Annals of Sri Lanka Department of Agriculture. 2006.8:219-227.

CLONING OF TRYPSIN INHIBITOR GENE FROM SOYBEAN AND TRANSFORMATION INTO TOBACCO

D.A. SHIRANI[1], S. BHAT, R. BHAT[2], M.S. KURUVINASHETTI[2], P.U. KRISHNARAJ[2], H.M. VAMADEVAIAH[2] and K. KRISHNAMURTHY[2]
[1]*Agricultural Research Station, Sita Eliya, Nuwara Eliya*
[2]*University of Agricultural Science, Dharwad, India*

ABSTRACT

Plant serine protease inhibitors (SPIs) have been found to be effective in developing transgenic crop plants which confer resistance to insects particularly to Lepidoptera. The genes encoding these proteins have less problems, related to codon bias, RNA processing and protein stability. Therefore, a study was initiated to isolate and clone a serine protease inhibitor gene from soybean (*Glycine max*). Using specific primers, gene encoding kunitz trypsin inhibitor of soybean (654bp) was amplified and cloned into pTZ57R/T vector. The clones were confirmed through PCR amplification and restriction digestion analysis. Further, the clones were sequenced and analyzed *in sillico*. Cloned trypsin inhibitor gene of soybean showed 99 and 100 per cent homology with reported kunitz inhibitor genes of soybean at nucleotide and protein level respectively. Further, to facilitate transformation of crop plants, soybean kunitz trypsin inhibitor gene was sub cloned into a plant transformation vector pHS100 at *Bam*H1 and *Xba*1 restriction sites. The recombinant clone pHS-SBC1 was mobilized into *Agrobacterium tumefaciens* LBA4404 by triparental mating. The *A. tumefaciens* with recombinant clone pHS-SBC1 was used to transform tobacco and the presence of inserts in kanamycin resistant plants was confirmed through PCR with specific primers.

KEYWORDS: Cloning, Soybean, Trypsin inhibitor gene, Plant transformation.

INTRODUCTION

Protease Inhibitors (PIs), which are ubiquitous in nature are one of the prime candidates with highly proven inhibitory activity against insect pests and able to suppress enzymatic activity of phytopathogenic micro organisms and nematodes. The expression of these genes is induced upon mechanical wounding and insect/pathogen attack. Plant protease inhibitors are an ideal choice for heterologous expression as they have no or less problems related to the expression in the transgenic plant. Moreover, the protease inhibitors have practical advantages over other inducible chemicals which are products of complex biochemical pathways. By transferring a single defensive gene from one plant to another either with their own promoter or with constitutive promoters, genetically modified plants can be obtained (Ryan, 1990). Serine class of protease inhibitors is effective against the serine protease in insect gut from many families, particularly Lepidoptera. A large number of plant serine protease inhibitor genes have been cloned and expressed in transgenic plants which confer resistance to lepidopterans and other families of insects (Marchetti *et al.*, 2000; Balestrazzi *et al.*, 2004; Williams *et al.*, 1997; Leo *et al.*, 1998; Johnson *et al.*, 1989; Qu *et al.*, 2003;

Sane *et al.*, 1997; Xu *et al.*, 1996; Gatehouse *et al.*, 1997; Li *et al.*, 1998; Alpteter *et al.*, 1999; Lee *et al.*, 1999 and Cipriani *et al.*, 1998).

The mode of action of these inhibitors on the digestive enzymes of the pest is not always a simple inhibition of proteolytic activity. Broadway and Duffey (1986) have shown that the mode of action of PIs is to cause destructive hyper-production of trypsin coupled with insufficient dietary availability of sulphur-containing amino acids (i.e. methionine) needed for enzyme synthesis. As a result of which, the insects become weak with stunted growth and ultimately die.

