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PCR amplification and cloning of proteinase inhibitor gene in cotton (*Gossypium species*)

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The main objective of the work was to see presence of the three different Proteinase inhibitor genes of soybean Viz. Kti3, CI-II and PI-IV genes in cotton by synthesizing specific primer from already published data. DNA was extracted from different cotton spp. and was used for PCR amplification by the different specific primers for the Kti3, CI-II and PI-IV genes. Results have shown expected fragments of 651 bp for Kti3 gene and 250 bp for CI-II and PI-IV genes. These PCR fragments were were electroeluted from the Agarose gels and then used for cloning. These genes were then cloned in plasmid vector. Selection of transformed colony was done on the basis of Blue-White screnning. Plasmid was isolated and results were reconfirmed by doing PCR amplification of the Kti3, CI-II and PI-IV genes using specific primers. Results showed that the expected fragments were successfully cloned in the plasmid

Key words : Proteinase Inhibitor, Cotton, Protinase Inhibitor, Polymarase Chain Raction, Kunitz Trypsin Inhibitor, DNA isolation

INTRODUCTION

BOTANICALLY, cotton belongs to the family Malvaceae and the genus *Gossypium*. This genus comprises of about 50 species among which 4 are commercial cultivated. They are *Gossypium arboreum, G. herbaceum, G. hirsutum and G. barbadense*In India alone on an average 6-8 insecticidal sprays are applied in cotton crop. More than 10% of the world's pesticides and nearly 25% of the world's insecticides are used in cotton farming (Khadi, 2003). In general, transgenic plants develop using proteinase inhibitors digestive enzymes with a view to control crop pest and designed not to kill the insect that feed, but to retard their development. Presumably, this is the fundamental difference between this strategy and chemical pest control and use of Bt toxins are aimed at complete control through pest mortality. PIs are primary gene products and they are excellent candidates for engineering pest resistant into plants. Inhibitor genes of plant origin are particularly promising. Gatehouse *et al.* (1985) reported that insects belonging to both Lepidoptera and Coleoptera can over express existing gut proteinases or induce the production of new types that are insensitive to the introduced PIs to overcome the deleterious effect of PIs ingestion .The first were transgenic plants using cowpea trypsin inhibitor cDNA clone. Hilder *et al.* (1987). The different PIs that have been used for development of insect resistant transgenic plants are cowpea serine PIs (CpTI), potato serine inhibitors (PI-I and PI-I), sweet potato inhibitors, rice cysteine PIs, soybean Kunitz trypsin inhibitors, corn cysteine PIs, mustard trypsin inhibitor (TI), tobacco PI and bean a- amylase inhibitors. Strong inhibitory activity against insect gut proteases of *Sitioplilus zeamais* and a wide inhibitory spectrum against various cysteine proteinases of insects Irie *et al.* (1989). A cDNA clone of multidomain PI from *Nicotiana spp* was transfer into tobacco and peas under the control promoter from ribulose 1,5-biphosphate carboxylase gene . Transformed tobacco plants with gene coding tomato and potato inhibitor proteins and the transgenic plants found resistant to *Manduca sexta* Johnson *et al.* (1989). Field trials carried out in the US showed that the expression of CpTI on tobacco provided significant protection in field against *Helicoverpa zae* (Hoffman *et al.* (1992). The gut digestive enzymes are not the only targets effected by PIs, they can also effect the water balance, molting and enzyme regulation of insects Boulter (1993). Mcmanus *et al.* (1994) transferred the chymotrypsin inhibitor gene from potato to tobacco and these plants were also resistant to *Chrysodeixis eriosoma*. Shade *et al.* (1994) reported that the transgenic pea expressing bean á-amylase inhibitor from bean (*Phaseolus vulgaris*) confers resistance to the bruchid beetle, *Callosobruchis maculates* and *C. chinensis*. Shroeder *et al.* (1995) reported that bean alfa-amylase inhibitor-expressing pea was also resistant to *Bruchus pisorum.* The expression of bean á-amylase inhibitor in azuki bean conferred resistance to three species of bruchids (Ishimoto *et al.* 1996). However the bi-functional á-amylase / proteinase inhibitor genes are more useful for developing insect- resistant transgenic plants.

