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Development and molecular characterization of transgenic Pigeon pea carrying *cry2Aa* for pod borer resistance

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Abstract
Pigeonpea is an important legume which belongs to the family *Fabaceae*. It is an excellent source of seed protein content of about 21 per cent. Pod borer or *Helicoverpa armigera* a lepidopteron insect is the most serious and widespread pest of pigeonpea. Two popular varieties Asha and BSMR736 were used for tissue culture independent *Agrobacterium* mediated transformation. The plant to row progeny screening of 216 primary transformants in T_1 generation using cry2Aa specific PCR assay, identified seventeen transformants. Insect bioassay and Cry protein estimation using ELISA conducted simultaneously showed larval mortality ranging from 20% to 70% and the Cry2Aa content from 0.04 to 1.45 µg/g of fresh leaf tissue. Reduction in larval weight and length ranged from 13.32% to 47.13% and 42.33% to 64.95% respectively. Southern blot analysis indicated the single copy integration in selected events. The selected transgenic events can serve as resource for the development of pyramided transgenic pigeonpea in future.

Keywords: Agrobacterium, *in planta* transformation, *Helicoverpa armigera*

Introduction
Pigeonpea is an important pulse crop which is widely cultivated throughout the tropical and sub-tropical regions. It belongs to the family *Fabaceae*. Pigeonpea is a diploid crop with 2n=22 and with a genome size of 808Mbp. In India Madhya Pradesh stands first in the pigeonpea production followed by Maharashtra and Karnataka. In India pigeonpea is cultivated in an area of 38,84,886 ha with production of 28,48,993 tonnes and yield of 7,334 kg/ha (FAO, 2016) [14]. In Karnataka pigeonpea is cultivated in an area of 0.65 million hectare with production-0.26 million tonnes and yield of 406 kg/ha which accounts for 10.69% of total pigeonpea production in India. In Karnataka pigeonpea is largely grown in northern parts, especially in Gulbarga and Bidar districts. Gulbarga (Kalburgi) is known as "pigeonpea bowl" of Karnataka. India alone has to import 200,000 tonnes of grains annually to cope with the ever growing market demand of dry and split pigeonpea seeds (dahl) (Shiferaw *et al*. 2008) [12]. The major reason behind fall in production is the susceptibility of this crop to the devastating lepidopteran pest *Helicoverpa armigera*. *Helicoverpa* causes heavy losses up to 60 per cent with an annual loss estimated to be US $ 400 Million in pigeonpea (Kumar and Morya 2016) [15]. Since traits/genes conferring resistance to pod borer is not available in the primary gene pool of pigeonpea, application of transgenic technology has been considered as one of the feasible options to minimize the problem as it has already been exploited commercially in cotton and a few other crops. The use of genetic transformation technology in crops allowed them to express genes that encode the δ-endotoxins/cry toxins (James 2005) [7] which imparts resistance against lepidopteron pests. cry2Aa toxin one of the several cry toxins reported earlier has been previously used in the development of genetically modified rice and chickpea (Bashir *et al*. 2004, Chen *et al*. 2005, Acharjee *et al*. 2010) [4, 5, 2]. Mode of action of cry2Aa was found to be different from *cry*1Ac owing to the limited sequence homology and different receptor binding epitopes (Morse *et al*. 2001) [8]. These Cry1Ac and Cry2Aa proteins interacted with different receptor binding sites in the insect gut epithelium, where there were no chance of cross-reactivity between these two proteins (Hernandez-Rodriguez *et al*. 2008) [6]. Moreover, Cry1Ac-resistant larvae did not survive on Bt cotton plants producing Cry2Ab protein (Tabashnik *et al*. 2008) [16]. The Cry2Aa proteins of *B. thuringiensis* are promising candidates for management of resistance development in insects due to its distinct structural features and mode of action compared to most commonly used Cry1Ac proteins (Morse *et al*. 2001) [8]. Therefore pigeonpea transgenics carrying cry2Aa were developed in this study with a future aim to utilize them in management of resistance development by combining with other cry genes.

