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Cloning and Expression of Trypsin Inhibitor Gene *Ti* from Pea (*Pisum sativum* L.) cv. 'Arkel' in *Escherichia coli* DH5α Cells

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ABSTRACT

Protease inhibitors (PIs) play key regulatory roles in many biological processes. The single gene advantage associated with these inhibitors make them ideal candidates for gene transfer to produce pest resistant recombinant plants. The aqueous extracts from pea (*Pisum sativum*) displayed prominent trypsin inhibitory activity. For cloning of trypsin inhibitor gene (ti) into bacterial hosts, cDNA was first prepared from the RNA isolated from pea seedlings. Amplification of the *Ti* gene was carried out using two sets of primers, the 5′-primer contained *Eco*RI restriction sequence, and the 3′- primer contained *Hind*III restriction sites. The purified amplicons were cloned into pet 27b+ expression vector using *Eco*RI and *Hind*III restriction enzymes. The constructed vectors were transformed into *Escherichia coli* DH5α cells. The recombinant cells were grown in LB medium containing kanamycin and treated with IPTG to induce the expression of the cloned *Ti* gene. The expression profile of the cells revealed highly intense 12.6 kDa band in the induced samples. The gel print technique and Dot blot assay revealed that the protein showed significant inhibitory activity towards trypsin and enzyme assay with synthetic substrates showed that the protein caused 90 ± 3% inhibition of trypsin.

Keywords: insect resistance, pest management, phytophagous insects, plant defense, protease inhibitor, transgenic plants

INTRODUCTION

Naturally occurring protease inhibitors (PIs) play key regulatory roles in many biological processes. They are of common occurrence in the plant kingdom and have been described in storage tissues as well as in the aerial parts of plants (Leo *et al*. 2000; Habib and Fazili 2007; Nadaraja *et al*. 2010; Pandey and Jamal 2010; Bajuk *et al*. 2011). They are also induced in plants in response to injury or attack by insects or pathogens (Quilis *et al*. 2007; Philippe *et al*. 2009). Widely distributed throughout the plant kingdom, these anti-metabolic proteins play key roles in defense against herbivores and pathogens (Jongsma and Bolter 1997; Zavala *et al*. 2004) The defensive capabilities of plant PIs rely on inhibition of proteases present in insect guts or secreted by microorganisms, causing a reduction in the availability of amino acids necessary for their growth and development (Lawrence and Koundal 2002).

A number of inherited diseases are caused due to abnormalities in PIs. These include different forms of emphysema, epilepsy, hereditary angioneurotic oedema and Netherton syndrome (Bitoun 2000; Lomas *et al*. 2002; Lehesjoki 2003; Ritchie 2003) Some of these diseases may be susceptible to treatment with the PIs administered as drugs, with synthetic inhibitors that take over their function, or with natural inhibitors made available by gene therapy (Krol *et al*. 2003; McKay *et al*. 2003; Rawlings *et al*. 2004). A Kunitz-type inhibitor EcTI from seeds of the Brazilian plant *Enterolobium contortisiliquum* was shown to be cytotoxic against human tumor cells without affecting normal tissue remodeling fibroblasts (Nakhata *et al*. 2011).

The indiscriminate and excessive use of chemical pesticides has resulted in reduction in beneficial insect population and development of resistance in insect pests. Integrated pest management (IPM) employs multi-component pest control strategies which include development of transgenic crops that express an insecticidal protein (Ter *et al*. 2010). It is now possible to identify, clone and insert genes from any organism into the crop plants to confer resistance to insect pests without any biological barriers (Huang *et al*. 2007; Quilis *et al*. 2007; Khadeeva *et al*. 2009). Considerable progress has been made in developing transgenic plants with toxin genes from *Bacillus thuringiensis* (*Bt*) in different crops. However, wide spread use of just one or a few genes is not advisable and there is a need to identify alternative genes for deployment through transgenic crops to control insect pests (Tabashnik 1994; Hilder and Boulter 1999; Sharma *et al*. 2000). PIs, being single gene products, are preferred over other products of complex biochemical pathways. Genetically modified plants can be readily obtained by transferring single defense related gene from one plant to another (Boulter 1993; Marchetti *et al*. 2000). It has been demonstrated that transgenic tobacco plants constitutively expressing the trypsin inhibitor (TI) gene exhibit resistance to insect pests *Spodoptera litura* and *Helicoverpa armigera* and show enhanced tolerance to stress induced by salt treatment, pH variability and exposure to other solutes (Dunaevskii *et al*. 2005; Huang *et al*. 2007; Shan *et al*. 2008; Khadeeva *et al*. 2009; Srinivas *et al*. 2009).