Soybean kunitz trypsin inhibitor (*SKTI*) which belongs to serine class of protease inhibitor with molecular mass of 18-22KDa inhibits both trypsin and chymotrypsin. It is a single polypeptide chain of 181 amino acids including two disulphide bonds (Kunitz, 1946). Using this gene, few transgenic crop plants have been developed.

Transgenic tobacco plants expressing high levels of *SKTI* have shown greater resistance than cowpea trypsin inhibitor gene (*CpTI*) which has been widely used in developing transgenics against *H. virescens* (Gatehouse *et al.*, 1993). Transgenic rice developed with *SKTI* was found to be resistant against brown plant hopper (*Nilaparvata lugenes*) (Lee *et al.*, 1999).

Though transgenic crops developed with δ-endotoxin genes from *Bacillus thuringiensis* are fairly widespread, several reports have shown the possibility of developing resistance against Bt (Tabashnik, 1994 and Michaud, 1997) in the insect populations. In order to delay the resistance, one of the strategies would be the use of alternative genes for developing transgenic plants either singly or in combination with other insecticidal genes.

Considering the fact that plant derived genes efficiently express in the transgenic plants, this study was conducted to clone an alternative insect resistant gene trypsin inhibitor from soybean and to transform it into the model system tobacco.

MATERIALS AND METHODS

The present study was undertaken to clone trypsin inhibitor gene from soybean (*Glycine max*), sub clone the gene into a plant transformation vector and *Agrobacterium* mediated transformation of tobacco with cloned trypsin inhibitor gene. The materials used and methods employed are as follows;

Plant material and PCR amplification

The total DNA of soybean variety JS335 was isolated by following CTAB protocol (Doyle and Doyle, 1987) with some modifications. Complete coding sequence of trypsin inhibitor gene was amplified by PCR using DNA from soybean variety JS335 as template and with a set of gene specific primers (Cipriani *et al.*, 1998) and used for cloning into pTZ57R/T vector and the plant transformation vector pHS100 (BARC, Bombay).

Forward: 5'-ATGAAGAGCACCATCTTC-3' (18 mer)
Reverse: 5'-TCACACACTGCGAGAAAG-3' (18 mer)

PCR was carried out in a eppendorf master cycler gradient in 20 µl reaction volume containing 100ng DNA template, 25mM MgCl2, 5pM of each primer, 2.5mM dNTPs, 1X Taq polymarase buffer and 1.0U Taq DNA polymerase (Bangalore Genei) for 40 cycles.

Gel elution of the PCR amplicon

The single sharp amplicon at 650 bp was separated out using sharp sterile scalpel blade and collected in a sterile pre-weighed 2.0 ml microcentrifuge tube. The Qiagen gel extraction kit was used to elute the DNA from the agarose block as described in user manual.

Cloning of PCR

The purified PCR fragment of size ~650bp amplicon was ligated to pTZ57R/T cloning vector (2886 bp) as described in InsT/A clone™ PCR product cloning kit (K1214) form MBI, Fermentas, USA.

For ligation, an optimal molar ratio of ends vector: insert (1:3) was computed and the components of ligation mix were added to 0.5 ml micro centrifuge tube and was incubated at 16°C for 16 hrs. The competent cells of *E. coli* DH5α were transformed with 10µl of ligation mixture.

The recombinant clones were identified by blue/white assay in the Luria agar plates with Amp_{50}, X-gal, and IPTG after overnight incubation at 37°C. The positive clones were named as pTZ-SB clones.

Confirmation of clones

The confirmation of the presence of cloned fragment in the recombinant vector was done by PCR amplification and through comparative restriction analysis of selected clones and the control vector to ensure the presence of insert with *Xba*I and *Bam*HI enzymes.

Sequencing of clones

pTZ-SBC3 clone was sequenced using M13 primers employing primer walking technique at Bangalore Genei Private Ltd., Bangalore. The sequence was subjected to analysis using BLAST algorithm available at http://www.ncbi.nlm.nih.gov.