Duan *et al.* (1996) introduced PI-II gene from potato in several Japonica rice verity and this transgenic plants were found to insect resistant in green house trials. Wound inducible PI-II promoter with the first intron of rice actin I gene was able to give high-level expression of PI-II gene in transgenic rice plant. These transgenic plants expressing PI gene were found resistant to pink stem borer (*Sesamia inferens*). Thus, introduction of plant derived PI gene in to serial plant was successful for control of insect pests. Xu *et al.* (1996) reported the constitutive expression CpTI genes in transgenic rice plants, which confined resistant to two species of stem borers. Expression of CpTI gene, driven by the constitutive active promoter of the rice actin - I gene resulted in high-level accumulation of CpTI protein in transgenic rice plants. The trypsin inhibitor in transgenic plants was biologically active and the plants showed increased resistance against two species of stem borers. Sane *et al.* (1997) isolated cowpea trypsin inhibitor gene and cloned the fragment in a plant expression vector coupled with CaMV 35S promoter and NOS terminator and used for tobacco transformation. The efficiency of transgenic tobacco plants express CpTI against *Spodoptera litura* in feeding trials under laboratory conditions and found reductions to extent of 50% in the biomass of *S. litura* larvae fed on transgenic leaving expressing 3-5 µg CpTI /g fresh leaves. Yeh *et al.* (1997) reported the transgenic tobacco plants expressing the sweet potato TI and conferred resistance against *S. litura.*Transfer of sweet potato gene to cauliflower. cDNA gene encoding a cysteine PI isolated from rice was introduced into tobacco, potato, poplar, oilseed-rape but only results reporting the toxicity of such plants against a beetle feeding on poplar have been published. PI genes from different sources have been transferred to rice and insect resistance has been tested in detail. Schuler *et al.* (1998) reported that although a number of transgenic crop plants have been developed to determined the

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usefulness of different PI, the detail analysis of this transgenic expressing different PIs is not available or not published due to patent applications. To date, no crop expressing PIs transgene has been commercialized. Gatehouse (1999) showed that transgenic petunia, Birch and lettuce plants expressing PI and PI-II where resistant to Lepidopteran and Orthopteran insects.

Lee *et al*. (1999) developed insect-resistant wheat by transferring the gene of barley TI (BTI). However only early-instars larvae were inhibited in transgenic leaves did not have a significant protective effect against leaf-feeding insects. Corn cystatins (CC) cDNA under the control of AMV 35S promoter in rice plants showed a high level of expression in both rice seeds and leaves. About 2% of the total proteins in transgenic rice plants were found to be CC proteins.

Lawrence *et al.* (2001) reported a protease inhibitor gene isolated from a native variety of cowpea in laboratory was used to transform pigeonpea through *A. tumefaciens* mediated genetic transformation. CaMV 35S promoter containing kanamycin resistance as plant selection marker drove the gene. The molecular analysis of the *in vitro* cultured transgenic pigeon pea plants confirmed the integration and stable expression of the protease inhibitor gene. The first generation of these transgenic pigeon pea plants is been maintained in glasshouses for insect feeding experiments and primarily insect bioassays showed encouraging results against pod borer (*Helicoverpa armigera*) (Lawrence and Koundal, 2001).

These studies have conclusively demonstrated the feasibility of genetic engineering of insect resistant by expressing PIs gene. In the present investigation following objectives were set to isolate the proteinase inhibitor gene (s). Primer based amplification of Proteinase inhibitor (PI) genes Cloning and transformation of PI genes

MATERIALS AND METHODS

The plant materials used

For these studies viz., *G. hirsutum* cultivars viz., Anjali (LRK – 516) (plate-1), LRA – 5166, Bt-162, NHH – 44 and Suvin, *G. arboreum* cultivar *viz.,* AKA - 8401 (plate-2) were collected from the Crop Improvement Division, CICR, Nagpur. Seeds were germinated for 24 - 36 hours and total genomic DNA was isolated.

3.5.0. Primers

The primers were synthesized from Qaigen (the details are presented in the following text).

1. Code - KTi 3

Forward:5'-GGATCCATGAAGAGCACCATCTTCTTTCTCTTTC-3'
Reverse:5'-CAGCTGTCACTCACTGCGAGAAAGGCCATG-3'

2. Code - C-II

Forward:5'-GGATCCATGGAACTGAACCTCTTCAAAAGTGAT-3'
Reverse:5'-GAGCTCCTAGTCATCATCTTCATCACTGGA-3'

3. Code - PI-IV

Forward:5'-GGATCCATGTGTATTCTGAGCTTCCTCAAAAGT-3'
Reverse:5'- GAGTCTCTAGTCATCTCTGGACTTGCAAGG-3'

