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Constitutive expression of protease inhibitor gene isolated from black gram (*Vigna mungo* L.) confers resistance to *Spodoptera litura* in transgenic tobacco plants

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Plant insecticidal proteins like protease inhibitors have been reported to confer insect resistance in transgenic plants. We have cloned a protease inhibitor gene of black gram (*Vigna mungo* L.) and developed transgenic tobacco plants expressing the protease inhibitor constitutively under the 35S promoter by *Agrobacterium tumefaciens* mediated genetic transformation. A full-length open reading frame (ORF) of 545 nucleotides codes for a serine protease inhibitor protein with a deduced sequence of 180 amino acids contributing to a predicted molecular mass of 20.2 kDa. The *Vigna mungo* protease inhibitor (*VmPI*) is basically a Bowman-Birk type proteinase inhibitor (BBI) of double headed type; family of plant serine protease inhibitors that block trypsin or chymotrypsin. Comparison for conserved domain in the protein by National Centre for Biotechnology Information (NCBI) conserved domain (CD) search showed that the regions from 113-168 amino acid residues have the conserved domain of which 9 residues compose reactive loops on the conserved domain of BBI. The transgenic tobacco plants developed were confirmed by Southern, northern hybridization, polymerase chain reaction (PCR) and reverse transcriptase-PCR (RT-RCR) analysis. When transgenic T_1 tobacco plants were analyzed for insecticidal bioassays, the larval growth of *Spodoptera litura*, the tobacco cutworm was found severely retarded as compared to their growth on control plants. Therefore, black gram protease inhibitor could be used as an alternative individually or in combination with other insecticidal genes for the development of insect-resistant transgenic crop plants.

Keywords: Insect resistance, *Vigna mungo*, protease inhibitor, *Agrobacterium tumefaciens*, transgenic tobacco

Introduction

Over the past two decades, there have been significant advances in biotechnological approaches to improve resistance to insect pests. Major types of proteins shown to confer resistance to insect pests in several transgenic plants come from the soil borne bacteria *Bacillus thuringiensis* (*Bt*) endotoxins[1]. The *Bt* crops have been commercialized in many countries, but there is concern that constitutive expression of these insecticidal proteins may lead to selection of resistance among insect population. Hence, there is a need to identify alternative genes for deployment through transgenic crops to control insect pests. A group of plant derived proteins shown to exert lower selection pressure to insect pests in transgenic plants are the protease inhibitors (PIs). The use of PIs is regarded as a viable alternative alone or in combination with the *Bt*- endotoxins for developing resistance to insect-pests of the major crop plants.

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Protease inhibitors (PIs) are generally small proteins which are mainly abundant in storage tissues such as tubers and seeds, but are also found in the aerial parts[2-3]. Protease inhibitors are widely distributed throughout the plant kingdom and they play important roles in the defense against herbivores and pathogens[4-6]. They form complexes with proteases, thereby inhibiting proteolytic activity, and also serve as storage proteins in plant storage organs. A direct proof of the protective role of PIs against insect herbivores was provided by Hilder *et al*[7]. Since then a large number of transgenic plants have been developed using PIs. The protease inhibitors are divided into four classes, i.e. serine, cysteine, aspartic and metallo-protease inhibitors. Of these, the most abundant are serine PIs and are present in seeds, leaves and tubers of several members of the Fabaceae, Poaceae and Solanaceae[8]. Furthermore, it has been demonstrated that mechanical wounding, insect chewing and microbial infection enhanced the level of PIs significantly in local as well as remote tissues[9-11].

The protease inhibitors have been used to enhance resistance to insect pests in transgenic plants due to their small size, abundance, stability and high specificity for a particular class of digestive enzymes of insects[12]. Also, the genes of plant origin have added advantages, as they are correctly transcribed, translated and processed in the host plant after genetic transformation without any codon on biasness. From a biosafety stand point, the transgenic plants expressing plant PIs are shown to have no adverse effects and most of the inhibitors can be easily inactivated by cooking. Therefore, introduction of PI genes into host crops can be regarded as a safe strategy from the food safety stand point. In this study, to evaluate the prospects of protease inhibitor from *Vigna mungo* (*VmPI*) in conferring insect-resistance to genetically modified crops. The *VmPI* has been isolated and introduced into tobacco (*Nicotiana tabacum*) *via Agrobacterium* mediated genetic transformation. We report the response of transgenic tobacco plants on resistance to *Spodoptera litura and* development of larval growth.

