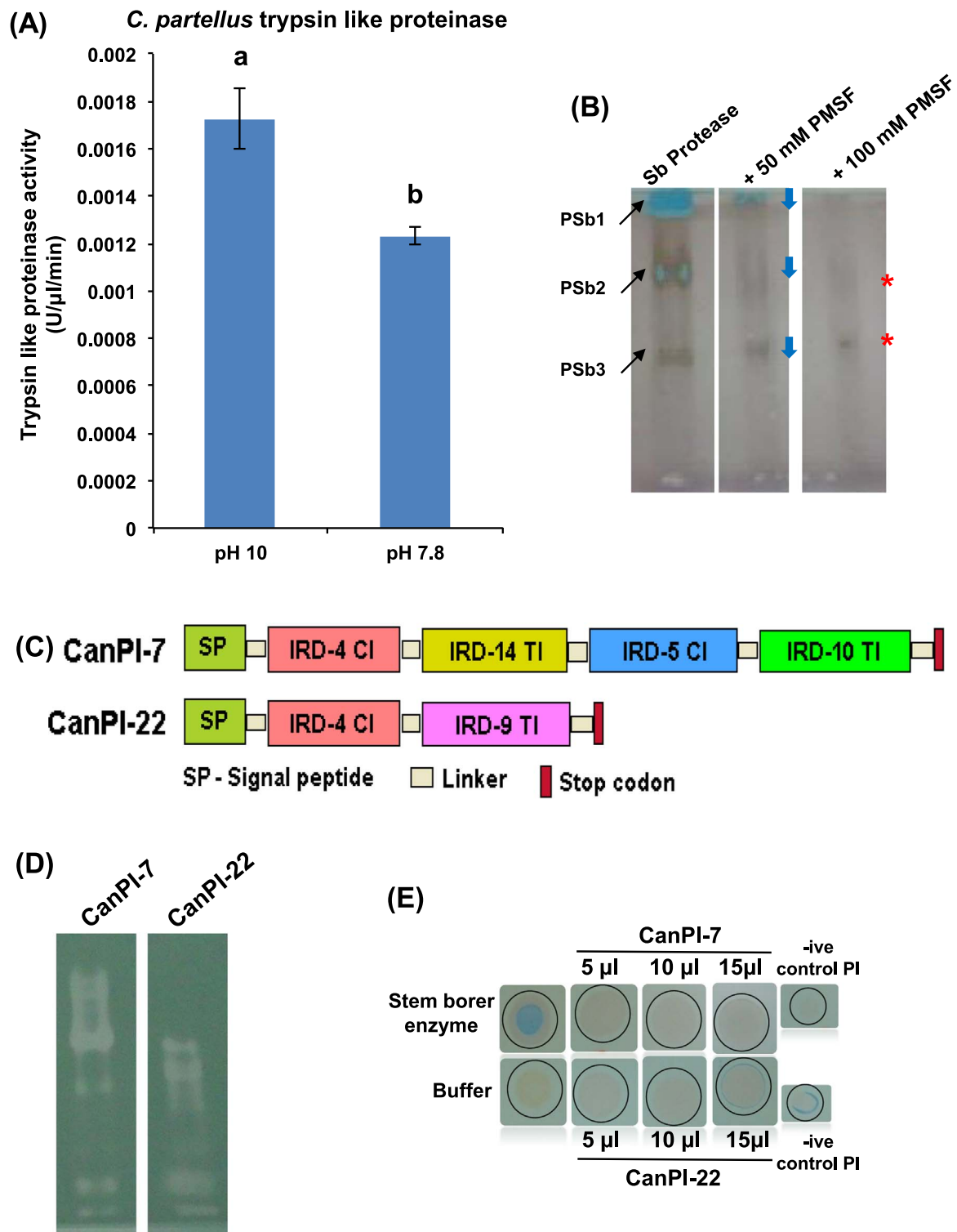
Fig. 1. Characterization of *C. partellus* proteinases and its inhibition by CanPI (A) Trypsin like proteinase activity of *C. partellus* was characterized at pH 10 and pH 7.8 using BApNA assay with incubation time increased upto 60–120 min at 37 °C. Analysis of variance (ANOVA) was performed and means of the treatments were separated by Tukey's honest significant difference (HSD) (p≤0.05) (B) *In gel* visualization of *C. partellus* proteinases activity with different concentrations of PMSF, a serine protease inhibitor was carried out by the gel X-ray film contact print technique (GXCT) on Native PAGE (12%) with incubation time of around 15 h at room temperature. Protease isoforms of stem borer were named as 1, 2, 3. Downward arrow indicates inhibition of proteinases activity by PMSF and star symbol indicates the PMSF uninhibited forms of proteinases. (C) Schematic representation of recombinant CanPI displaying their Trypsin (TI) and Chymotrypsin (CI) inhibitory repeat domains (IRDs). CanPI-7 has two CI and two TI-IRDs and CanPI-22 has one TI and one CI-IRD. The figure is modified from Mishra et al., 2010. (D) GXCT visualization of recombinant CanPI on Native PAGE (12%). (E) Dot blot assay was used to test CanPI-7 and CanPI-22 for their potential to inhibit *C. partellus* proteases. *C. partellus* enzyme extract was used as a positive control and only buffer (Glycine NaOH, pH 10) as a negative control.