Materials and methods

Pigeonpea varieties *viz*., ICPL 87119 (Asha), moderately resistant to fusarium wilt and sterility mosaic disease and BSMR 736 which is resistant to sterility mosaic disease were taken for transformation. Seeds of these cultivars were obtained from Agriculture Research Station (ARS), Gulburga. Agrobacterium strain LBA4404 carrying cry2Aa gene (kindly provided by by Dr. P. Ananda Kumar, NRC on Plant Biotechnology, IARI, New Delhi as a part of ICAR-NASF funded pulse project) was used for transformation. The confirmation of the presence of cry2Aa gene in *A. tumefaciens* strain LBA4404 was done by PCR amplification of the plasmid DNA. *In planta* transformation method reported by Rao *et al*. (2008) [9] for pigeonpea using embryonic axis as explants with some modification was used for transformation. The seeds were surface sterilized with 70% ethanol and allowed to germinate overnight in sterile distilled water. The seed coat from germinated seeds were removed and embryonic axis attached to single cotyledon were injured using fine needle and infected with *Agrobacterium tumefaciens* strain LBA 4404 harbouring binary vector for 30 min. Vaccum infiltration treatment was given for 20 min at 450 mm Hg to increase the proximity between *Agrobacterium tumefaciens* and embryonic axis. The explants were kept in dark for two days at 25± 2°C for cocultivation on moist germination paper. The well responded seedlings of seven days old were transferred to plastic trays containing sterile coco peat and hardened in green house condition for two weeks period. The well-developed healthy seedlings also called as primary transformants (T_0 generation) were transplanted in earthen pots carrying media mixture of soil and FYM and allowed to grow in transgenic containment facility till harvesting. The putative transformants were identified by sowing the harvested seeds from each primary transformant (T_0) in plant to row progeny manner (up to 30 seeds). The progenies were tested using cry2Aa gene specific PCR assay and transgenic plants (T_1 generation) were identified. Genomic DNA was isolated from young leaves of the putative transgenic and control plants following the modified protocol of Agbagwa *et al.* (2012). The DNA was checked for its purity and intactness and then quantified. The crude genomic DNA was run on 0.8 per cent agarose gel stained with ethidium bromide following a standard method (Sambrook *et al*. 1989) [10] and was visualized in a gel documentation system. The DNA was quantified by using nanodrop Spectrophotometer. PCR components included nuclease free water: 13 µl**,** taq buffer with Mg^{2+} (10X): 2 µl, dNTP's (2mM): 2 µl**,** Primers (forward and reverse: 5 pmol): 2 µl and DNA (100ng):1 µl. The total volume prepared was 20 µl. PCR amplification consisted of pre-denaturation at 94°C for 5 min; denaturation at 94°C for 1min, annealing at 64°C for 30s; extension at 72°C for 2 min, repetition for 32 cycles; and final extension at 72°C for 10 min. The PCR products were separated on 1.2 % agarose gel. Cry 2Aa gene specific primer used was Forward: 5'-GTGGATGGAGTGGAAGAG-3' and Reverse: 5'-GAAGAGGGACCAGATGG-3'.The *Helicoverpa armigera* larvae were collected from fields in the Main Agriculture Research Station (MARS) Dharwad and reared at 26-28 °C and 60% relative humidity in insect culture room. The chickpea artificial diet was used to feed the different growth stages of *H. armigera*. The adult male and female moths are allowed to mate and the first instar larvae obtained were used for bioassays. The transgenics along with control plants were subjected to insect bioassay to assess their resistance to *H. armigera*. The insecticidal spray for pigeonpea plants were stopped prior to one month of conducting bioassay. Fully expanded leaves from 80 days old plants were inserted into water agar gel (2%) in jam bottles. On each leaves ten first instar larvae were released. Larval mortality was recorded at 24, 48, 72, 96 and 110 hr. The larval length and weight recorded on the sixth day of bioassay. The larvae, which failed to show any movement, were considered as dead. The mortality in transformed plant tissues was corrected with Abbott's formula (Abbott 1925) [1]. Three replications were maintained throughout the experiment and standard deviation was calculated. The experimental design used for statistical analysis was completely randomized block design.