Materials and Methods

Plant Material and Insects

Seeds of black gram (*Vigna mungo* L.) cv. Uttara were collected from Indian Institute of Pulse Research, Kanpur, India. Seeds were sown in plastic trays and seedlings were raised in dark condition, and 10 days old etiolated seedlings were used for genomic DNA isolation. The second instars larvae of *S. litura* were obtained from Division of Entomology, IARI, New Delhi, India.

VmPI Gene Isolation and Construction of Expression Vector

Genomic DNA was extracted from young etiolated seedlings of black gram by CTAB method[13] and used as a template for *VmPI* gene isolation by polymerase chain reaction (PCR) approach using mung bean (*Vigna radiata*) protease inhibitor (PI) gene specific primers since PIs are predicted to lack introns[14]. The primers were designed along the flanking regions of known trypsin-type mung bean PIs sequences (DQ417203) and the primers used were: Forward, 5'GCGGTACCTTCAAAACTGTCATGAATTCAG C 3´ and Reverse, 5' CTCTAGATTAGTCATCA-TCTTTATCCATGG3´. To facilitate directional cloning of PI gene *Kpn*I and *Xba*I sites were included in the forward and the reverse primers, respectively. PCR was performed using standard conditions and *Pfu* DNA polymerase (Fermentas, USA). The PCR product was cloned into pGEM-T Easy vector (Promega, USA) and at least three clones were sequenced. After ensuring sequence fidelity, it was compared with publicly available sequences by NCBI BLASTn search (http://www.ncbi.nlm.nih.gov) and analyzed by sequence alignment (BioEdit, USA).

Genetic Transformation of Tobacco

For genetic transformation of tobacco, seeds of tobacco (*N. tabacum* var Havana) were surface sterilized with 0.1% $HgCl_2$ for 2 - 3 min, washed with sterile water and inoculated on 1/2 Murashige and Skoog (MS) media for germination and *Agrobacterium*-mediated genetic transformation protocol was used as standardized earlier in our laboratory[15]. To overexpress *VmPI* in tobacco, the plasmid designated as pGEM-T-*VmPI* was digested with *Kpn*I and *Xba*I and sub-cloned into the corresponding sites of the binary vector pBinAR. The resultant recombinant vector pBinAR-*VmPI* was transformed into *Agrobacterium tumefaciens* strain GV3101 by a freeze thaw method. The plant transformation was done and the transformants were selected using 200 mg/l kanamycin. From the T_o transgenic lines, the T_1 transgenic were developed and analysed by molecular analysis.

Molecular Analysis of Transgenic Plants

Total genomic DNA was isolated from leaves of T_0 and T_1 transgenic and wild type tobacco plants[13]. The presence of transgene in transformed and wild type plants was confirmed by PCR using both neomycin phosphotransferase II (*nptII*) gene specific primers (Forward: 5´ GATGGATTG CACGCAGG 3´ and Reverse: 5´GAAGGCGAT AGAAGGCG 3´) and gene specific primers (Forward 5´ GCGGTACCTTC- AAAACT GTCATGAA TTCAGC 3´ and Reverse 5´ CTC TAGATTAGT CATCATCTTTATCCATGG 3´). Transgene expression analysis of PCR positive plants was done by semi-quantitative single step reverse transcriptase-polymerase chain reaction (RT-PCR) using both gene specific and action primers (Forward: 5´ AGACCTTCA ATGTGCCTGCCATG 3´ and Reverse 5´ GCTCAGCAG AGGTGGTGAACATG 3´). Further T_0 transgenic plants were subjected to Southern analysis and T_1 transgenics to northern analysis. A 20 µg aliquot of genomic DNA from *N. tabacum* was digested to completion with *Bam*HI and *Kpn*I (Fermentas) enzymes. The restricted fragments were subjected to electrophoresis on a 0.8% agarose gel. After neutral capillary blotting of electrophoresed DNA fragments on a Nylon N+ membrane, Southern hybridization

was carried out using standard molecular methods[16]. For RNA blot analysis, total RNA (20 µg) was separated by electrophoresis through a 1.2% formaldehyde agarose gel and then transferred to a Hybond N+ nylon membrane[17]. The hybridization was performed as described above. All the probes used in Southern and northern blot analyses were labeled using [α - 32p] dCTP by random primer labeling.