4. Discussion

Designing molecular/biotechnological means of pest control is challenging for many economically important pests as their physiology and interactions are not known. *C. partellus* is one such pest. Study of digestive physiology of *C. partellus* - an oligophagous pest specialized on monocotyledonous crops, revealed presence of larval proteinases that are active at alkaline pH, corroborating with other Lepidopteron
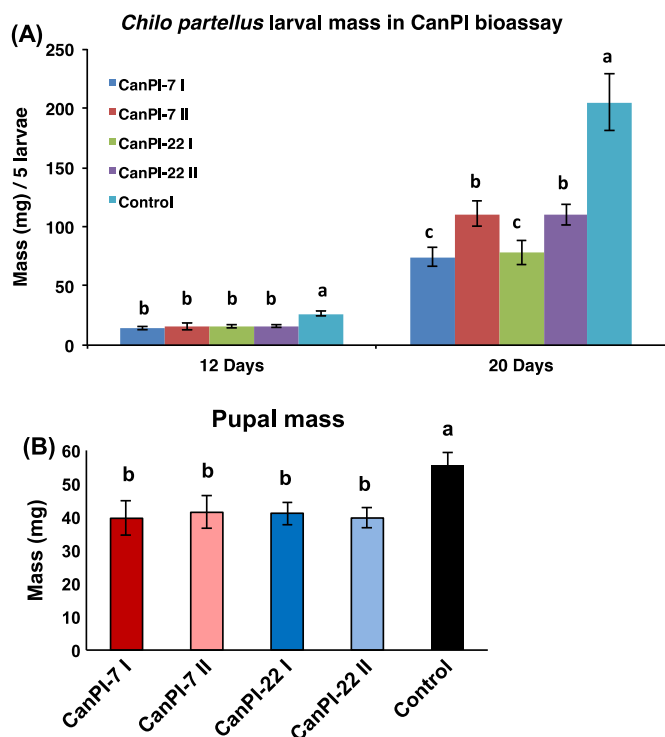
Fig. 2. *C. partellus* – CanPI bioassay (A) Four test diets with incorporation of recombinant PIs namely CanPI-7 I, CanPI-7 II, CanPI-22 I, CanPI-22 II and control diet without PI were used to rear *C. partellus* larvae. Average larval mass was recorded on 12 and 20 days after treatment. Data was analyzed using ANOVA and means of the treatments were separated by Tukey's honest significant difference (HSD) (p≤0.05) (B) Pupal mass of *C. partellus* larvae raised on CanPI incorporated diets and control diets.

