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Study of putative Transformants of transgenic pigeonpea for *Helicoverpa armigera*

Sonal Kambale, Bhagyashree Bahatkar, Santosh Gahukar, Amrapali Akhare and Abhilasha Kharkar

Abstract
Pigeonpea [*Cajanus cajan* (L.) Millsp] is the major grain legumes of the semi-arid tropics. Its production and productivity are constrained by several diseases and pests but the major pest attacking Pigeonpea is Helicoverpa armigera which is highly susceptible to the insecticidal proteins of Bacillus thurengenesis (Bt). A codon-optimized chimeric Cry1Aabc gene of Bt, driven by a constitutive promoter was introduced in pigeonpea (PKV Tara) by in-planta and *in-vitro* Agrobacterium mediated genetic transformation. In present investigation confirmation of putative transformants using gene specific marker and detached leaf, an assay was carried out. Out of 73 plants tested, 4 plants were confirmed to be PCR positive using Cry1Aabc and nptII gene specific markers. For detached leaf bioassay with neonate larvae of H. armigera, 4 plants which were confirmed through PCR amplification with gene specific markers were used. The results of leaf detached bioassay showed significant level of Cry protein expressed in positive plants which were able to reduce the weight of larvae and leaf damage. The positive plants selected for the study show potential for providing tolerance against H. armigera.

Keywords: Transgenic pigeonpea, genetic transformation, Cry1Aabc gene, Helicoverpa armigera

Introduction
Among various species of *Helicoverpa* found world-wide, *H. armigera* (Lepidoptera, Noctuidae) is the most serious pest harboring over 181 plant species belonging to 45 families. Its management is very difficult because of its high mobility, survival rate under adverse conditions, capacity to complete several generations in a year and ability to develop resistance against insecticides. Due to *H. armigera* attack, serious and extensive yield losses have been reported in some legumes from 28-40%, ensuring economic loss up to 300 million dollars annually (Arshad Ali *et al*., 2009). Attempts to develop pod borer resistance genotype by conventional breeding approaches have not been successful because of low genetic variability and incompatibility associated with wild species (Nene and Sheila, 1990). The non availability of source for resistance in cultivated species has to lead the plant breeders to explore the feasibility using alternative biotechnological approaches for the improvement of pigeonpea for pod borer resistance. Development of transgenic expressing insecticidal proteins in productive cultivars is one of the promising strategies followed in many crops including pigeonpea. Genes confirming insect resistance to plants have been obtained from various microorganisms like *Bt* from *Bacillus thuringiensis*, isopentyl transferase from *Agrobacterium tumefaciens*. Progress in engineering insect resistance in transgenic plants has been achieved with the insect control protein gene of *Bacillus thuringiensis* which is an entamocidal bacterium that produces an insect control protein. *Bt* gene code for *Bt* toxins. The insect toxicity of *Bt* residues in large *Cry* proteins, these *Cry* proteins are ingested by insects, they are dissolved in alkaline juices present in midgut lumen. The toxic fragments bind to receptors present in brush borders of midgut epithelial *B. thuringenesis* cells. As a result, the brush border develops pores, permitting influx into epithelial cells of ions and water, which causes their swelling and eventual lyses. Numbers of methods are available to transfer desirable foreign genes to plants. Of this *Agrobacterium* mediated transformation is more efficient as it results in integration of Well-defined DNA sequences, potentially low copy number, high expression of the introduced genes and preferential integration into actively transcribed regions. The advances in recombinant DNA technology have made it achievable to clone and express the toxin genes to confer resistance to insect pests. Looking to the potential of pigeonpea transgenic in enhancing insect resistant variety the present study reports two popular transformation systems i.e. in-planta and in-vitro Agrobacterium mediated genetic transformation for development of

transgenic pigeonpea lines for improved plant protection against the pod borer.

Material and Methods

Materials

The genetically pure seed of PKV-Tara was obtained from Senior Research Scientist, Pulses Research Unit Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola.

Agrobacterium strain

Agrobacterium tumefaciens strain EHA-105 harboring Cry1Aabc gene was procured from National Research Center on Plant Biotechnology, New Delhi Methodology

In-planta transformation

Infection to the growing embryonic axis of germinated seeds and floral bud was done with bacterial culture. The injured seeds and floral buds were treated with *Agrobacterium* culture with tobacco leaf extract for 60mins. The observation was recorded for the growth of plants after embryonic axis infection and development of pod from infected flower. The survived plant material was maintained in a transgenic house. The seed harvested from these plants was screened through PCR confirmation.

Confirmation of putative Transformants

Molecular screening

Total genomic DNA was extracted from the young leaves by Sambrook *et al.* (1989) [11]. Amplification of DNA extracted from plants was carried out by gene specific primers at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 60 s, 54 °C for 60 s for annealing and 72 °C for 5 min, with a final extension at 72 °C for 10 min. PCR products were resolved on 1.5% EtBr stained agarose gel in 1× TBE buffer. Gel images were documented using the Gel Documentation system (Bio Rad Gel Doc, USA).