Proteinase Inhibitory Activity Assay

Inhibition of trypsin by leaf extracts from transgenic tobacco plants (T_1 lines 4, 5, 8 and 9) was assayed following the method of Bacon *et al*[16]. Fifty microgram of tobacco total soluble protein was incubated with 0.1 µg of trypsin for 10 min at 37ºC. Wild type tobacco plant was used as negative control in the bioassay.

Detached Leaf Bioassays of Transgenic Plants

The efficacy of transgenic plants against insect attacks was tested by detached leaf bioassays with second instars larvae of *S. litura.* Healthy leaves from two month old wild type and T_1 transgenic plants were challenged with early second instars of *S. litura.* The leaves were embedded in 3% agar-agar in 110 mm sterile Petri plates. This technique keeps the leaves in a turgid condition for about 1 week. The experiments were run in triplicates for each individual transgenic line and control plant with each leaf placed in 110 mm sterile Petri plates. Five early second instars larvae were released and initial weight of larvae was recorded. The survival of insects within the Petri plate was monitored at an interval of 24 hours and insects were weighed after 3 and 6 days of feeding. All larvae (both dead and surviving) were removed, their size was recorded and also the extent of leaf damage was measured by leaf area meter.

Results and Discussion

Analysis of *VmPI*

A full-length open reading frame (ORF) of *VmPI* of black gram was obtained using a PCR-based approach and cloned into T/A cloning vector pGEM-T Easy. Its sequence consisted of 545 bp (NCBI GenBank accession no. HQ629949) (Fig. 1a). The ORF encoded a predicted polypeptide of 180 amino acid residues (Fig. 1b) with a calculated molecular mass of 20.2 kDa and an isoelectric point of 8.7. After ensuring sequence fidelity, it was compared with publicly available sequences by NCBI BLASTn search (http://www.ncbi.nlm.nih.gov) and analyzed

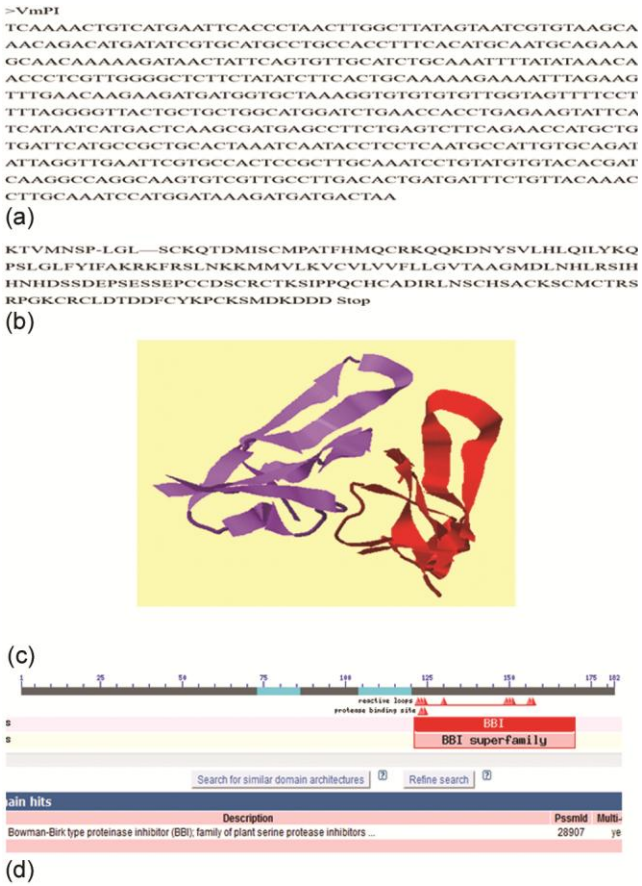

Fig. 1 — Nucleotide sequence (a), deduced amino acid sequence (b), 3D structure (double headed Bowman Birk type) (c), comparison of conserved protein domain (d), of black gram protease inhibitor.