DNA isolation

DNA was isolated by Dellapotta *et al.* (1983) germinated seeds were crushed in liquid nitrogen into fine powder Then added 2 ml. of extraction buffer in small aliquots and transferred to a centrifuge tube and 250 µl of 20% SDS and 20% PVP were added and mixed the contents properly. The tube was kept in water bath at 65°C for 30 min Then the tube was taken out and 3ml of 7.5 M Ammonium acetate was added and kept in frreezer. The content were centrifuged at 15000rpm, 4.0°C for 15 min.To the aqueous layer, 6/10th volume of isopropyl alcohol was added and kept at -20°C overnight for complete precipitation of DNA.Spinning at 15000rpm for 15 min pelleted the DNA. The pellet was dissolved in 25 µl of TE buffer and 10 µg of RNase was added and incubated for 15 min. at 37° C. Spinning at 15000rpm for 15 min pelleted the DNA. The DNA was dissolved with 100µl of TE buffer and added equal volume of phenol, chloroform & isoamyl alcohol mixture and mixed properly by inverting the tube 4 to 5 times. Centrifuged it at 15000rpm for 15 min. Carefully pulled out the aqueous layer in a fresh tube leaving the inter phase. Added equal volume of ice-cold ethanol and pelleted the DNA by centrifugation. Dissolved the pellet in suitable volume of TE buffer, reprecipitated the DNA with ethanol, it is spanned and vacuum dried the pellet. Dissolved the pellet in suitable volume of TE buffer. Estimated the DNA contents and checked the purity by UV spectrophotometer at 280nm.

PCR reaction.

PCR amplification was carried out in a 25 µl solution containing *Taq* polymerase and 10X PCR buffer (both from BANGLORE GENEI ,INDIA); (4) Agarose (HIMEDIA, INDIA) and PCRs were performed for each different primers and PCR reaction mixes consisted of 2.5 ul of 10X reaction buffer (100 mM Tris pH 9,15mMMgCl2,500M KCL and 0.1% Geletin, 3 ul dNTPs (200 uM of each), 2 ul of 5 pica moles/ul primer, 1 ul of 50 ng/ul genomic DNA and 0.8U/ul *Taq* polymerase and remaining water. Only one DNA sample and two forword and reverse primers were added to any single reaction. All reaction volumes were 25 ul overlaid with a drop of mineral oil. A thermo cycling programme was standardized by repetitive use of different programes laboratory. An initial denaturing step of 3 min at 94 ^{0}C, 2 cycles of 1 min. at 94 ^{0}C, 1 min at 65 ^{0}C, 1.min c at 72 ^{0}C, 20 cycles of 1 min. at 94 ^{0}C, 1 min at 60 ^{0}C, 1.min at 72 ^{0}C, 20 cycles of 1 min. at 94 ^{0}C, 1 min at 55 ^{0}C, 1.min at 72 ^{0}C, 1 cycle (final extension) 7 min at 72 ^{0}C, then kept at 4 ^{0}C. PCR machine was used for all the above raction is Biometra. A standard ramp set was used for all ractions. Electrophoresis was performed in 5mm thick agarose gels (2%HIMEDIA) with 0.5 X TBE buffer for 2 h at 5V/cm, constant voltage. The gels were stained with 10mg/ml Ethidium bromide, visualized on a 302 nm UV transilluminator and photographed with a Gene Genus gel documentation system (SYNGENE).The DNA was eletroeluted from 1.5% agarose and viewed the DNA on UV light to confirm complete elution of DNA. After Electroelution the DNA was purified and precipited by adding ammonium acetate(7.5M) and 2 volume of ethanol.

Transformation into E. coli and confirmation of the results

Cloning of eluted fragments was done by using QUIAGEN PCR cloning kit. The competent cells were warmed at room tempreture and 2 ul ligation raction mixture was was added/tube and kept for incubation on ice.tube swere heated at 42^0 C for 30 sec and incubated for 30 sec.added 250 ul of SOC medium and then100 ul transformation mixture used for plating

LB medium was prepared and 750ul of Kanamycin was added .For selection of transformants by blue For Blue /White colony screening added 100 µl of X-gal stock solution and 450 µl of IPTG stock solution. 100µl of each transformation mixture was used for plating. On to LB agar plates and kept it for incubation at room temperature at 27 ^{0}C. White transformed colony was selcted for making LB broth .next day cells were harvested and Lysozyme and SDS were used for plasmid was isolation. Confirmation of the results was done by isolating plasmid and reamplification of plasmid DNA by using specific primers in PCR.