Subcloning into plant transformation vector

The plasmid DNA of pTZ-SBC3 and the plant transformation vector pHS100 was isolated in large quantities and sequentially restricted with *Xba*I and *Bam* HI to facilitate directional cloning and these were ligated to get recombinant clones in order to transform the trypsin inhibitor gene into plant. These clones were confirmed by PCR amplification and restriction analysis. The positive clones were named as pHS-SB clones.

***Agrobacterium* Transformation**

The confirmed clones were further mobilized into *Agrobacterium tumefaciens* strain LBA4404 by triparental mating using pRK2013 as helper plasmid in DH5α. The transconjugants were selected on selection medium. Plasmids were isolated from recombinant *Agrobacterium* and the presence of trypsin inhibitor insert was confirmed by PCR amplification.

Tobacco transformation

The *Agrobaterium* containing construct; pHS-SBC1 was used for tobacco (*Nicotiana tabacum* L. cv. White burley) transformation with the protocol mentioned by Hooykaas and Schilperoort (1992) with some modifications. DNA was extracted from Kanamycin resistant plants and checked through PCR with gene specific primers.

RESULTS AND DISCUSSION

Cloning of trypsin inhibitor gene

In the present study, the amplified ~650bp amplicon was cloned into linearised pTZ57R/T and transformants were screened through Blue-white screening assay. The positive clones were confirmed through PCR (Fig. 1) and restriction analysis (Fig. 2). The sequence was subjected for homology search at nucleotide level and at protein level using BLASTn and BLASTp programs available at http://www.ncbi.nlm.nih.gov. and the cloned 654bp long insert encoding 217 amino acids was found to have 99% homology with reported soybean inhibitor genes. *In silico* analysis of

translated amino acid sequence (216 amino acids aligned) showed 100% homology with amino acid sequences of trypsin inhibitor subtype A and *Tia* Kunitz trypsin inhibitor of soybean.

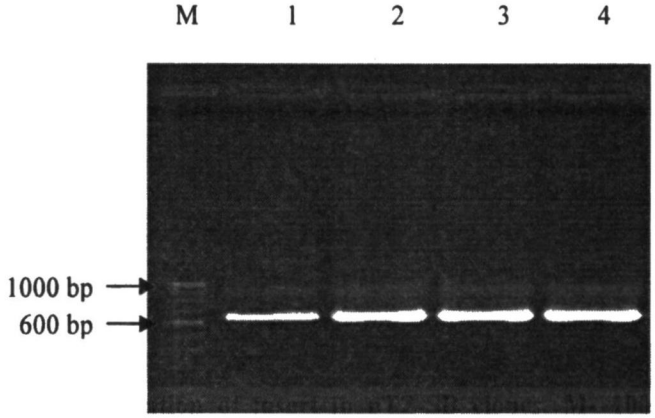

Figure 1. PCR confirmation of insert in pTZ-SB clones. M- 100bp DNA ladder, 1- positive control (soybean genomic DNA), 2- pTZ-SBCI, 3- pTZ-SBC2 4- pTZ SBC3.

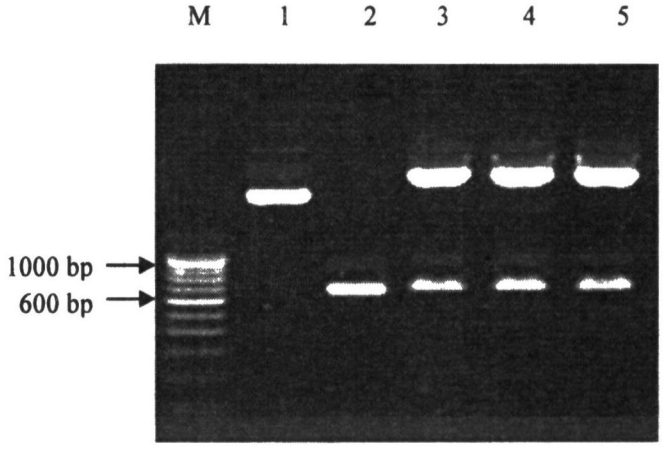

Figure 2. Restriction analysis of pTZ-SB clones with *Xba*1 and *Bam*H1
M- 100bp DNA ladder, 1- vector: pTZ57R/T, 2- PCR product, 3- pTZ-SBC1, 4- pTZ-SBC2, 5- pTZ-SBC3.

