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Cloning of Trypsin Inhibitor Gene from Cowpea and Yard Long Bean and Transformation of Tobacco

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ABSTRACT. *Trypsin inhibitor, a serine protease inhibitor is known to play an important role in the defence mechanism on Lepidopteran insects. Trypsin inhibitor genes from cowpea (Vigna unguiculata L.) and yard long bean (Vigna sesquipedalis L.) were cloned using gene specific primers. They were subsequently sub-cloned into a plant transformation vector pHS100 containing CaMV35S promoter. Agrobacterium tumefaciens (LBA4404 strain) with recombinant pHS100 vector carrying trypsin inhibitor gene from cowpea was used to transform tobacco. Presence of the transgene in kanamycin resistant plants was confirmed by Polymerase Chain Reaction (PCR).*

INTRODUCTION

Protease inhibitors are small proteins which represent a class of well studied compounds of plant defence and are abundant in storage organs such as seeds and reserve organs. Expression of protease inhibitor genes has been detected in leaves of several plant species following wounding suggesting their role in protecting plants from insect attack and microbial infection. Protease inhibitors suppress the enzymatic digestion of proteins in the insect midgut and it also creates destructive hyper-production of trypsin coupled with insufficient dietary availability of sulphur containing amino acids needed for enzyme synthesis (Broadway and Duffey, 1986). As a result of which, the insects become weak with stunted growth and ultimately die. Protease inhibitors as insect control proteins have proved to enhance resistance in transgenic plants (Hilder *et al.*, 1987). Inhibitor genes of plant origin are particularly promising as they are not likely to have problems in expression, when inserted into other plant genomes. Considerable progress has been made in developing transgenic plants with toxin genes from *Bacillus thuringiensis* (*Bt*) in different crops (Sharma *et al*., 2000; Hilder and Boulter, 1999). However, there are distinct possibilities of development of resistance to *Bt* (Tabashnick, 1994). Resistance development against the foreign insecticidal protein should be minimal in any of the sustainable transgenic development. One of the strategies would be the use of alternative genes either singly or in combination.

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Cowpea trypsin Inhibitor (*CpTI*) is a serine class protease inhibitor which belongs to a well known family; Bowman-Birk trypsin inhibitor. It has been found effective against the serine proteases present in gut of many insects such as *Spodoptera litura*, *Heliothis virescens* and *Manduca sexta* belonging to Lepidoptera (Sane *et al*., 1997). Constitutive expression of *CpTI* in cauliflower (Lingling *et al*., 2005), rice (Xu *et al*., 1996), potato (Gatehouse *et al*., 1997), strawberry (Graham *et al*., 1997), cotton (Li *et al*., 1998), pigeon pea (Lawrence and Koundal, 2001) and wheat (Alpteter *et al*., 1999) has been found effective against their major insect pests.

With this background, a study was conducted to clone trypsin inhibitor genes from cowpea and yard long bean and to transfer cowpea trypsin inhibitor gene into the model system tobacco to check its expression in the plant system.

MATERIALS AND METHODS

Plant material and PCR amplification

Total DNA was isolated from cowpea variety Banavasi local and a land race of yard long bean following Cetyl Trimethyl Ammonium Bromide (CTAB) protocol (Doyle and Doyle, 1987). Complete coding sequence of trypsin inhibitor gene from cowpea and yard long bean were amplified by PCR amplification using a set of specific primers. The primers were designed online at http://www.Changbioscience.com based on the sequence information available at GenBank. Restriction sites were added to facilitate directional cloning into the plant transformation vector, pHS100 (Kamble *et al*., 2003). The primers used were; forward 5'GCTCTAGAT GATGGTGCTAAAGGTG 3'and reverse 5'GCGGATCCTTACTCATCATCTTCATCCC 3' (restriction sites are underlined). PCR conditions were standardized to amplify a single sharp amplicon.

PCR amplification

Amplification reaction was done in a thermocycler in a final reaction mixture of 20 µL containing 20 ng of DNA template, 1 µL from each primer (5p moles), 1.5 U of Taq DNA polymerase, 1x assay buffer, 125 µM of dNTP and 1.25 mM of $MgCl_2$. Polymerase Chain Reaction was performed with the following amplification programme; initial denaturation at 940^{o}C for 5 min, followed by one minute each of 94^{o}C, specific primer annealing temperature of 55^{o}C and extension at 72^{o}C for 39 cycles.