In view of the tremendous biological significance associated with these inhibitors, and the fact that our natural resources have not been fully exploited in this context, we chose to carry out the analysis of pea (*Pisum sativum*) seeds for their protease inhibitory potential. Following this, the TI gene *Ti* was isolated, cloned and expressed in *Escherichia coli* with the purpose of making the inhibitor readily available for potential use in biology and medicine.

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Original Research Paper

MATERIALS AND METHODS

Plant Material

Garden pea (*Pisum sativum* L.) cv. 'Arkel' seeds were grown as described by Carbonell and García-Martínez (1985). Pea seeds were allowed to imbibe by placing them on top of sterile cotton swabs previously saturated with 70% ethanol. Seeds were then immersed in 5% (v/v) aqueous sodium hypochlorite for 5 min and rinsed 3-4 times in autoclaved water and left at room temperature for one week to grow. The plant seedlings were separated from seeds and were stored at −80°C until use.

Chemicals and reagents

*Eco*RI and *Hind*III, *Taq* DNA polymerase, Proteinase K, oligo(dT), dNTP, 100 bp ladder and 1 kb ladder, *Murine leukemia virus* (MULV) reverse transcriptase were purchased from Fermentas, Leon-Rot, Germany. Genoprep mRNA kit, PCR purification kit, plasmid midiprep kit and ethidium bromide were purchased from Qiagen, Valencia, USA. Kanamycin, trypsin, chymotrypsin, isopropyl-1-thio-β-galactopyranoside (IPTG), Coomassie brilliant blue and bromophenol blue were obtained from Sigma Chemical Co., St. Louis, USA. *E. coli* DH5α was the product of Invitrogen, New York, USA. Folin-Ciocalteu's phenol reagent was purchased from Sugma Chemical Co. St. Louis, USA. pET 27b+ vector was obtained from Merck, Middlesex, UK. All other reagents used were molecular biology grade chemicals.

Cloning strategy

Two sets of primers were designed based on the *Ti* gene encoding the mature chain of the Ti protein (GenBank: AJ414577.1; Page *et al.* 2002) 5′- GGCGAATTCATGGAGTTGATGAATAAGAAG GC-3′ (sense) and 5′- CGAAGCTTGTTCTTAATGACCTCCTCC ACCTCAGAG-3′ (antisense). The underlined letters denoted the sites targeted by the restriction enzymes *Eco*RI and *Hind*III For first-strand synthesis, total RNA was isolated from 100 mg of pea seedlings using Genoprep mRNA kit. The RNA was dissolved in DEPC water. To generate cDNAs, 0.1 µg of total tissue RNA was transcribed at 37°C for 60 min in a 30-µl reaction volume containing 0.25 µg oligo (dT) plus 200 U Moloney MULV (Mo MULV) reverse transcriptase. The PCR mixture contained 60 ng cDNA, 1 ml of 10X reaction buffer, 5 µl of 2 mM dNTP, 1 µl of 1U/µl of *Taq* DNA polymerase and 1 µl of 100 pm/µl of each primer. The PCR reaction was performed on a Biometra thermocycler (Goettingen, Germany) and the cycling conditions were as follows: one cycle at 95°C for 5 min, 30 cycles at 95°C for 30 sec, 57.5°C for 1 min and 72°C for 10 min followed by one cycle at 72°C for 10 min. The PCR product was run on a 1.5% agarose gel containing ethidium bromide and visualized under ultraviolet (UV) light.

Cloning of *Ti* gene

PCR product of the correct size (345 bp) were excised from the agarose gel and purified using a PCR purification kit. The purified product and pET 27b+ vector (courtesy IIT Mumbai) were cut with the restriction enzymes *Eco*RI and *Hind*III at 37°C for 2 h. They were then ligated using T_4 DNA ligase for 16 h at 4°C. *E. coli* DH5α (courtesy Indian Institute of Technology, Mumbai) was transformed with the resulting vector by heat shock in which the cells and vector were mixed and placed on ice for about 10 min and then incubated at 42°C for 45 s. The resulting white colonies were confirmed as containing the inserted sequence by colony PCR and restriction enzyme analysis. A colony containing the intact inserted sequence was identified and cultured in 5 ml Luria Broth (LB) with 10 µg/ml kanamycin. The plasmid was extracted using the plasmid midiprep kit and was cut by *Eco*RI and *Hind*III.