insect proteases. *C. partellus* crude protease extracts showed high activity at pH 10 compared to pH 7.8. The local tissue/ site-specific pH differences may exist in the larvae and would play a role in optimizing enzyme activities, though this needs to be investigated further. However, as compared to the polyphagous insect *H. armigera*, *C. partellus* has several fold lower gut proteolytic activity and fewer protease isoforms [37]. Interestingly the non-host proteinase inhibitors from *C. annuum* CanPI-7 and -22 were highly effective in retarding larval growth and development in both the insects, *C. partellus* and *H. armigera*. The CanPIs that are known for their variable IRDs displayed differential inhibition of the *C. partellus* proteinases. CanPI-22 IRDs showed strong inhibition of *C. partellus* proteinases as compared to CanPI-7 in the *in silico* molecular docking studies. IRD-protease interactions in the larvae might be responsible for induced up-regulation of CanPI specific proteinases. Importantly *C. partellus* induced upon ingestion of CanPI-7 or CanPI-22 were sensitive to CanPI-7 but not to CanPI-22. This indicates the negative influence of strong proteinase inhibition (CanPI-22) on the insect physiology and its consequences in rendering the PI ineffective. While it positively supports the use of CanPI-7 with strong antibiosis effect on *C. partellus* and ability to retain inhibition of the induced proteinases.

Phytohormones such as jasmonic acid induce plant defense response against insect pests by elevating secondary metabolites and proteins [30]. PI based approach to control *C. partellus* would be effective and/or complementary in developing tolerance/resistance in crop plants. Studies in an insect closely related to *C. partellus*, indicated that co-expression of maize serine PI and a potato carboxypeptidase inhibitor in rice, conferred resistance to *C. supressalis* [38]. Upon ingestion both the CanPIs resulted in causing adverse effect on *C. partellus*. Both CanPI-7 and-22 showed consistently higher proteinase inhibition and growth retardation in *H. armigera* [26,34,39]. As expected high PI dose had a more pronounced effect on *C. partellus* larval mass. However, the effect on pupal mass, larval/pupal period
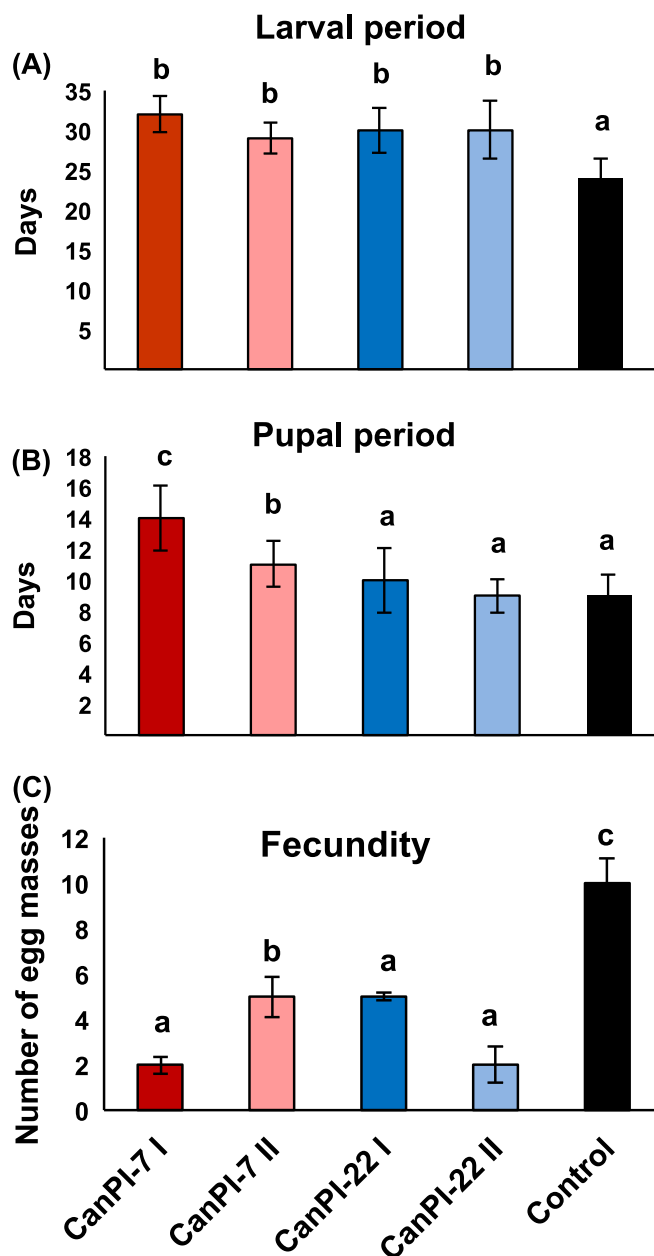