Gel elution of the PCR products and their cloning

The amplicons (~325 bp) were eluted from the gel and cleaned using gel extraction kit (QIAGEN) as described in user's manual of the kit. The purified PCR fragments were ligated to pTZ57R/T cloning vector (2886 bp) separately as described in InsT/A clone^{TM} PCR product cloning kit (K1214) from MBI, Fermentas, USA. The recombinant clones were identified by insertional inactivation of lacZ' expression (blue/white assay). The positive clones were named as pTZ-CpC and pTZ-YlbC.

Confirmation of clones

The confirmation of the presence of cloned fragments was done by PCR amplification of clones with respective primers. The total DNA of cowpea and cloning vector were used as positive and negative controls respectively in the PCR. The confirmation was also done through comparative restriction analysis of selected clones and the control vector to ensure the presence of inserts using *Xba*I and *Bam*HI enzymes.

Sequencing of clones

The complete coding sequence of the trypsin inhibitor genes of cowpea and of yard long bean cloned in pTZ57R/T was sequenced using M13 primers at Bangalore Genei Pvt Ltd., Bangalore. The sequences were subjected to homology analysis using Basic Local Alignment Search Tool (BLAST) algorithm available at http://www.ncbi.nlm.nih.gov.

Sub-cloning into plant transformation vector

The DNA of the plant transformation vector pHS100 (Kamble *et al*., 2003) and pTZ-CpC1 and pTZ-YlbC1 recombinant clones were isolated using alkaline lysis protocol. The vector pHS100 and plasmids of pTZ-CpC1 and pTZ-YlbC1 were sequentially digested with *Xba*I and *Bam*HI enzymes to facilitate directional cloning. Then the trypsin inhibitor inserts were ligated to linearized pHS100 vector and *E. coli* (DH5α strain) cells were transformed to get recombinant clones. The clones were confirmed through PCR and restriction digestion analysis. The positive clones were named as pHS-CpC and pHS-YlbC.

Agrobacterium transformation

The plant transformation vector containing trypsin inhibitor genes from cowpea and yard long bean *i.e.* pHS-CpC1 and pHS-YlbC1 were mobilized to *Agrobacterium tumefaciens* strain LBA4404 by triparental mating using pRK2013 as helper plasmid. The trans-conjugants were propagated on YEMA (Yeast Extract Mannitol Agar) selective medium. Plasmids were isolated from recombinant clones and confirmed through PCR amplification.

Tobacco transformation

For expression studies in plant system, the model system tobacco (variety white burley) was transformed with the *Agrobacterium* clone having construct from pHS-CpC1 by using protocol mentioned by Hooykaas and Schilperoort (1992) with some modifications. *Agrobacterium tumefaciens* LBA4404 with recombinant vector, pHS-CpCl was grown in YEMA with streptomycin (100 μg/mL), rifampicin (25 μg/mL) and kanamycin (50 μg/mL) as selection at 28^{o}C for 24 hrs. Young leaves of tobacco (5 - 6 week old plants) were surface sterilized and were cut into 1 cm² discs. The leaf disks were infected in *Agrobacterium* culture treated with acetosyringone at 200 μM and cultures were left for 20 min for agro-infection. The explants were dried and placed on solid MS (Murashige and Skoog, 1962) medium without any hormone and kept in dark for 2 days for co-cultivation. There after leaf disks were transferred to solid MS medium containing benzyladenine (1 mg/L) and Naphthalene acetic acid (0.1 mg/L) for callus induction and cephotaxime (200 mg/L) to eliminate further growth of *Agrobacterium*. Kanamycin (100 μg/mL) was added to medium

for selection. The cultures were incubated at 25^{o}C and 16 hrs photoperiod for 3 - 4 weeks. The shoots and excised calli were sub-cultured and incubated for another 4 weeks at same culture conditions. Young shoots were rooted in hormone free MS medium with 200 µg/mL cefotaxime and 200 µg/mL kanamycin as selection. DNA was extracted using rapid method from kanamycin resistant plants and checked through PCR with gene specific primers.