by sequence alignment (BioEdit, USA). It showed 93% homology with *Cicer arietinum* trypsin inhibitor. The comparison with the other isolated protease inhibitors from different plants showed that the *VmPI* is basically a Bowman-Birk type proteinase inhibitor (BBI) of double headed (Fig. 1c) type of plant serine protease inhibitors that block trypsin or chymotrypsin proteases. A ccomparison for conserved domain in the protein by NCBI-CD search showed that the regions from 113-168 amino acid residues have the conserved domain of which 9 residues compose reactive loops on the conserved domain of BBI (Fig. 1d) similar to trypsin inhibitor Kunitz family of protease inhibitors. Southern blot analysis using the amplified *VmPI* gene fragment as a probe revealed hybridizing fragments of 3-4 different sizes, suggesting that several similar sequences are present in the black gram genome, indicating that *VmPI* belongs to an oligogene family in black gram.

Development of Transgenic Tobacco Over Expressing the *VmPI* Gene

In order to evaluate the functional significance of *VmPI* in transgenic tobacco plants, the plasmid designated as pGEM-T-*VmPI* was digested with *Kpn*I and *Xba*I to release 545 bp full length ORF and sub-cloned into the binary vector pBinAR under the transcriptional control of the constitutive cauliflower mosaic virus (CaMV) 35S promoter. The binary vector was mobilized into *Agrobacterium* and was used in transformation of tobacco employing the leaf disc co-cultivation method[16]. A total of 10 putative T_o transgenic plants were identified through genomic DNA based PCR and RT-PCR screening (Fig. 2A) of which five independent transgenic plants were confirmed through different molecular approaches. The levels of *VmPI* transcript in T_1 transgenic plants were also analyzed by RT – PCR. The expression of a single *VmPI* transcript was observed in all the T_o transgenic plants with varied expression levels. Transgenic plants 3 and 6 represented a low expression level, while moderate expression was found in plants 2 and 7 and relatively higher expression was found in plants 1, 4, 5, 8, 9 and 10. The T_o and T_1 seeds were raised via self-pollination and T_1 seeds were used in functional characterization. The homozygous lines were identified on the selection medium containing 100 mg/l kanamycin in the T_1 generation. The T_1 transgenics were also analyzed by PCR and RT-PCR (Fig. 2B). The *VmPI* over expressing T_o plants were found to contained one to several copies of the transgene (s) based on Southern blot analysis (Fig. 3A) and single copy transgenic plants were identified for further analysis. The expression of *VmPI* in the selected T_1 lines was also analyzed by northern blotting technique (Fig. 3B).

Estimation of Trypsin Protease Inhibitor Activity

The functional role of trypsin PIs is to inhibit the trypsin proteases that normally degrade proteins. The trypsin inhibitor activity was higher in transgenic compare to wild type plants. There was significant difference in trypsin protease inhibitor activity between transgenic (line 5) and the wild type plants (Fig. 3C).

Enhanced Insect Resistance Exhibited by Transgenic Plants

The insect resistance of T_1 plants expressing *VmPI* protein was tested using detached leaf bioassays with early second instar larvae of *S. litura*. Prominent differences in the amount of leaf area consumed by the larvae were noticed between the wild type and transgenic plants. The *S. litura* consumed the whole leaf in 6 days (Fig. 4A). The measurements (leaf area damaged and insect weight gained) were taken at the end of the bioassay. High expression plants efficiently controlled second instar larvae of *S. litura*, while some leaf damage was observed in the low expression transgenic plants (Fig. 4B). The larval growth was