The per cent reduction in larval weight was calculated as follows

$$\frac{\text{Average larval weight in control plant} - \text{Average larval weight in transgenic plant}}{\text{Average larval weight in control plant}} \times 100$$

The per cent reduction in larval length was calculated as follows

$$\frac{\text{Average larval length in control plant} - \text{Average larval length in transgenic plant}}{\text{Average larval length in control plant}} \times 100$$

Estimation of Cry protein content

The quantitative estimation of Cry protein in the transgenics was carried out using commercially available Quanti-ELISA plates pre-coated with specific Cry antibody from Envirologix Pvt. Ltd., Hyderabad, India. The sandwich ELISA was carried out according to the manufacturer's instructions. The spectrophotometric measurements were taken by setting microtiter plate reader to 450 nm. Based on the slope obtained from standard graph generated from calibrators the Cry protein concentrations were calculated by using the formula given below

Concentration of Cry protein conc. (µg/mg) =

$$\frac{(\text{Absorbance in test sample} - \text{Absorbance in negative sample}) \times \text{Slope} \times 5}{\text{Weight of sample (gm)}}$$

Southern blot analysis

Transgenic plants which showed relatively high mortality of larvae and Cry protein content were subjected for southern blot analysis. Genomic Southern blot analysis was carried out using the DIG-High Prime DNA Labeling and Detection Kit (Roche Diagnostics, Mannheim, Germany, cat. no. 11745832910) with further modifications.

Result

Development of transgenic pigeonpea carrying cry2Aa gene

Of the 1500 explants subjected for *A. tumefaciens* infection, 216 primary transformants could be established in transgenic containment facility. All the 216 primary transformants were allowed to grow till plant maturity and T_1 seeds were collected. The plant to row progeny screening of 30 seeds from each primary transformants, seventeen PCR positive plants were identified in T_1 generation with cry2Aa specific primers (Fig.1). Out of seventeen plants, twelve were originated from primary transformants designated as *cry2Aa*177, three from cry2Aa178 and two from cry2Aa179. The identified PCR positive plants were grown till maturity and T_2 seeds were harvested.

Characterization of developed transgenic lines in T_1 generation

The insect bioassay of seventeen cry2Aa transgenic plants showed significant variability in larval mortality in leaf. It was noticed that the larval mortality in case of leaf tissues ranged from 20% to 70% (Table.1). The highest larval mortality was recorded in 2Aa177-23 (70%) followed by 2Aa177-26 (Fig. 2) while lowest (20%) in 2Aa 177-36. Reduction in larval weight ranged from 10.72% to 47.13% (Table.1). The highest reduction in larval weight recorded in 2Aa 177-23 while lowest in 2Aa 178-6. Reduction in larval length ranged from 42.33% to 64.95% (Table.1). The highest reduction in larval length recorded in 2Aa177-23 followed by 2Aa177-26 (Fig. 3) while lowest in 2Aa 177-36. The transgenic pigeon pea designated as 2Aa 177-23 was found superior among all other transformants.

Simultaneously ELISA was also conducted (Fig.4) and the transgenic plants showed significant variation in Cry2Aa protein levels among the different transgenic lines. The Cry2Aa protein level in leaf tissue ranged from 0.04 to 1.45 µg/g of fresh leaf tissue (Table.1). Highest Cry2Aa protein level was detected in 2Aa 177-23 while the lowest was in 177-36. A positive correlation observed between insect morality and Cry2Aa protein accumulation with r^2= 0.9521(Fig.5). Southern blotting showed single hybridization signal in 2Aa177-23 and 2Aa-177-26 (Fig.6) indicating single copy integration. Size of the band showing the signal was unique these transformants.