RESULTS AND DISCUSSION

Complete coding sequences (CDS) of trypsin inhibitor gene from cowpea and yard long bean were amplified with gene specific primer and cloned into pTZ57R/T. The positive clones were further confirmed through PCR (Plate 1) and restriction digestion analysis (Plate 2).

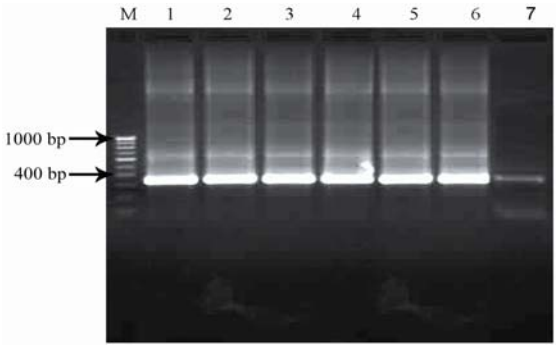

Plate 1. PCR confirmation of trypsin inhibitor insert in pTZ-Cp and pTZ-Ylb clones.
Note: M- 100bp DNA ladder, 1- pTZ-CpC1, 2-pTZ-CpC2, 3- pTZ-CpC3, 4-pTZ-CpC4, 5-pTZ-YlbC1, 6- pTZ-YlbC2, 7- Positive control (Cowpea genomic DNA).

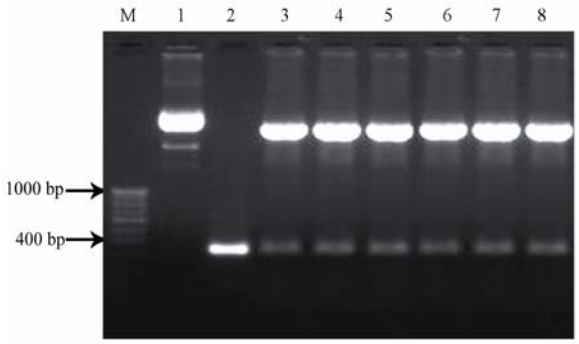

Plate 2. Restriction analysis of pTZ-Cp and pTZ-Ylb clones with *Xba*1 and *Bam*H1.
Note: M100bp DNA ladder, 1- vector: pTZ57R/T, 2- PCR product, 3- pTZ-CpC1, 4-pTZ-CpC2, 5- pTZ-CpC3, 6-pTZ CpC4, 7- pTZ-YlbC1, 8- pTZ-YlbC2.

BLAST search indicated that the cloned CDS of trypsin inhibitor genes from cowpea and yard long bean had 99% homology with published sequences of trypsin inhibitor of cowpea having 324 bp as coding sequence and as well as with *Phaseolus vulgaris* trypsin proteinase inhibitor. There were only 2 bp difference *i.e.* at 151st and 161st positions between trypsin inhibitor genes of cowpea and yard long bean. Since most of the

trypsin inhibitor from cowpea and other legumes such as *Phaselolus* and mung bean belong to the same Bowman Birk familiy, extensive homology is expected among its members (Laskowski and Kato, 1980). The open reading frame (ORF) was 324 bp long in both the trypsin inhibitor genes and *in silico* translation gave a polypeptide of 107 amino acids. Translated amino acid sequences of trypsin inhibitor from two different sources showed only one amino acid difference at 54th position of the peptide. Amino acid sequence of trypsin inhibitor from cowpea was subjected to tBLASTn and 100% homology was observed with *Phaseolus vulgaris* trypsin proteinase inhibitor (AY059390), *Vigna unguiculata* subsp. sesquipedalis trypsin inhibitor (AY204566) and *V. unguiculata* RNA for trypsin inhibitor fIV (VUTRYPIFV). Amino acid sequence of trypsin inhibitor from yard long bean showed 98% homology with *Phaseolus vulgaris* trypsin proteinase inhibitor (AY059390), *V. unguiculata* subsp. sesquipedalis trypsin inhibitor (AY204566) and *V. unguiculata* RNA for trypsin inhibitor fIV (VUTRYPIFV). Conserved domain search results revealed that the length of CDS is 55 residues and is homologous to the Bowman Birk type proteinase inhibitor (BBI family). Trypsin inhibitor gene from cowpea has been deposited at GenBank of NCBI (Accession number: EF541135).