The rpsBLAST result showed that the *SKTI* peptide has a single STI (soybean trypsin inhibitor) domain and has shown 99.4% conservation in the protein. Kunitz (1946) has characterized the trypsin inhibitor gene of soybean as a single polypeptide chain of 181 amino acids including two disulphide bonds. In the deduced amino acid sequence of the cloned *SKTI*, there were 4 cysteine residues at 64^{th}, 111^{th}, 161^{st} and 170^{th} positions to facilitate intrachain disulphide bridges. It is clear from the deduced amino acid sequence of cloned *SKTI* that the gene and the peptide are exactly similar to the known soybean kunitz trypsin inhibitor.

Subcloning into plant transformation vector

Sequence result of the pTZ-SBC3 clone showed that the insert was in correct orientation. Therefore, the insert was released from pTZ-SBC3 clone and directionally cloned into pHS100 plant transformation vector (Fig. 3) under CaMV35S constitutive promoter at *Xba*I and *Bam*H1 sites and transformed *E. coli* DH5α cells. The confirmed clone pHS-SBC1 was mobilized into *Agrobacterium tumefaciens* LBA4404 by triparental mating. Plasmid DNA isolated from these transformants showed positive for PCR (Fig. 4) and restriction analysis (Fig. 5). The clone pHS-SBC1 was mobilized into *Agrobacterium tumefaciens* strain LBA4404 through tri-parental mating using *E.coli* containing pRK2013 as helper plasmid.

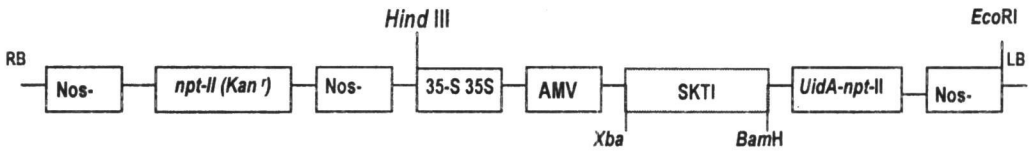

Figure 3. T-DNA cassette of pHS100 plant transformation vector containing *SKTI* gene.

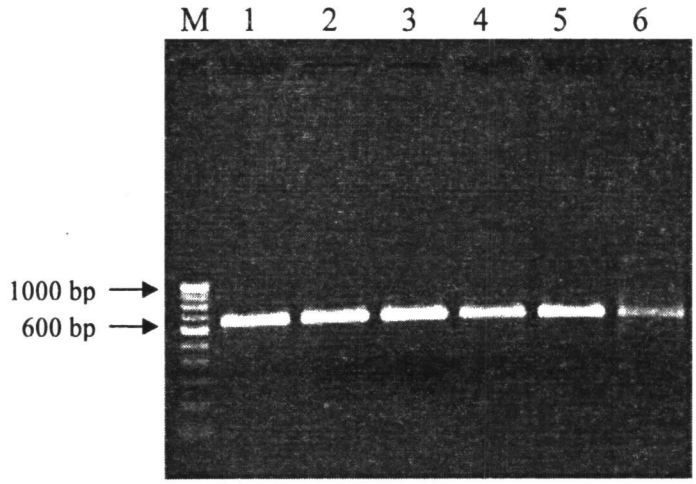

Figure 4. PCR confirmation of SKTI insert in pHS-SB clones M- 100bp DNA ladder, 1 – pHS-SBC1, 2- pHS-SBC2, 3- pHS-SBC3, 4-pHS-SBC4, 5-pHS-SBC5, 6-positive control (pTZ-SBC3).