Expression of recombinant *Ti* in *E. coli*

E coli DH5α transformed with the plasmid containing pET 27b+ *Ti* recombinant vector was grown in 100 ml LB medium with 10 µg/ml kanamycin. After the OD at 600 nm was 0.4-0.5, 1 ml of culture was stored at 4°C which served as un-induced control sample. The expression of the fusion protein was induced by the addition of 0.5 mM IPTG and incubated with vigorous shaking at 220 rpm. At various time periods during incubation, 1 ml of culture was transformed to a microfuge tube and IPTG induced cells were collected by centrifugation at 12,000 rpm for 1 min at room temperature. Supernatants were removed by aspiration. The pellet was re-suspended in 10 volumes of lysis buffer (0.5 mM Tris HCl buffer, pH 8.0) and then sonicated by an ultrasonic disrupter (Model WW-04711-75, Cole-Parmer, Illinois, Chicago, USA). The pellet was washed four times with phosphate-buffered saline (PBS) and air dried to remove all PBS. Pellet was re-suspended in 100 µl of 1X SDS gel loading buffer (0.25 M Tris HCl buffer, pH 6.8 containing 1g% SDS and 0.02 g% bromophenol blue), boiled at 100°C for 3 min and centrifuged at 12,000 rpm for 1 min at room temperature and the supernatant was collected and SDS PAGE was carried out on 17% gel with the protein bands stained with Coomassie brilliant blue.

Gel X-ray film contact print method

The activity of the induced TI was checked by gel X-ray film contact print method. When the electrophoresis was complete, the gel was removed and placed in Tris HCl buffer, pH 7.6 for 15 min. Tris HCl buffer was then replaced by 0.1% trypsin solution and left at room temperature for 15 min. The gel was then briefly rinsed in Tris buffer to remove the excess trypsin. An X-ray film was placed over the gel and incubated at 37°C for 10 min. The X-ray film was removed and washed with double distilled water and rubbed gently with tissue paper. TI band appeared as un-hydrolyzed gelatin against the background of hydrolyzed gelatin which produced a clear background.

Dot blot assay

The activity of the over-expressed TI protein was assayed by dot blot method (Pichare and Kachole 1994). 5 µl of trypsin solution (4 µg/100 µl in 0.1 M Tris HCl buffer, pH 7.6) were mixed with 5 µl of TI protein and incubated at 4°C for 5 min. This solution was spotted on X-ray film with respect to control. A clear zone is observed in the control as trypsin causes degradation of the gelatin coating on the X-ray film. In the test solution the presence of inhibitor causes inactivation of the trypsin and thus reduces the clear zone or causes disappearance of the clear zone.

Protease inhibitor assay

Trypsin and chymotrypsin activity of the expressed inhibitor protein was assayed by the method of Kunitz (Kunitz 1947). 0.2 ml of the protein was added to 0.2 ml of 40 µg/ml trypsin solution, pre-incubated at 37°C for 10 min. The volume was made up to 1 ml with 20 mM sodium phosphate buffer, pH 7.0. One ml of 2% casein solution was added to the mixture after re-incubating it at 37°C for 10 min. The reaction was allowed to take place at 37°C for 10 min and was the stopped by addition of 1 ml of 10% TCA solution. The substrate blanks were prepared in a similar manner except that the order of addition of casein and TCA was reversed. In sample blanks the inhibitor protein solution was replaced by 0.2 ml of buffer (20 mM sodium phosphate buffer, pH 7.0). Acid-insoluble material was removed by centrifugation at 2500 rpm for 15 min. The supernatant was analyzed for acid soluble peptides with Folin-Ciocalteu's phenol reagent.

RESULTS

RT-PCR results of Ti cDNA

Total RNA was isolated from pea seedlings. After reverse transcription, PCR resulted in an amplification fragment with a length of 345 bp as shown in lane B of **Fig. 1**, lane A represents 100 bp ladder. Amplicon was then purified using PCR purification kit. The purified amplicon was electrophoresed on a 1.5% agarose gel as shown in **Fig. 2** in which lane A represents multiple bands corresponding to 100 bp ladder and lane B shows a single band corresponding to 345

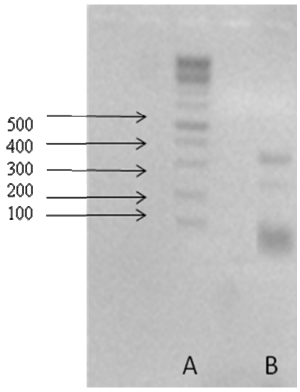

Fig. 1 RT-PCR results for *Ti* cDNA on 1.5% agarose gel. Lane A: 100 bp DNA marker; lane B: Ti amplification fragment indicating 345-bp amplicon.