Sub-cloning into plant transformation vector

Directional cloning of trypsin inhibitor genes of cowpea and yard long bean into pHS100, was achieved by digesting recombinant pTZ57R/T plasmids with *Xba*I & *Bam*HI. The inserts were purified and ligated with X*ba*I and *Bam*HI cut pHS100 vector, under CaMV35S constitutive promoter and transformed to E. *coli* (DH5α strain). Recombinant clones were confirmed through PCR (Plate 3) and restriction digestion analysis (Plate 4). The confirmed clones; pHS-CpC1 (Figure 1) (having insert from cowpea) pHS-YlbC3 (insert from yard long bean) were mobilized into *Agrobacterium tumefaciens* LBA4404 via tri-parental mating using *E. coli* and pRK2013 as helper strain. The trans-conjugants were picked on YEMA containing kanamycin (50 µg/mL), stereptomycin (100 µg/mL) and rifampicin (25 µg/mL). The recombinant *A. tumefaciens* (pHS-CpC1) and *A. tumefaciens* (pHS-YlbC3) were confirmed through PCR amplification of the plasmids obtained from *Agrobacterium.*

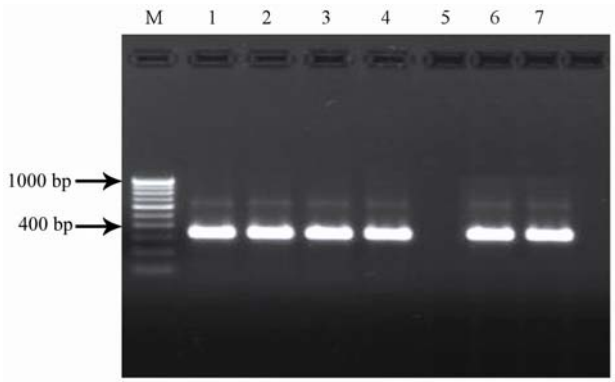

Plate 3. PCR confirmation of trypsin inhibitor insert in pHS-Cp and pHS-Ylb clones.

Note: M- 100bp DNA ladder, 1 – pHS-CpC1, 2- pHS-CpC2, 3- pHS-CpC3, 4-pHS-CpC4, 5-blank, 6- pHS-YlbC1, 7- pHS-YlbC2.

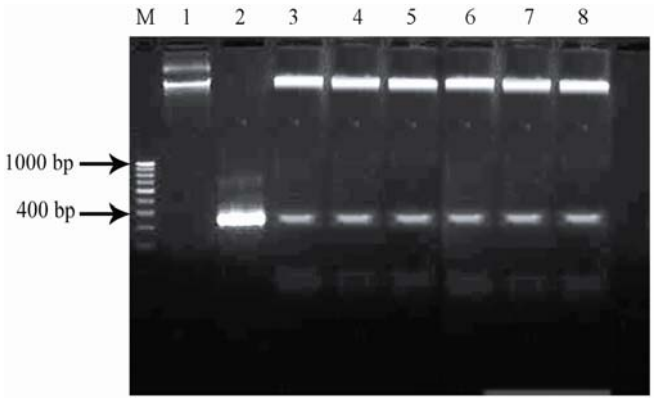

Plate 4. Restriction analysis of pHS-Cp and pHS-Ylb clones with *Xba*1 and *Bam*H1.

Note: M- 100bp DNA ladder, 1- vector: pHS100, 2-PCR product, 3-pHS-CpC1, 4-pHS-CpC2, 5-pHS-CpC3, 6-pHS-CpC4, 7-pHS-YlbC1, 8-pHS-YlbC2.