Fig. 3. Effect of CanPI ingestion on larval, pupal period and fecundity of *C. partellus* (A) Larval period; (B) Pupal period and (C) fecundity of *C. partellus* reared on CanPI incorporated artificial diets namely CanPI-7 I, CanPI-7 II, CanPI-22 I, CanPI-22 II and control. Data was analyzed using ANOVA and means of the treatments were separated by Tukey's honest significant difference (HSD) (p≤0.05).

and fecundity did not display a dose dependent influence on *C. partellus*. Mahajan et al. (2013) highlighted the importance of optimizing the dose of PIs for effective antibiosis in insect larvae [26]. It is important to note that *C. partellus* treated with low inhibitor diet of CanPI-7 or -22 was equally effective in delaying its growth and significantly reducing its fecundity. The reduction in pest fecundity is crucial as it has detrimental and long-term effects on the population build-up of the subsequent generations. Mortality in *C. partellus* raised on CanPI-7 or -22 diets was not significantly high indicating that CanPIs as a growth inhibitor might not put strong selection pressure on *C. partellus*. These experiments with recombinant CanPIs and *C. partellus* have been carried out using artificial diet and under controlled laboratory conditions and they provide promising leads. Experiments using CanPI expressing transgenic plants and *C. partellus* under polyhouse conditions are necessary for further validating the
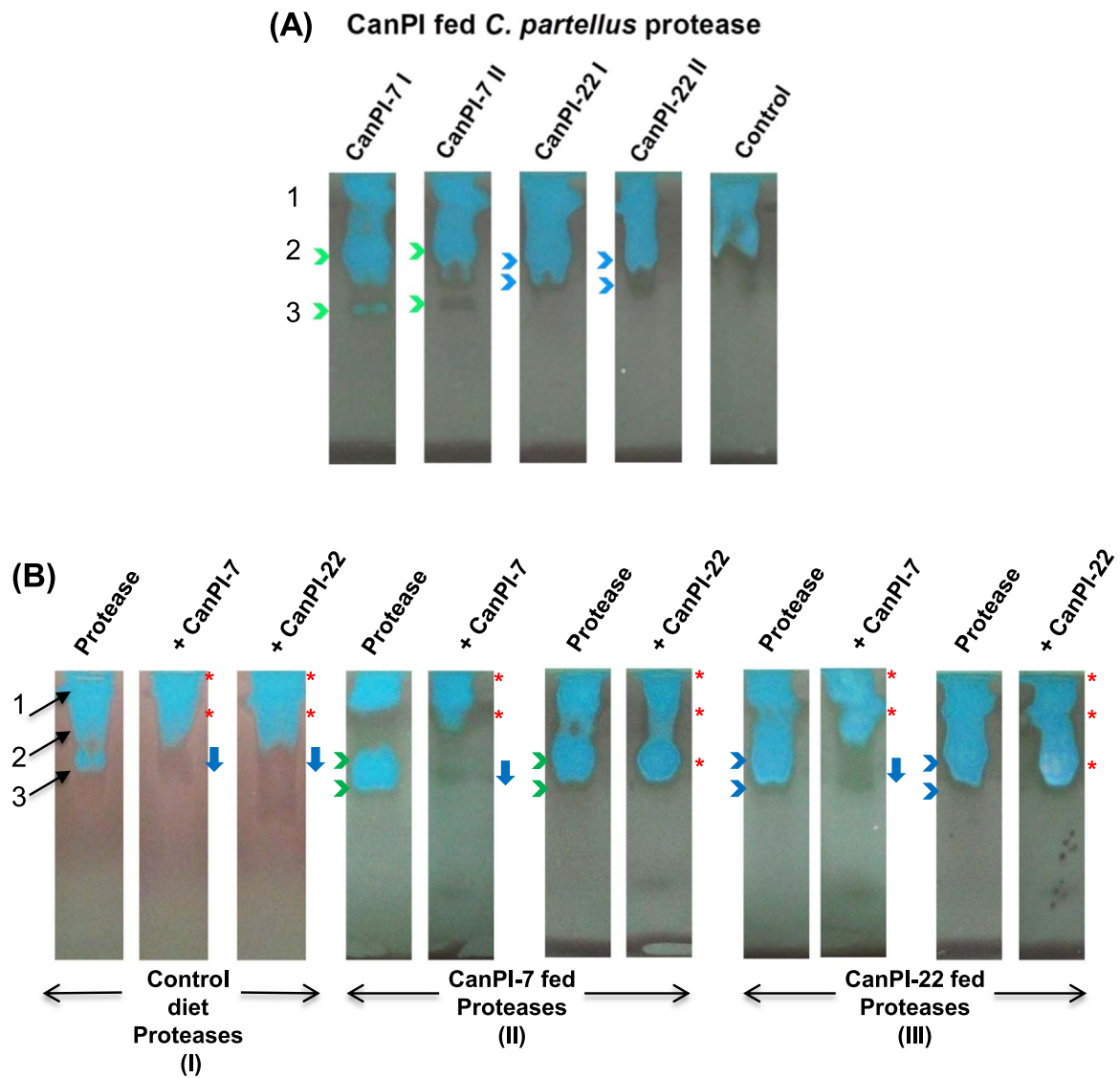
Fig. 4. CanPI ingestion induced changes in *C. partellus* proteases (A) Proteases activity profiles of *C. partellus* larvae raised on CanPI and control diets were resolved on 12% native PAGE visualized using GXCT with around 15 h incubation at room temperature. Green and blue coloured arrows indicate the protease isoforms induced in *C. partellus* larvae by CanPI-7 and CanPI-22 ingestion respectively. (B) *C. partellus* protease from control (I) CanPI-7 fed (II) and CanPI-22 fed (III) were treated *in vitro* with CanPI-7 and -22, the mixture was resolved on native PAGE (12%) followed by visualization of protease profiles by GXCT with more than 15 h incubation at room temperature. Green and blue coloured arrows indicate the protease isoforms induced by CanPI-7 and CanPI-22 respectively. The downward arrow indicates inhibited protease isoforms where as star indicates the uninhibited protease isoforms.