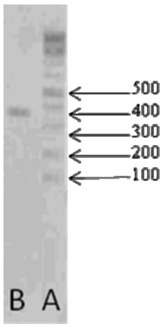

Fig. 2 Electrophoretic pattern of purified amplicon on 1.5% agarose gel. Lane A: 100 bp DNA marker; lane B: purified 354 bp product.

bp amplicon. The amplicon corresponded to a translation product of 12.6 kDa polypeptide. The amplicon was cloned into *E. coli* DH5α with pET 27b+ as vector.

Restriction digestion of 345 bp amplicon and pET 27b+ vector

The amplicon as well as vector were treated with *Eco*RI and *Hind*III and electrophoresed on 1.5 and 1.2% agarose gels, respectively. The banding pattern of insert is shown in **Fig. 3** in which lane A represents 100 bp ladder and lane B represents 345 bp digested insert. Similarly the banding pattern of vector is shown in **Fig. 4** in which lane A represents 1 KB ladder, lane B represents undigested plasmid producing two bands and lane C produces only single band representing digested plasmid. After digestion the bands of the insert and vector were purified using DNA purification kit.

Ligation and transformation

The vector and the insert were then ligated using T4 DNA ligase. After ligation the mixture was used to transform DH5α. *E. coli* DH5α cells by standard $CaCl_2$ treatment followed by heat shock at 42°C. The transformed bacterial cells were selected by growing them in a medium containing kanamycin and incubated at 37°C for 16 h. Kanamycin resistant colonies were stored at –80°C in 10% glycerol.

Plasmid isolation

The plasmids were isolated from these transformed cells and electrophoresed on 1.2% agarose gel and corresponds in size to that of vector plus insert (5414 bp + 345 bp) as shown in **Fig. 5** in which lane A shows multiple bands representing 1 kb ladder and lane B shows a single band representing 5759 bp corresponding to that of vector plus insert.

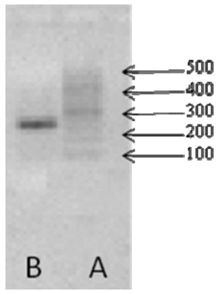

Fig. 3 Restriction enzyme digested 345 bp insert on 1.5% gel. Lane A: 100 bp DNA marker; lane B: *Eco*RI and *Hind*III digested 345 bp insert.

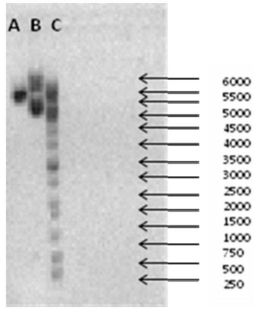

Fig. 4 Agarose gel electrophoretic pattern of double digested plasmid on 1.2% gel. Lane A: 1 kB DNA marker; lane B: uncut plasmid; lane C: *Eco*RI and *Hind*III digested plasmid.

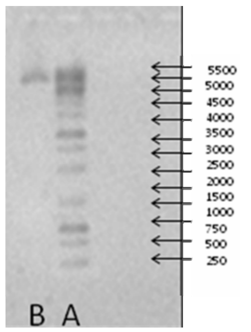

Fig. 5 Plasmid containing 345-bp insert on 1.2% gel. Lane A: 1 kB DNA marker; lane B: plasmid-containing insert isolated from *E. coli* strain DH5α cells.

Restriction enzyme analysis of pET 27b+ - *Ti* recombinant plasmid

For the pET 27b+ - *Ti* recombinant plasmid cut by *Eco*RI and *Hind*III, the results on 1.2% agarose gel revealed two bands of 5414 bp corresponding to vector and 345 bp corresponding to insert as shown in lane B and lane A represents 1 kB ladder, thus confirming the presence of insert (**Fig. 6**).

Expression of recombinant *Ti* protein in *E. coli*

For the analysis of *Ti* protein, the proteins were separated by 17% SDS PAGE and stained with Coomassie brilliant blue (**Fig. 7**) A clear dark blue band was detected at about 12.6 kDa, the predicted size of the *Ti* protein.

Gel X-ray contact print method

The activity of the induced trypsin inhibitor was checked by gel x-ray contact print method as shown in **Fig. 8** in which lane A shows single light band representing un-induced sample, lane B shows single intense band representing 12.6 kDa TI protein pointing to the fact that the induced inhibitor is present in active conformation preventing the action of trypsin on the gelatin coating of the X-ray film.

Fig. 6 Restriction map of pET 27b+ - Ti recombinant plasmid on 1.2% agarose gel. Lane A: 1 kB DNA marker; lane B: pET 27b+ vector containing 345-bp insert digested with *Eco*RI and *Hind*III.

