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DEVELOPMENT OF TRANSGENIC PIGEONPEA EXPRESSING *cry1Ab* THROUGH AGRO-INFECTION OF FLORAL BUDS

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Pigeonpea [*Cajanus cajan* (L.) Millsp., 2n = 22], a legume crop belonging to family Fabaceae, is an important source of protein (22%) in human diet and is also used for fodder, fuelwood and green manure (Krishna *et al.*, 2011). It is cultivated in more than 50 tropical and sub-tropical countries with India ranking first in area and production (Kamble *et al.*, 1998; FAO, 2018). Pigeonpea production is hampered by various stresses. Among the different stresses, spotted or legume pod borer, *Maruca vitrata* Fab. (Crambidae: Lepidoptera) causes up to 24.50 per cent pod damage in early maturing cultivars (Taggar *et al.*, 2019). Management of *M. vitrata* with insecticides is difficult as the larvae feed in a concealed manner by making a webbed mass of inflorescence (Murdock, 2002; Taggar, 2014). Attempts to develop insect resistant pigeonpea through conventional breeding have not been successful due to limited resistant sources in the crossable crop germplasm and incompatibility with wild species (Choudhary *et al.*, 2013). In this regard, integration of *Bacillus thuringiensis cry* gene(s) through transgenesis seems to be a promising approach for development of insect resistance. *Cry1Ab* expressing transgenic cowpea has been reported to efficiently control *M. vitrata* (Adesoye *et al.*, 2008; Mohammed *et al.*, 2014). According to Srinivasan (2008), Cry1Ab protein is most effective (LC_{50} 0.21 ppm) in controlling *M. vitrata* as compared to Cry1Aa, Cry1Ac, Cry1Ca and Cry2Aa with LC_{50} of 0.81, 1.67, 0.48 and 1.06 ppm, respectively.

Recalcitrancy for *in vitro* shoot regeneration coupled with genotypic specificity is a major concern for using *in vitro* transformation approach in pigeonpea (Mohan and Krishnamurthy, 1998). Agro-infection of pricked seed embryo axes is a genotype independent method of *in planta* transformation that avoids *in vitro* shoot regeneration. However, the primary transformants obtained using this method are chimeric (Keshamma *et al.*, 2012). Thus, the main emphasis of the present study was to generate pigeonpea transformants expressing *cry1Ab* following *Agrobacterium*-mediated floral dip method. The method is easy to perform and known to produce transgenic plants that do not display chimerism. The healthy seeds of early maturing cultivar AL201 were sown in earthen pots (containing soil, cocopeat and field manure in 3:1:1 ratio) in a transgenic glasshouse. The plants were watered twice a day during summer season and once a day during winter season. At flowering stage, ten morphologically healthy plants were shifted to transgenic screenhouse maintained at 28°C/24°C temperature during day/night, photoperiod of 10 h and relative humidity of 70%.

pCambia 3301 vector in *Agrobacterium tumefaciens* strain LBA4404 obtained from Dr. Rohini Sreevathsa, ICAR - National Institute for Plant Biotechnology, New Delhi was used for genetic transformation. It carried *cry1Ab* gene under the control of Ubiquitin promoter and Nos terminator, *bar* and *GUS* as selectable and scorable marker genes, respectively (Fig 1a). Besides, it had *nptII* for plasmid selection.

A single *Agrobacterium* colony was confirmed and used to inoculate sterilized liquid YEP medium (10 ml, prepared by dissolving 10 g yeast extract, 10 g peptone and 5 g NaCl in 1 L distilled water; pH 7.0) supplemented with kanamycin (100 mg/L) for plasmid selection. The suspension was incubated at 28°C overnight with shaking at 250 rpm to obtain primary culture that was diluted in 1:100 ratio and incubated for 18 to 24 h with shaking to raise secondary cultures (OD_{600} 0.6-0.8). These were centrifuged at 4500 rpm for 10 min at room temperature to pellet bacterial cells, which was dissolved in re-suspension solution containing sucrose (5.0% w/v) and Silwet L-77