Table 1: Performance of transgenic pigeonpea carrying cry2Aa in T_1 generation

Event No.	Per cent corrected mortality					Per cent reduction in larval weight	Per cent reduction in larval length	Average Cry2Aa protein level (µg/g)
	First day (24hrs)	Second day (48hrs)	Third day (72hrs)	Fourth day (96 hrs)	Fifth day (110 hrs)			
Asha control	0.0	0.0	0.0	0.0	0.0			-0.01
177-21	0.0	0.0	10.0	20.0	30.0	16.46	52.68	0.37
177-22	0.0	3.3	23.3	53.3	53.3	28.31	55.94	0.88
177-23	10.0	10.0	50.0	70.0	70.0	47.13	64.95	1.45
177-26	3.3	10.0	46.7	66.7	66.7	36.59	55.99	1.33
177-28	0.0	3.3	23.3	53.3	53.3	28.84	50.06	0.88
177-29	0.0	6.7	26.7	56.7	56.7	32.00	52.19	1.24
177-34	3.3	6.7	36.7	63.3	63.3	36.59	56.09	1.31
177-35	0.0	0.0	0.0	30.0	33.3	16.46	47.28	0.54
177-36	0.0	0.0	0.0	20.0	20.0	15.58	42.33	0.04
177-37	0.0	40.0	0.0	40.0	40.0	13.85	51.65	0.80
177-40	0.0	40.0	20.0	40.0	40.0	14.42	50.11	0.77
177-42	0.0	0.0	0.0	30.0	33.3	14.12	51.30	0.74
178-6	0.0	0.0	0.0	26.7	26.7	10.72	45.51	0.07
178-7	0.0	0.0	0.0	30.0	33.3	13.86	45.92	0.64
178-14	0.0	0.0	0.0	33.3	33.3	13.32	46.09	0.58
179-9	3.3	10.0	30.0	60.0	60.0	35.05	56.09	1.28
179-11	3.3	10.0	30.0	56.7	56.7	33.33	54.12	1.14
SD	0.0	0.4	0.7	1.9	1.9	1.36	1.02	0.04
CD(5%)	0.0	0.7	1.5	3.8	3.8	2.76	2.07	0.09
CD(1%)	0.0	1.0	2.0	5.1	5.1	3.69	2.77	0.12
CV	0.0	5.6	5.2	5.5	5.3	7.32	2.56	7.25

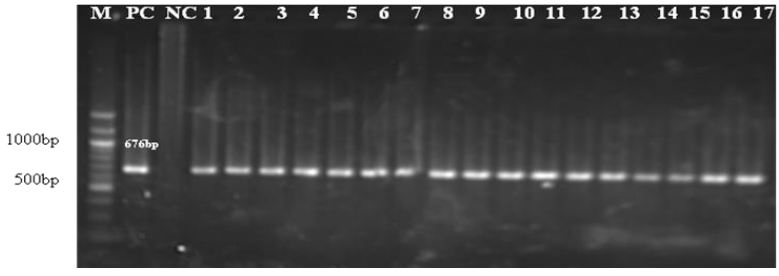

Fig 1: PCR confirmation of progeny of primary transformants in T_1 generation M-100bp ladder, PC-positive control, NC-negative control, lane1-17 – positive Transformants

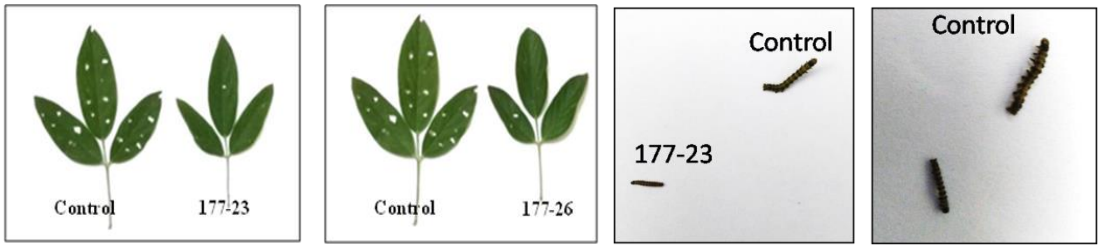

Fig.2 Fig. 3

Fig 2: Detached leaf bioassay performed on T1 transgenic pigeonpea events using *Helicoverpa armigera* first instar larvae

Fig 3: Reduction in *H. armigera* larvae growth at the end of detached leaf bioassay period (5 days)