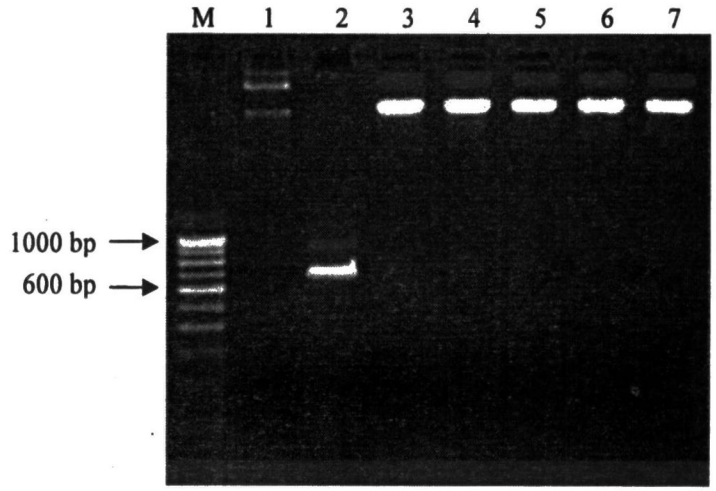

Figure 5. Restriction analysis of pHS-SB clones with *Xba*1 and *Bam*H1 M- 100bp DNA ladder, 1- vector: pHS100, 2-PCR product, 3-pHS-SBC1, 4-pHS-SBC2, 5-pHS-SBC3, 6-pHS-SBC4, 7-pHS-SB5.

Tobacco transformation

The *Agrobacterium,* with the construct of pHS-SBC1 was used to transform tobacco by following protocol from Hooykaas and Schilperoort (1992). The surviving green shoots which showed resistance to kanamycin at 200µg/ml were rooted with selection and transferred to sterilized peat. DNA was isolated from putative transformants and checked for the presence of insert. More than 80% of the checked plants were PCR positive for the gene (Fig. 6).

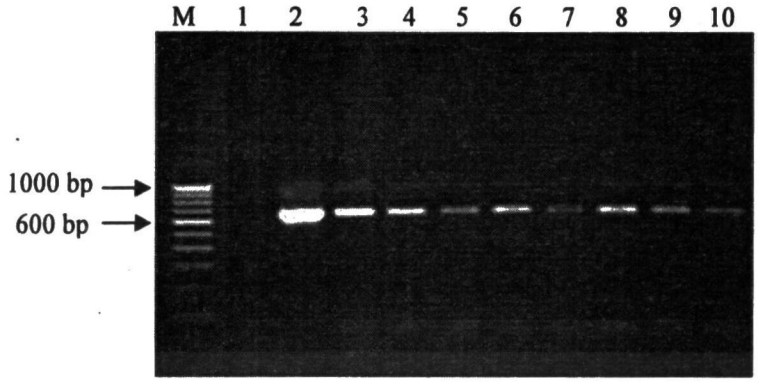

Figure 6. PCR confirmation for the presence of *SKTI* insert in tobacco M-100bp DNA ladder, 1-negative control (untransformed tobacco), 2- positive control (pHS-SBC1), 3 to 10-positive plants.

CONCLUSIONS

Serine protease inhibitor genes have proved to be very important in developing transgenic crop plants either singly or in combination with other insecticidal genes like *cry* genes of *Bacillus thuringiensis*, lectins and other protease inhibitor genes. In the present study, soybean trypsin inhibitor gene was cloned to a plant transformation vector and tobacco (the model system) was transformed. Expression of the gene in the plant system has to be confirmed before deployment in the transgenic plant development. Since the gene does not have introns, the expression can also be tried in the prokaryotic system.