RESULTS AND DISCUSSION

DNA Isolation And PCR Analysis

The total genomic DNA was isolated in all the selected cultivars as described by Dellapotta *et al.* (1983) with minor modifications. The DNA was purified and the intact DNA was subjected to

quantitative and qualitative checks.DNA was quantified and used for PCR analysis .Using three different primers as mentioned above, the target genes of proteinase inhibitors were amplified in BIOMETRA thermal cycler. Since, the primer was 34bp length, and then amplification program in the thermal cycler was standardized with different program, especially annealing temperature, which plays crucial role in the amplification with lengthy primers. The PCR program amplified the expected gene length is presented in the figure 2.The annealing temperature was gradually reduced from high temperature 60° C to 55° C for efficient amplification of the targeted gene and timing was given constantly 2 minutes. The amplified product was resolved in 1.5% agarose gel electrophoresis. After the run, the gel was stained with ethidium bromide and observed under the UV- trans-illuminator and results were documented. The primers KTi_3, C-II and PI - IV primers amplified the expected 600bp and last two primers amplified 250 bp respectively and it was confirmed with the standard marker 1Kb ladder. The results were shown in the plates. The template DNA with KTi_3 primer amplified ~650bp in all the cultivars of *G. hirsutum* and *G. arboreum* . The template DNA with C-II and PI-IV primer amplified ~250bp in LRA-5166, BT-162, and NHH 44, and AKA – 8401 (plate – 6 and 7). We isolated cotton proteinase inhibitor (serine types) genes from seeds of *G. hirsutum* cultivars like Anjali (LRK - 516), LRA - 5166, Bt-162, NHH - 44 and *Suvin* and *G. arboreum* cultivar-AKH-8401, using the soybean genes KTi_3, C-II and PI-IV primers. As Marchetti *et al.* (2000) reported, we have amplified approximately the same size genes of 0.650 Kb for KTi_3 primer and around 0.250 Kb for C-II and PI-IV primers(pictures not shown) in both the species of *G. arboreum* and *G. hirsutum* cultivars. In general terms the evidence collected in present studies is similar to that reported by Marchetti *et al.* (2000). The three different genes isolated from *G.hirsutum* and *G. arboreum* cultivars demonstrated that those different levels of insect resistance might be achieved by developing different transgenic cotton plants Our results clearly indicated that the proteinase inhibitor genes present in all the commercially cultivated cotton varieties of *G.hirsutum* and *G. arboreum.* Although the genes are present in the cotton plants but they are induced by insect attack or wounding. The main objective of the work is to isolate the proteinase inhibitors genes and attaching a constitutive promoter to the gene, which in turn produces throughout the plant parts during its life cycle, and control the bollworms at neonate larval stage.

To the best of our knowledge we provide here the evidence of isolation of proteinase inhibitors genes in different Indian cultivars of cotton genome. It is noteworthy that this result was

Fig. 1 : PCR amplification by Kti3 Primer 1=Marker,2= Anjali (LRK – 516),3= LRA–5166; 4= Bt-162; 5=NHH – 446; 6 = Suvin; 7 = AKA – 8401

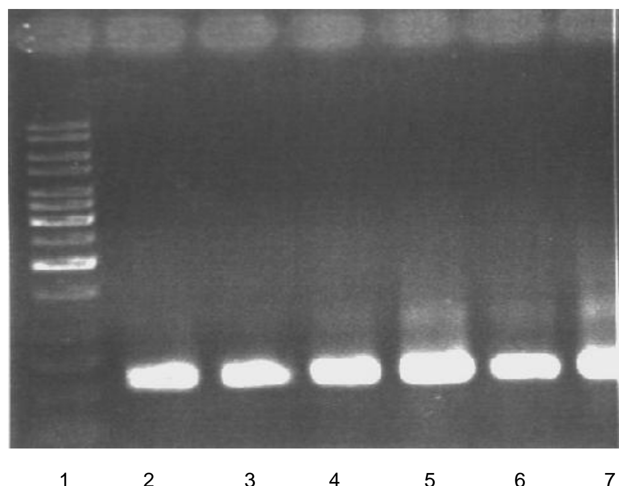
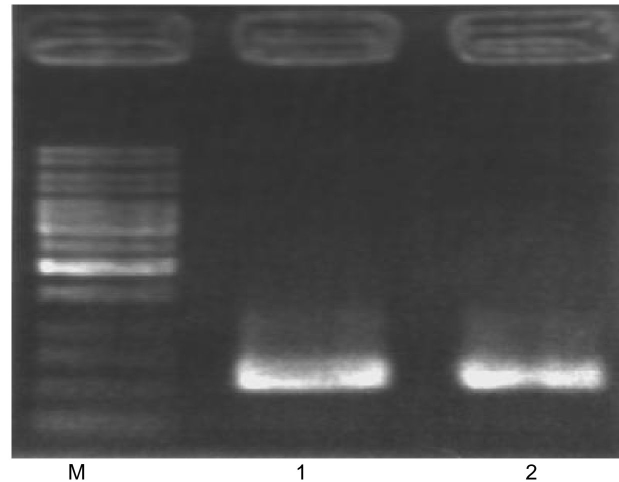


Fig. 2 : Plasmid PCR reamplification by Kti3 Primer 1=Marker, 2= Anjali (LRK – 516)



achieved by using soybean proteinase inhibitor gene sequences (Marchetti *et al.,* (2000). The amplified gene was eluted efficiently, dialyzed the DNA fragments, and precipitated the DNA for further studies on DNA sequencing.