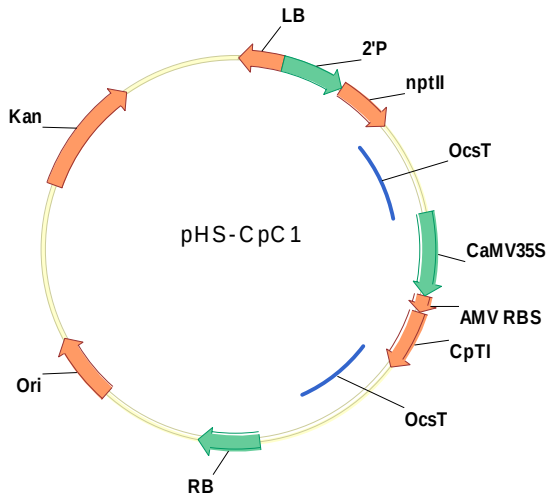

Figure 1. pHS100 plant transformation vector containing cowpea trypsin inhibitor gene.

Note: LB: Left border, 2'P :Manopine synthase 2' promoter, *npt*II : Coding region of Neomycin Phosphotransferase II (Kanamycin resistance), OcsT: Octopine synthase terminator, CaMv35S: Cauliflower mosaic virus 35S promoter, AMV RBS: Alfalfa mosaic virus ribosome binding site, *CpTI*: Cowpea trypsin inhibitor gene, RB: Right border, Ori: Replication origin, Kan: Prokaryotic kanamycin resistant gene expression cassette.

Tobacco transformation

Tobacco variety white burley was transformed with the method described by Hooykaas and Schilperoort (1992). The green plants surviving in the rooting medium with 200 mg/L kanamycin were transferred to sterilized peat and acclimatized in the green house. DNA was extracted from nine plants and checked through PCR with gene specific primers. Eight from the nine kanamycin resistant plants checked were PCR positive and only one was

false positive (Plate 5). False positive plants could originate because of the leaky expression of plant selection marker genes such as *npt*II and *hpt* (eg. under CaMV35S or *nos* promoters) in *Agrobacterium* leading to bacterial overgrowth during selection (Veluthambi *et al.*, 2003).

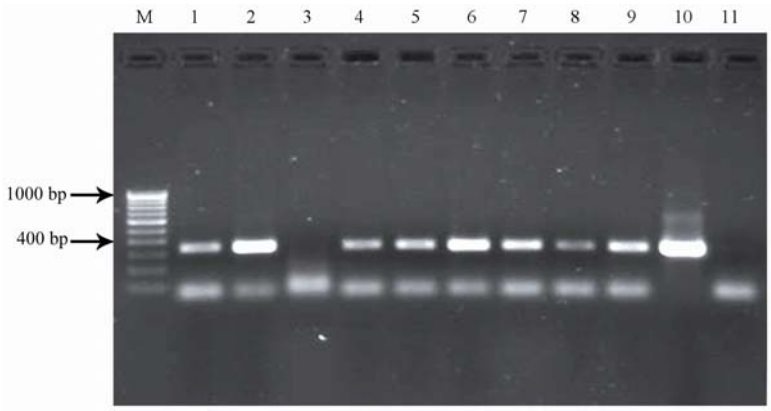

Plate 5. PCR confirmation for the presence of *cowpea trypsin inhibitor* insert in tobacco.

Note: M-100bp DNA ladder, 1-2-positive plants, 3- PCR negative plant 4-9- PCR positive plants, 10-positive control (pHS-CpC1), 11- negative control (untransformed tobacco).

CONCLUSIONS

Trypsin inhibitor genes can be employed in developing transgenic crop plants either singly or in combination with other insecticidal genes. In this study, trypsin inhibitor genes from cowpea and yard long bean were cloned to pTZ57R/T vector and sequenced. The sequence analysis showed 99% homology with reported trypsin inhibitor genes at nucleotide level, 100% homology (in case of cowpea trypsin inhibitor gene) and 98% homology (with trypsin inhibitor of yard long bean) at protein level. To facilitate the transformation of crop plants, the genes were sub-cloned into a plant transformation vector and tobacco was transformed. Presence of the gene was confirmed by *nptII* and gene specific PCRs. Expression studies on the transgene in tobacco is being carried out.

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REFERENCES

Alpteter, F., Diaz, I., Mcauslane, H., Gaddour, K., Carbonero, P. and Vasil, I.K. (1999). Increased insect resistance in transgenic wheat stably expressing trypsin inhibitor CMe. Mol. Breed. 5: 53 - 63.