Detached leaf Bioassay

Insect culture

H. armigera larvae used in laboratory bioassay experiments were maintained at Biotechnology Centre, Dr. P.D.K.V. Akola. The H. armigera culture was maintained on semi synthetic diet. The H. armigera neonates were reared in groups of 200 to 250 in Petri dishes having a 2 to 3 mm layer of artificial diet on the bottom. After 5 days the larvae were transferred individually to twelve-cell well plates to avoid cannibalism. The pupae were removed from cell wells, sterilized with 2 percent sodium hypochlorite solution for 2 minutes and kept in a group of 20 in plastic jars containing soil rite. Upon emergence, 10 pairs of adults were released inside an ovipositor cage. Adults were provided with an adult diet 10% honey solution on a cotton swab for feeding. Muslin cloth used for the females to lay eggs was hanged inside the cage as an oviposition substrate. After eggs hatching the larvae were moved to artificial diet and the neonate's larvae were used for bioassay.

Detached leaf bioassay

The detached Leaf bioassays were performed in the laboratory at temperature 27±2 ºC and 65-75 relative humidity (RH) with a photoperiod 12:12 (L: D. hrs). The larva was reared on an artificial diet and 5 days old larvae were used for detached leaf bioassay. The third fully expanded leaf from the top of the pigeonpea plant was selected for the bioassay. The experiment was carried out in 3% agar containing Petri plate. The solidified agar was used as a substratum for holding pigeon pea leaf in a slanting manner inside the Petri plate. The observations were recorded after 5th day as average larval weight in mg; percent weight reduction as compared to control, percentage of leaf area damaged and mortality rate.

Result and Discussion

Confirmation of Putative Transformants

PCR confirmation with NPT II and Cry1 Aabc gene specific primers

For confirming integration of gene of interest through PCR, seeds were collected from each plant. The DNA sample of one representative seed from each plant was extracted and subjected for PCR confirmation with npt II and Cry1 Aabc gene specific primers. Out of 73 DNA samples, four samples were found to be positive with both Cry1Aabc (Plate 1) and nptII (Plate 2) gene specific primers at 750bp and 1000bp respectively.

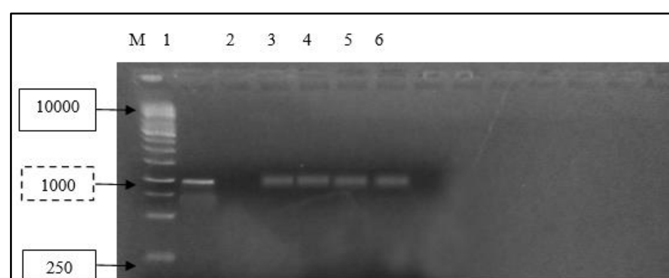


Plate 1: PCR confirmation of putative T1 plants with Cry1Aabc gene specific primer

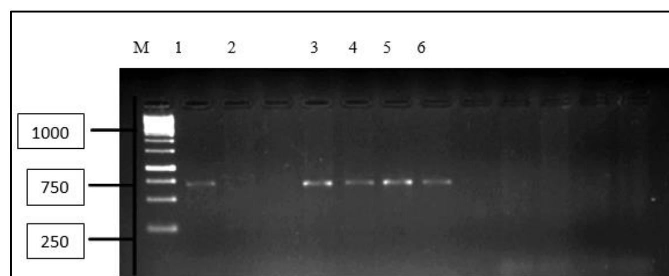


Plate 2: PCR confirmation of putative T1 plants with nptII gene specific primer M: 1Kb marker 1: Positive control (plasmid) 2: Negative control 3-4: Putative sample positive (Floral bud infection) 5-6: Putative sample positive (Embryonic axis infection)

Pigeonpea is less explored with regard to the development of transgenic methods. Generally, poor responses of the investigated genotypes/cultivars to tissue culture initiation and plant regeneration has necessitated development of transformation systems that target apical and axillary meristems in the embryonal axis and plant regeneration plants via meristem culture (Shivaprakash *et al*. 1994; Franklin *et al*. 1998; Geetha *et al*. 1999; Mohan and Krishnamurthy 1998; Singh *et al*. 2002, 2004) [14, 4, 16, 17].

Similarly, Rohini and Sankara Rao reported positive events and increase transformation frequency in peanut and safflower when infected with Agrobacterium that was added successive rounds of experiments have produced some T0 plants. Supartana (2006) in-planta transformation method for rice (Oryza sativa L) using A. tumefaciens strains M-21 and LBA 4404 harboring pIG121-Hm binary vector. The results of the experiment with pBI-121-GUS are very encouraging and indicative of the appropriate conditions followed for

inplanta transformation using pBinAR-Cry1Aabc mobilized into Agrobacterium tumefaciens strain EHA 105.