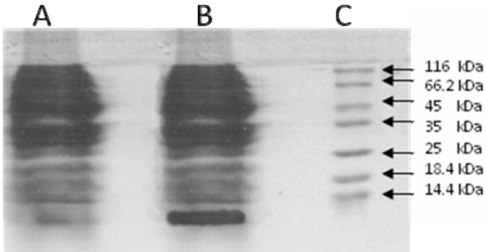

Fig. 7 SDS gel electrophoretic pattern of recombinant DH5α cells on 17% polyacrylamide gels. Lane A) uninduced DH5α cell extract; lane B) induced DH5α cell extract; lane C) molecular weight marker.

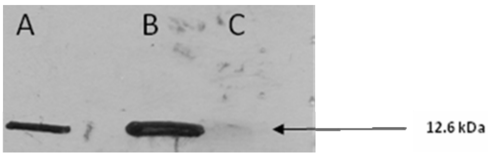

Fig. 8 Gel X-ray film contact print photograph recombinant DH5α cells. Lane A) uninduced DH5α cell extract; lane B) induced DH5α cell extract; lane C) molecular weight marker.

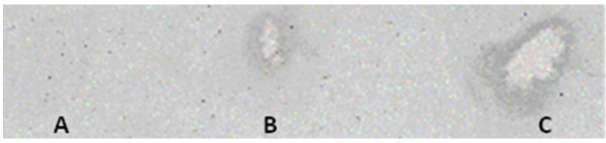

Fig. 9 Dot blot assay for protease inhibitor activity. A) plasmid plus trypsin; B) plasmid only; C) trypsin only.

Dot blot assay

The activity of the over expressed TI protein was assayed by dot blot method with respect to control as shown in **Fig. 9**, suggesting that the expressed inhibitor is fully active in inhibiting the activity of trypsin.

Protease inhibitor assay

PI assays were performed in which the expressed inhibitor protein was found to be highly active against trypsin showing 92.97% inhibition. The inhibitor also showed activity against chymotrypsin but the degree of inhibition was only 15.85% (data not shown).

DISCUSSION

PIs in plants have been associated with stress tolerance, defence against herbivores and pathogens, antimicrobial activity and stabilization of recombinant proteins (Jongsma and Bolter 1997; Zavala *et al*. 2004; Quilis *et al*. 2007; Kim *et al*. 2009; Philippe *et al*. 2009; Goulet *et al*. 2010). The use of transgenic crop varieties and hybrids developed with endotoxin genes from *Bt* are nowadays fairly widespread, but development of resistance by insect populations has been a point of concern (Tabashnik 1994; Michaud 1997). The development of transgenic plants resistant to different kinds of pests and other biotic stresses, on one hand increases the productivity and on the other hand also minimizes the use of hazardous chemicals and pesticides, and thus causing neutralization of their negative effects without compromising upon their potential benefits (Srinivasan *et al*. 2009; Ter *et al*. 2010; Shan *et al*. 2010). Efforts are underway to develop transgenic plants containing combination of genes with biocontrol potential from different sources (Khadeeva *et al*. 2009; Ter *et al*. 2010). Among them, PI genes can serve as primary candidates that would augment their defense potential against prospective pests. The genes from plant origin have the advantage of being correctly transcribed, translated and processed in recombinant plants.

The possible role of PIs in plant protection was investigated as early as 1947 by Mickel and Standish (1947). PI genes, like the *Bt* genes, have practical advantages over genes encoding insecticidal metabolites with complex pathways. Transfer of a single gene from one plant species to another and expressing it from its own wound inducible or constitutive promoters could impart resistance against insect pests (Boulter 1993) PIs also exhibit a very broad spectrum of activity, including suppression of plant pathogenic fungi and nematodes (Williamson and Hussey 1996). The over expression of PIs, many of which have a higher content of cysteine and lysine residues can augment the nutritional quality of the recipient plant.

In an effort to isolate and develop alternative genes for pest control, and to make the PIs easily available and in sufficient quantities that could serve as potential therapeutic targets, cloning and characterization of *ti* gene from pea (*Pisum sativum*) into bacterial hosts was carried out. The expression profile of the recombinant cells revealed a highly intense band that was active against proteases and synthetic substrates and possessed considerably higher inhibitor activity against trypsin.

CONCLUSION

The expression profile of the recombinant cells points towards a considerably higher level of expression of the inhibitor that is active against proteases and synthetic substrates. These inhibitors thus can serve as important candidates to augment the defense potential against insect pests, and thus deserve to be included as important components of Integrated Pest management strategies.

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