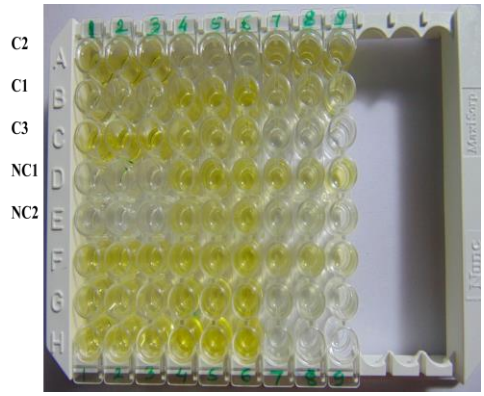

Fig. 4

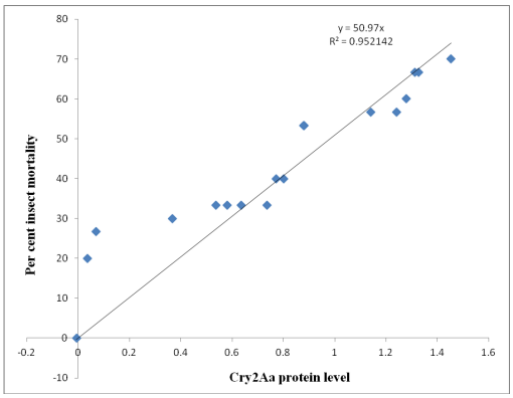

Fig. 5

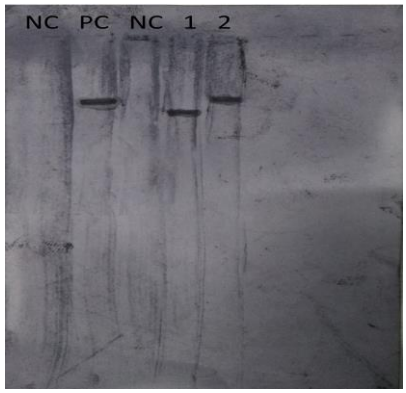

Fig. 6

Fig 4: Quantitative estimation of Cry2Aa protein through ELISA in pigeonpea transgenics in T_1 generation. row wise three replications: C1, C2, C3- calibrators, NC1-kit negative control, NC2-negative plant control remaining wells contain transgenic samples
Fig 5: Correlation between Cry2Aa protein level and per cent insect mortality in transgenic pigeonpea carrying cry2Aa
Fig 6: Southern blot analysis of selected transgenic pigeonpea events, NC-negative control, PC-positive control, 1-177-23, 2-177-26

Discussion

Since the *in vitro* regeneration frequency is low (Rao *et al*., 2008) [9] in pigeonpea, *Agrobacterium* mediated *in planta* transformation was used to generate pigeonpea transformants carrying cry2Aa. The frequency of PCR positive plants obtained in T_1 generation was 1.14%. Though the method has the advantage of tissue culture independent protocol, ease in generation of transgenic plants in a short time, independent of genotype- or variety, identification of transformants is laborious and time consuming. It can be noted here that, of the progeny of 216 primary transformants screened, only 17 PCR positive plants were obtained which were derived from only three primary transformants. Use of easily screenable marker such as herbicide resistance may facilitate quick identification of transformants just by spraying herbicide to the T_1 generation plants. The seventeen PCR positive plants varied significantly with respect to larval mortality and the Cry Protein content. Even some of the T_1 plants generated from the same primary transformants showed significant variation. Transformation is a cellular event; probably primary transformants are chimeric in nature. Site of integration in the plant genome, transgene copy number, internal cell environment, developmental stage of the plant etc., are known to influence the transgene expression. Also two PCR positive plants Viz., 2Aa177-23 and 2Aa-177-26 derived from the same primary transformants were on par with respect to larval mortality and the Cry protein content. However southern blot analysis revealed the independent origin of these transgenic lines.

Maximum mortality observed at the end of 5th day of insect bioassay among the 17 positive plants was only 70%. However Kumar *et al*. (2004) [11] reported 100% mortality with 650ng/ml of purified Cry2Aa protein. Acharjee *et al*. (2010) [2] observed differential resistance to pod borer larvae in chickpea transgenics carrying chimeric cry2Aa driven by Arabidopsis small subunitgene (*atsA*) promoter depending on the level of expression of Cry2Aa protein. Complete resistance to pod borer was observed in high expressing chickpea transgenics carrying modified cry2Aa. Since the transgenic plants generated here are not very efficient in controlling the *Helicoverpa armigera*, further effort is needed to generate transgenics with high resistance to pod borer. Also the events generated here in this study can be crossed with transgenics carrying *cry1Ac* or *cry1F* in order to increase the resistance against pod borer.

Conclusion

The transgenic plants generated here are not very efficient in controlling the *Helicoverpa armigera*, further effort is needed to generate transgenics with high resistance to pod borer. Also the events generated here in this study can be crossed with transgenics carrying *cry1Ac* or *cry1F* in order to increase the resistance against pod borer.

Acknowledgements

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