Table 1
List of primers[a].

| Sr. No.	Name	Given name		Primer sequence
1	CT002	CsuChy002	Fwd	5′ GAAGCCCCTTTGGACTACGGA 3′
2	CT002	CsuChy002	Rev	5′ CGGCGGCACCACCACATA 3′
3	CT005	CsuTry005	Fwd	5′ CACCCCAATTTCAACGACCTTCC 3′
4	CT005	CsuTry005	Rev	5′ CCAACCAGCAGCCCAGACTAC 3′
5	CT007	CsuTry007	Fwd	5′ AGGCATAACTGTGGGGGA 3′
6	CT007	CsuTry007	Rev	5′ GCGAAACCAAAGGAGCA 3′
7	CT009	CsuTry009	Fwd	5′ GGAGGAAACCCTACCACCATT 3′
8	CT009	CsuTry009	Rev	5′ GTGGGTTTGTCCCTGCTCAC 3′
9	CT012	CsuChy012	Fwd	5′ TCCTCACTGCTGCTCGTTG 3′
10	CT012	CsuChy012	Rev	5′ CGGAATGTGCGTTGGTTG 3′
11	G3PDH	G3PDH	Fwd	5′ GTTGTGCCTCACCAATTTGTCAG 3′
12	G3PDH	G3PDH	Rev	5′ GCCACCTTCAGCGATGTCG 3′
13	EF-1	EF-1	Fwd	5′ TGAACCCCCATACAGCGAATCC 3′
14	EF-1	EF-1	Rev	5′ TCTCCGTGCCAACCAGAAATAGG 3′