ACKNOWLEDGEMENT

The authors wish to express their gratitude to CARP, Sri Lanka for funding this research project under long term training program.

REFERENCES

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

Balestrazzi, A., M. Confalonieri, M. Odoardi, V. Ressegotti, G. Allegro, A. Tava and D. Carbonera, 2004. A trypsin inhibitor cDNA from a novel source, Snail Medic (*Medicago scutellata* L.): Cloning and functional expression in response to wounding, herbivore, jasmonic acid and salicylic acid. Plant Science 167:337-346.

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

Cipriani, G., D. Michaud, F. Brunelle, A. Golmirzaie and D.P. Zhang, 1998. Expression of soybean proteinase inhibitor in sweet potato. CIP Program Report (1997-98): 271–277.

Doyle J.J. and J.L. Doyle, 1987. Isolation of plant DNA from fresh tissue. Phytochemistry Bulletin 19:15-38.

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

Gatehouse, A.M.R., Y. Shi, K.S. Powell, C. Brough, V.A. Hilder, W.D.O. Hamilton, C. Newell, A. Merryweather, D. Butler and J.A. Gatehouse, 1993, Approaches to insect resistance using transgenic plants. Philosophical Transactions of the Royal Society, London 342:279-286.

Hooykaas, P.J.J. and Schilperoort, 1992. *Agrobacterium* and plant genetic engineering. Plant Molecular Biology 19:15-38.

http://www.ncbi.nlm.nih.gov.

Johnson, R., J. Narvaez, G. An and C. Ryan, 1989. Expression of proteinase inhibitor I and II in transgenic tobacco plants: Effects on natural defense against *Manduca sexta* larvae. Proceedings of National Academic Science, USA 86:9871-9875.

Kunitz, M., 1946. Crystalline Soybean Trypsin Inhibitor. Journal of General Physiology 29:149-154.

Lee, Soo In, Lee, Sung-Ho, Koo, Ja Choon, Chun, Hyun Jin, Lim, Chae Oh, Mun, Jeom Hee, Song, Yoo Han, Cho, and Moo Je, 1999. Soybean Kunitz trypsin inhibitor (SKTI) confers resistance to the brown plant hopper (*Nilaparvata lugens* Stael) in transgenic rice. Molecular Breeding 5(1):1-9.

Leo, F.D., M.A. Bonade-Bottino, L.R. Cecl, G. Galleranl and L. Jouani, 1998. Opposite effects on *Spodoptera littoralis* larvae of high expression level of a trypsin proteinase inhibitor in transgenic plants. Plant Physiology 118:997–1004.

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

Marchetti, S., M. Delledonne, C. Fogher, C. Chiaba, F. Chiesa, F. Savazzani and A. Giordano, 2000. soybean Kunitz, C-II and PI-IV inhibitor genes confer different levels of insect resistance to tobacco and potato transgenic plants. Theoretical and Applied Genetics 101:519-526.

Michaud, D., 1997, Avoiding protease-mediated resistance in herbivorous pests. Trends Biotechnology 15: 4–6.

Qu, L. J., J. Chen, M. Liu, N. Pan, H. Okamoto, Z. Lin, C. Li, D. Li, J. Wang, G. Zhu, X. Zhao, X. Chen, H. Gu and Z. Chen, 2003. Molecular cloning and functional analysis of a novel type of Bowman-Birk inhibitor gene family in rice. Plant Physiology 133:560–570.

Ryan, C. A., 1990. Protease inhibitors in plants: genes for improving defenses against insects and pathogens. Annual Review of Phytopathology 28:425-449.

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

Tabashnik, B.E., 1994. Evolution of resistance to *Bacillus thuringiensis*. Annual Review of Entomology 39:47–79.

Williams, D.L., W.C. Kain and R.M. Broadway, 1997. Isolation and characterization of a serine proteinase inhibitor cDNA (Accession No. U18995) from cabbage. Plant Physiology 114:747.

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