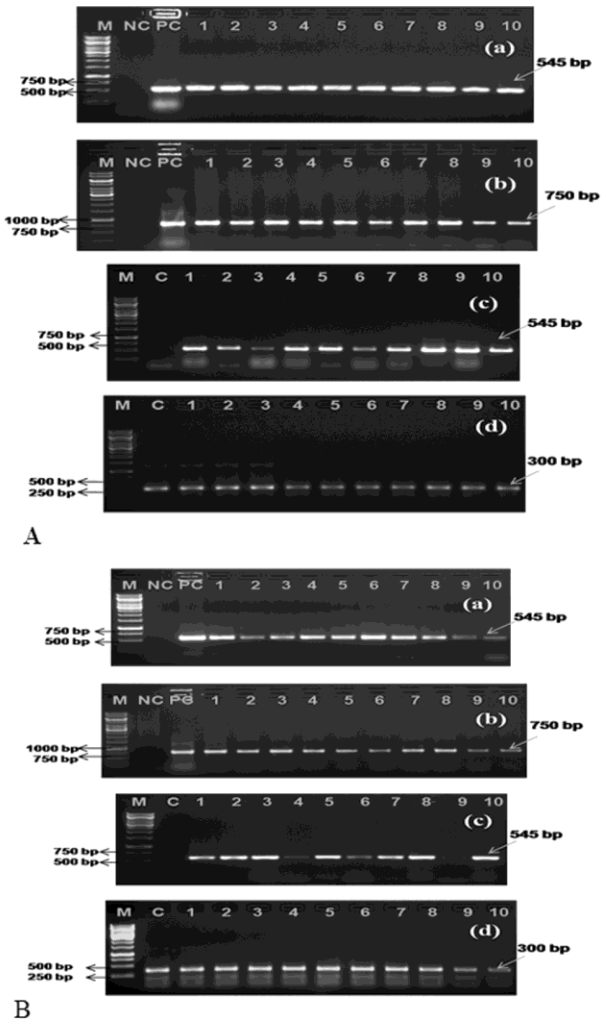

Fig. 2 — PCR and RT-PCR confirmation of T_o and T_1 transgenic tobacco plants; A. PCR confirmation of T_o transgenic plants, by gene specific primers (a), by *nptII* primers (b). M: 1 kb ladder, NC: negative control, PC: positive control, 1 to 10: transgenic plants; RT-PCR confirmation by gene specific primers (c), by actin primers (d).M: 1 kb ladder, c: control, 1 to 10: transgenic plants; B. PCR confirmation of T_1 transgenic plants, by gene specific primers (a), by *nptII* primers (b). M: 1 kb ladder, NC: negative control, PC: positive control, 1 to 10: transgenic plants RT-PCR confirmation by gene specific primers (c), by actin primers (d). M: 1 kb ladder, c: control, 1 to 10: transgenic plant M: λ *Hind*III marker, UR: unrestricted DNA, NC: negative control, 1 to 6 transgenic tobacco plants

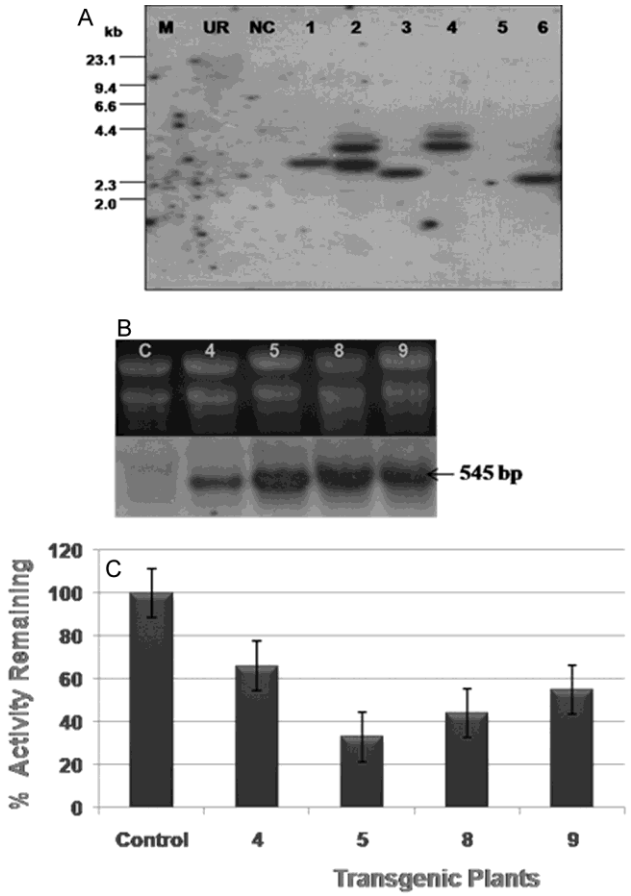

Fig. 3 — Molecular analysis of transgenic plants
A. Southern analysis of T_o transgenic tobacco plants. M: λ *Hind*III marker, UR: unrestricted DNA, NC: negative control, 1 to 6 transgenic tobacco plants
B. Northern analysis of T_1 transgenic tobacco plants. C: control plant, 4, 5, 8 & 9: transgenic tobacco plants.
C. Assay of protease inhibitor activities of T_1 transgenic tobacco plants. C: control plant, 4, 5, 8 & 9 transgenic tobacco plants.