Cloning And Transformation

The amplified gene was eluted out from the agarose gel and the process of dialysis was carried out using EDTA (0.5 M), ammonium acetate (2/3rd volume of 7.5M) and ethanol (2 volumes) and the DNA was precipitated. The contents were centrifuged and the DNA was pelleted out and dried. DNA was suspended into TE buffer for further studies on DNA sequencing. PCR amplified of target PI genes was eluted and fragments were subjected to cloning into QUIGEN cloning vector pDrive. The fragments approximately 600 bp for bp for Kti3 and 200 bp for CI-II for was successfully ligated in pDrive vectors. The ligated product was confirmed by gel electrophoresis and found that a single band where as the unligated control showed that two bands on 1.5% agarose gel The ligated pDrive vector was pushed in to QUIGEN competent cells. The transformed bacterial cells were selected on kanamycin medium (50 µg/ml). The transformed colonies shows white in colour due to insertion inactivation of *lacZ* gene, whereas non transformed bacterial colony shows blue in colour by reacting the substrate X-gal and IPTG. The results are presented in the plate. This result shows that successful cloning and transformation of PI gene in pDrive cloning vectors.The inserted DNA in pDrive vector was confirmed by primer based reamplification of PI genes when plasmid DNA was extracted and analysed the nucleotide sequence of Kti3 and CI-II were found to be identical to those amplified initially of approximately of 650 bp and 200 bp sequence.

QUIGEN cloning vector pDrive is used, commonly for instant cloning into cloning of amplified DNA fragment. The p Drive cloning vector provides superior performance through UA based ligation and allows easy analysis of cloned PCR product. In the present study the amplified PI genes was successfully cloned into the pDrive cloning vector and the integration was confirmed by gel electrophoresis Marchetti *et al* (2000) reported the cloning of Kti3, CI-II and PI-IV genes into pGEM-T vectors In the present study QUIGEN EZ competent cells were used as host for pDrive vector. The transformation was found high efficiency of about 73%(Bijola *et al.,* (1997) reported the same result when cloning of CI gene in Bluescript II KS (+) plasmid). The high transformation percentage of E.coli cells may be due to the less base pair length of all the two Kti3, CI-II genes of approximate 650 and 250 bp respectively and intronless nature of Kti3 and CI-II genes may help to get properly inserted without any extensive modifications.The transformed colonies with blue colour were

found at low temperature incubation this results show that the enzyme activity on substrate might be temperature controlled. The cloned pDrive vectors was analysed by reamplification of PI gene using Kti3 and CI-II. This confirmation reveals the PI gene integration in pDrive vector insertion of other unamplified primer sequence. The results showed that the expected size of 650 and 250 bp sequence of DNA amplification in the transformed bacterial cells .the results are clear that there was no insertion of or ligation of primer sequence. The further work on sequencing analysis will reveal the exact base pairs sequence of PI gene in cotton.

The further work on sequencing the amplified gene of KTi_3, C-II and PI-IV amplified in *G.hirsutum* and *G. arboreum* would provide the complete knowledge of the presence of various PI genes present in the cotton plants

SUMMARY AND CONCLUSION

In the present investigation, an attempt was made and succeeded to isolate three types of serine proteinase inhibitor genes from *G. hirsutum* and *G. arboreum* cultivars using soybean PIs primers. The expected fragment of corresponding genes to KTi_3, C-II and PI-IV primers were amplified, ~650bp and other two with ~250bp respectively.In the variety Suvin and LRA-5166 the PCR amplify Kti3, and CI-II of about 650 and 250 bp respectively and were cloned in pDrive vector which was confirmed by electrophoresis. After the reamplification of the isolated plasmid (pDrive of 3.85 kb) the expected fragments of 650 bp of Kti3 and 250 bp of CI-II were seen, which shows the successful cloning of inserted genes. At this point, we like to make a brief conclusion that the PI genes are present in cotton plants that need to be engineered for efficient expression to make viable transgenic cotton resistance against insect pest.

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