Detached leaf assay

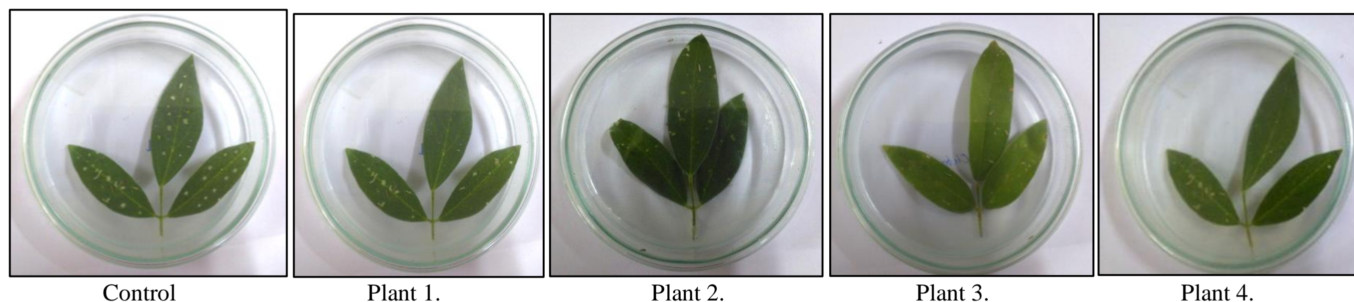
For detached leaf bioassay four plants that were confirmed positive previously by PCR amplification with nptII and Cry1Aabc gene specific primers were selected. 10 neonates of H. armigera larvae per replication were released on the pigeonpea leaves. Observation was recorded on total larvae live after 5 days, the weight of total larvae in (mg) after 5 days, average larval weight in mg, percent weight reduction as compared to control, percentage of leaf area damaged and survival rate (Table 1) As the Cry1Aabc expressions in transgenic plants were comparatively higher than in non transgenic plant.

Table 1: Observation of detached leaf bioassay of PCR positive plants for resistance to *Helicoverpa armigera*

Positive Plant No.	Average larval weight in mg.	Percent weight reduction as compared to control	Percentage of leaf area damaged	Survival Rate
1	2.9	46.9	28.3	70.3
2	3.2	41.3	41.7	65.3
3	3.8	30.6	30.0	60.3
4	3.7	33.1	40.0	68.3
Control	5.5	0.0	76.7	78.3

From the data of detached leaf assay carried out to test the effectivity of positive plants to control the population of H. armigera, it was revealed that the positive plants had expressed Cry protein in a sufficient concentration to reduce the weight of larvae and leaf damage as shown in Table 1. It was recorded that all 4 positive plants resulted in percent reduction in larval weight and leaf area damaged. The positive plant no.1 showed the highest weight reduction of 46.9%, followed by 41.3% in plant no. 2, 30.6% in plant no. 3 and 33.1% in plant no. 4. Similarly the positive plant no. 4 showed the highest reduction in leaf area damage followed by plant no. 2 with 41.7%, plant no. 3 with 30.0% and plant no.1 with 28.3%. The survival of the larvae is also reduced in the positive plants as compared to the control. Plant no.1 recorded 70.3% survival of the larvae whereas 65.3%, 60.0%, 68.3% survival was reported in plant numbers 2, 3 and 4 respectively.

The results revealed that the level of Cry protein expressed in positive plants is able to reduce the weight of larvae and leaf damage. Thus it was concluded from above results that positive plants selected for bioassay study have potential for providing tolerance against H. armigera. The further confirmation in subsequent generation is required to come to a more robust conclusion.



Control Plant 1. Plant 2. Plant 3. Plant 4.

Plate 3: Detached leaf assay for pigeonpea PCR positive plants for Helicoverpa armigera

Gopalswamy (2005) [5] also reported that the transgenic pigeonpea plant carrying Bt Cry1Ab and soyabean trypsin inhibitor genes were molecularly characterized for the presence of insecticidal genes and bioassay were conducted to test their efficacy against the gram pod borer Helicoverpa armigera under laboratory conditions. They observed that the Cry1Ab toxin level present in the leaves of transgenic plant could not inhibit the feeding by the larvae.

Krishna *et al.* (2011) [9] experimented on Agrobacterium-mediated genetic transformation, using embryonic axes explants of pigeonpea both pod borer resistant gene (Cry1Ac) and plant selectable marker neomycin phosphor transferase (nptII). The plant obtained were subjected to multi and no choice test to determine the behavioral responses and mortality through H. armigera bioassays on the leaf and relate their relationship with the expression of Cry1Ac protein which was found to be less in leaf as compared to the floral bud, anther, pod and seed.

Gaifulliana (2007) [3] reported that the levels of Cry1Ab or SBTI toxic proteins present in the transgenic pigeonpea plants were not sufficient to cause a substantial reduction in leaf feeding, survival and growth of H. armigera larvae. As a result, some plants though showed resistance to H. armigera the resistance was not manifested in the progenies, and therefore, there is a need to develop new events with high expression of Cry1Ab or Cry1Ac genes for controlling H. armigera damage in pigeonpea.

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