Broadway, R.M. and Duffey, S.S. (1986). Plant proteinase inhibitors: mechanism of action and effect on the growth and digestive physiology of larval *Heliothis zea* and *Spodoptera exigua*. J. of Insect Physiol. 32: 827 - 833.

Doyle, J.J. and Doyle, J.L. (1987). A rapid DNA isolation procedure from small quantities of fresh leaf tissues. Phytochem Bull. 19: 11 - 15.

Gatehouse, A.M.R., Davidson, G.M., Newell, C.A., Merryweather, A., Hamilton, W.D.O., Burgess, E.P.J., Gilbert, R.J.C. and Gatehouse, J.A. (1997). Transgenic potato plants with enhanced resistance to the tomato moth, *Lacanobia oleracea*: growth room trials. Mol. Breed. 3: 49 - 63.

Graham, J., Gordon, S.C. and Mcnicol, R.J. (1997). The effect of the CpTI gene in strawberry against attack by vine weevil (*Otiorhynchus sulcatus* F. Coleoptera: Curculionidae). Ann. of App. Biol. 131: 133 - 139.

Hilder, V. A., Gatehouse, A. M. R., Sheerman, S. E., Barker, R. F. and Boulter, D. (1987). A novel mechanism of insect resistance engineered into tobacco. Nature, 330: 160 - 163.

Hilder, V.A. and Boulter, D. (1999). Genetic engineering of crop plants for insect resistance; a critical review. Crop Protection, 18: 177 - 191.

Hooykaas, P.J.J. and Schilperoort, R.A. (1992). *Agrobacterium* and plant genetic engineering. Plant Mol. Biol. 19: 15 - 3 8.
http://www.ncbi.nlm.nih.gov

Kamble, S., Misra, H. S., Mahajan, S. K. and Eapen, S. (2003). A Protocol for Efficient Biolistic Transformation of Mothbean *Vigna aconitifolia* L. Jacq. Marechal. Plant Molecular. Biology Reporter. 21: 457 - 457.

Laskowski, M.J.R. and Kato, I. (1980). Protein inhibitors of proteinases. Annu. Rev Biochem. 49: 685 - 693.

Lawrence, P. K. and Koundal, K. R. (2001). *Agrobacterium tumefaciens* mediated transformation of pigeon pea (*Cajanus cajan* (L.) Millsp) and molecular analysis of regenerated plants. Current Science. 80(11): 1428 - 1432.

Li, Y.E., Zhu, Z., Chen, Z.X., Wu, X., Wang, W. and Li, S.J. (1998). Obtaining transgenic cotton plants with cowpea trypsin inhibitor. Acta Gossypii Sinica. 10: 237 - 243.

Lingling, L., Jianjun, L., Song-ming, L. L. and Bihao, C. (2005). Study on transformation of cowpea trypsin inhibitor gene into cauliflower *(Brassica oleracea L var botrytis).* Afr. J. of Biotech. 4 (1): 45 - 49.

Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarium, 15: 473 - 497.

Sane, V.A., Nath, P., Aminuddin and Sane, P.V. (1997). Development of insect- resistant transgenic plants using plant genes: Expression of cowpea trypsin inhibitor in transgenic tobacco plants. Current Sci. 72 (10): 741 - 747.

Sharma, H. C., Sharma, K.K., Seetartam, N. and Oritz, R. (2000). Prospects for transgenic resistance to insects. Electronic J. of Biotech. vol 3/issue 1/html.

Tabashnik, B.E. (1994). Evolution of resistance to *Bacillus thuringiensis*. Annu. Rev Entomol. 39: 47 - 79.

Veluthambi, K., Gupta, A. K. and Sharma, A. (2003). The current status of plant transformation technologies. Current Sci. 84:368 - 380.

Xu, D.P., Xue, Q.Z., Mcelroy, D., Mawal, Y., Hilder, V.A. and Wu, R. (1996). Constitutive expression of a cowpea trypsin inhibitor gene, CpTi, in transgenic rice plants confers resistance to two major rice insect pests. Mol. Breed. 2: 167 - 173.