also arrested, and the weight of larvae grown on plants 5 and 8 was less than half that of the larvae feeding on wild type plants (Fig. 4C). Survival of larvae was significantly low in the high expression plants. High expression plants exhibited an elevated level of tolerance, and the larvae were stunted and did not appear healthy (Fig. 4D) but the low expression plants behaved more like the wild type.

In this study, we report the cloning and characterization of a full-length ORF of a trypsin PI of black gram, which had close similarity to the PI of *Vigna radiata* (DQ417203). The molecular analysis showed that it belonged to an oligogene family. The common observation that the PIs are wound inducible[18-19] led to the studies focusing on their role in herbivore attack[20-21]. In the present study, the *VmPI* transgenics exhibited enhanced resistance towards second instar larvae of *S. litura*, thus justifying its basic function in herbivore resistance[21-22]. The up-regulation of PIs in different abiotic stress treatments was also noticed earlier[23-24], their possible involvement in inducing tolerance to abiotic stresses on over expression was also studied and drought tolerance was demonstrated in rice by the constitutive over expression of a rice protease inhibitor[25]. Lepidopteran insect-pests such as *H. armigera* and *S. litura* largely depend on serine proteases for digestion of food proteins. Therefore, the *VmPI* transgenics, which constitutively express the serine type trypsin PI, expected to be resistant to these pests, and the transgenic plants showed resistance to the second instars larvae of *S. litura*. Under the elevated stress conditions in plants, there is degradation of proteins by proteases which might be repressed in transgenics due to constitutive expression of *VmPI.* The elementary function of a PI is to inhibit the function of proteases leading to stability of the respective proteins, which would otherwise be degraded by the proteases under biotic stress. The transgenics had more trypsin PI activity than wild type plants.

The protease inhibitors have the potential to enhance the current *Bt* toxin technology because they target a broader range of pests, including nematodes and fungi[26]. There is a major concern that the effectiveness of *Bt* will be negated if field evolved *Bt* resistance becomes a more widespread problem[27]. A proposed management strategy for delaying insects´ development of resistance to plant protection transgenes, such as *Bt* toxins, is to deploy multiple insect control genes (such as PIs) with different modes of action in a single plant[28-29]. There is evidence that the combination of PIs with a sub-lethal dose *Bt* toxin has a strong negative effect on the growth and development of insects[30]. Several transgenic plants that express PIs have been produced and tested in order to increase the resistance against pathogenic organisms. In addition, SPIs and other PIs from different families have been used to minimize the proteolysis of recombinant proteins expressed in plants[31]. Several such successful examples have been mentioned in the literature and many of them have been implemented in the biotech agriculture fields[32-33]. Novel genes which are otherwise not possible to transfer by the classical plant breeding methods, can now be transferred to various crop