a Ge et al., 2012.
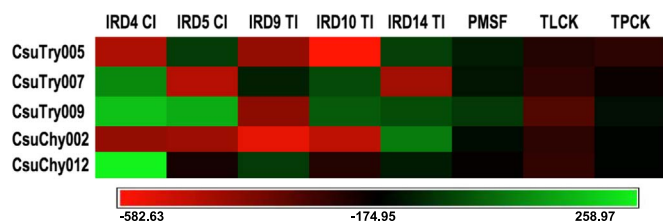
Fig. 5. Interaction of *Chilo* sp. proteases with CanPIs and its IRDs Heat map showing the minimum free binding energy of *C. suppressalis* trypsins, chymotrypsins with CanPI IRDs and substrate/inhibitors. The docking was carried out using PatchDock online server. The color strip below displays the values of minimum (red) to maximum (green) free energies representing high to low binding between the interacting molecules.

efficacy of CanPI for insect control.

Diversity of digestive proteases is an important factor contributing towards insect adaptation/ tolerance to defense proteins and inhibitors [37,40,41]. Upon ingestion of PI the insects are known to respond in a number of ways (i) by up regulating certain proteinases or (ii) expressing inhibitor insensitive proteinases [12]; which dependents on the type and amount of PI ingested [26]. *C. partellus* feeding on CanPI diet displayed a dose-dependent regulation of proteinase activity (Fig. 5A). Further characterization of proteinase isoforms is necessary to better understand *C. partellus's* digestive physiology.

Insights into *C. partellus* protease-PI interaction(s) were further highlighted through the induced up-regulation of different protease isoforms. CanPI-7 and CanPI-22 induced *C. partellus* proteinases were effectively inhibited by CanPI-7, while CanPI-22 was ineffective in inhibiting them. This suggests that CanPI-22 led to the up-regulation of proteases, which were insensitive to inhibition by CanPI-22. CanPI IRDs contributed to the qualitative characters of the inhibitor diet determining their influence on insect metabolism.

IRD-9 was not equally effective against proteases of *H. armigera* feeding on different natural diets [37]. CanPI-7 having a combination of 4 different IRDs, with trypsin and chymotrypsin inhibition specificities, has been reported to be effective across a range of inhibitor concentrations and proteases of *H. armigera* fed on different host plants [26,34,36,37]. The dynamic interaction between IRD/PI and *C. partellus* proteinase expression; determines the insect digestive physiology and eventually the sustainability of PI based insect control strategy. As noted, the strong inhibitor of most *C. partellus* proteinases in the form of CanPI-22 (with IRD-9) exerts pressure on the larvae to up-regulate inhibitor insensitive proteinases. On the other hand, a combination of diverse IRDs in CanPI-7, with moderate to low inhibition of *C. partellus* proteinases contributes towards the efficacy of the inhibitor which should be explored further for developing *C. partellus* control measures.

5. Conclusions

We investigated the digestive physiology of the sorghum pest *C. partellus*, its interaction with non-host proteinase inhibitors from *C. annuum* (CanPIs) and their potential to bring about the growth and developmental retardations in *C. partellus*. The two different PIs, CanPI-7 and CanPI-22, upon being ingested bring about retardation in larval growth and development. Each PI induces diverse proteinase isoforms in the pest; which are sensitive to CanPI-7 but not to CanPI-22. Molecular interaction studies by docking indicated that the CanPI-22 (IRD-9) was a strong inhibitor of *Chilo* sp. proteinases where as CanPI-7 IRDs were weak inhibitors, The correlation of experiments indicate that though ingestion of artificial diet incorporated CanPIs leads to antibiosis in *C. partellus* larvae; CanPI-7 prevents induction of inhibitor insensitive proteinases while CanPI-22 promotes it. So, CanPI-7 demonstrates the potential for its further development as a molecular tool for *C. partellus* control.

Appendix A. Transparency document

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2016.09.016.

Appendix A. Supporting information

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