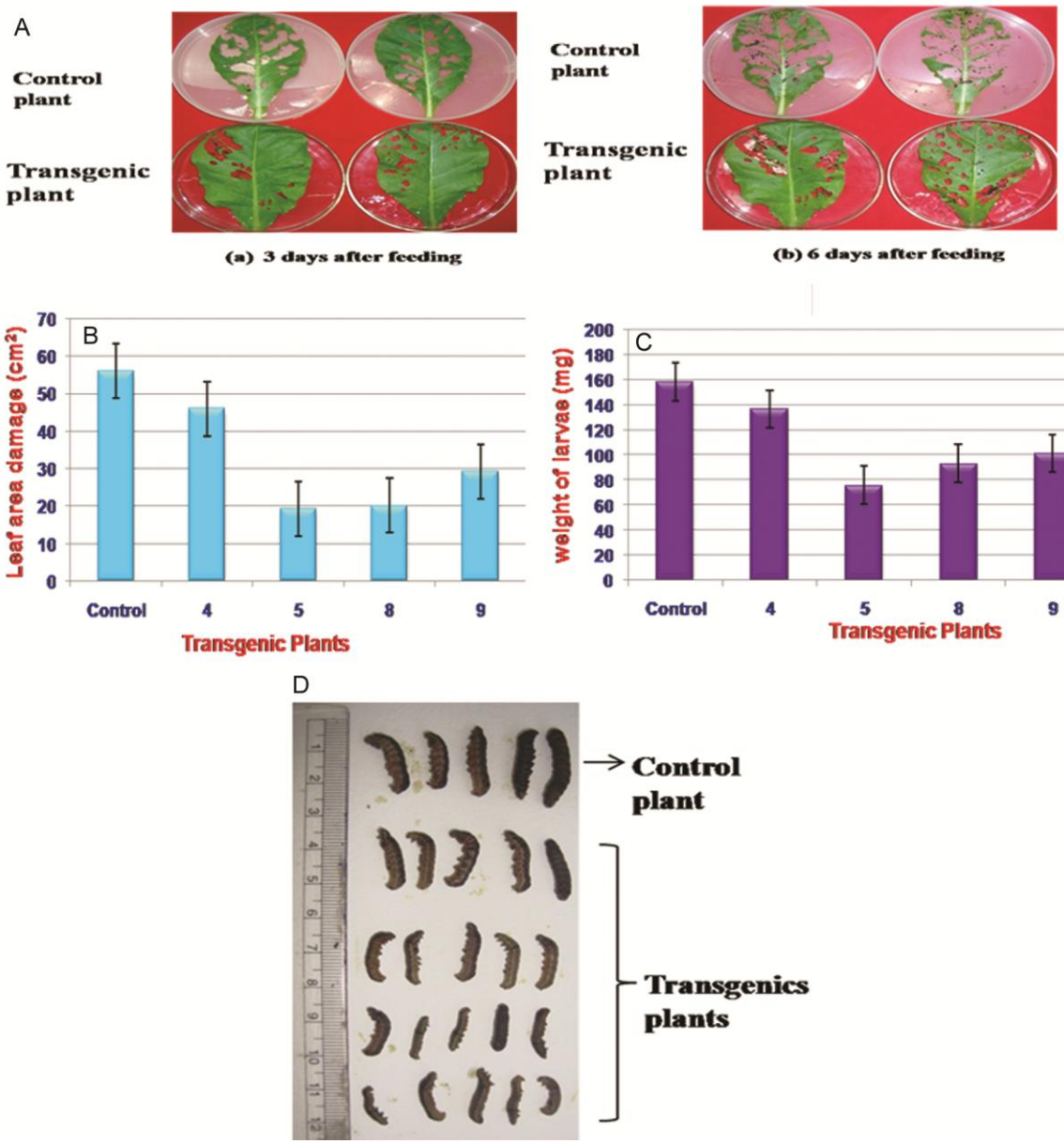

Fig. 4 — Insecticidal bioassay; A. Detached leaf insect bioassays using second instar larvae of *Spodoptera litura*; B. The leaf area damaged after 6 days of feeding. C: control plant, 4, 5, 8 & 9: transgenic tobacco plants; C. Weight of *S. litura* larvae after 6 days of feeding. C: control plant, 4, 5, 8 & 9: transgenic tobacco plants; D. *S. litura* larvae after 6 days of feeding on control and transgenic tobacco plants.

plants and the development of such crop plants will be useful for sustainable agriculture system[34-35]. We suggest that the *VmPI* is a suitable candidate gene either single or in combination with *Bt* gene for deployment of transgenic crop plants for durable resistance to biotic stresses for enhancing the crop productivity. The main protease inhibitor have been studied from the main families of leguminaceae and gramineae, however studies with the indigenous material is very limited. Having enormous potential in biotechnology application in strengthening the insect resistance in crops, the scope for exploration of the indigenous crops for protease inhibitor remains wide open. In addition, genomic and proteomic approaches will allow us to advance in the knowledge of the specific role of the different protease inhibitors which will result in the development of new strategies to improve the practical approaches of genetic engineering in crops improvement.

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