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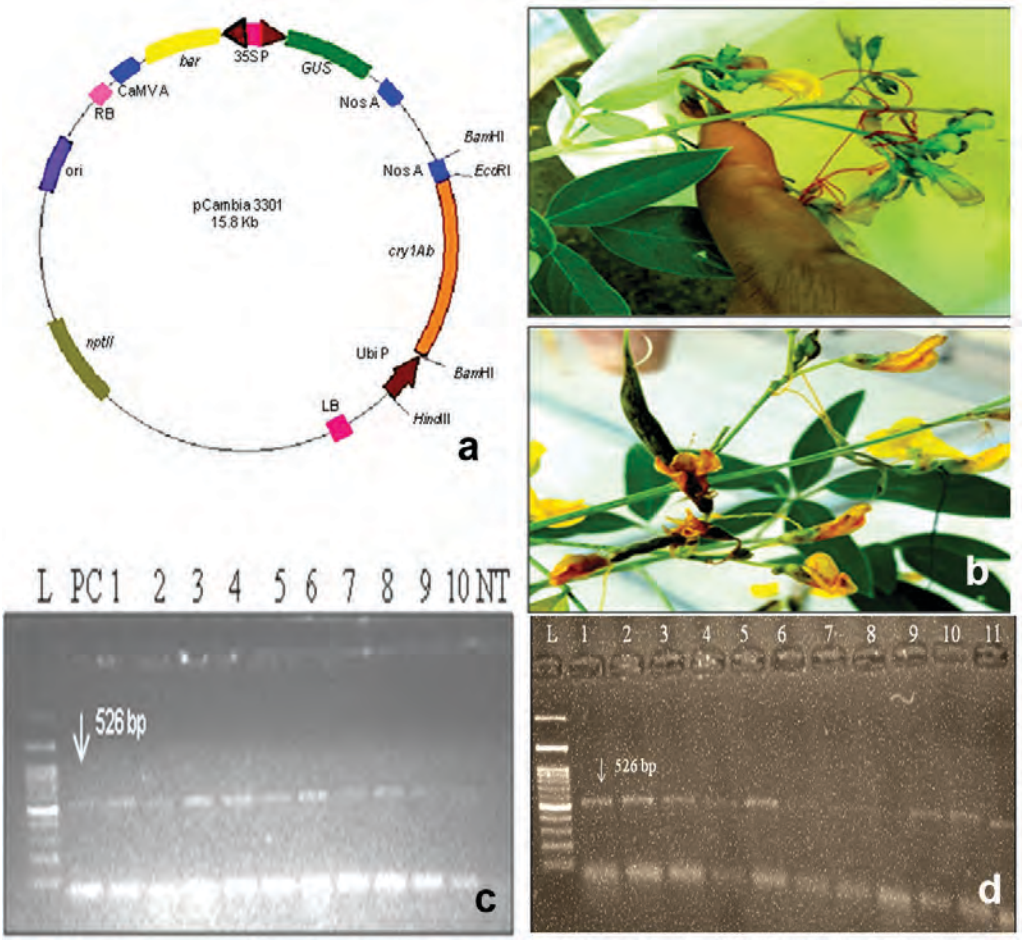


Fig. 1 **Vector map showing *cry1Ab* under the control of Ubiquitin promoter and Nos terminator. b. Genetic transformation of pigeonpea variety AL201: Dipping of tagged floral buds in *Agrobacterium* suspension (above), and (below) pods formed from treated buds. c. PCR analysis on putative T_1 plants. [L: 100 bp ladder (GeneRuler, USA); PC: plasmid control; NT: non-transgenic plant; 1 to 10: *cry1Ab*-positive samples]. d. PCR analysis on T_1 transgenic plants. [L: 100 bp ladder; 1 to 3: *cry1Ab*-positive apical, middle and basal leaf samples of AL201-4; 4 to 5: plasmid control; 6 to 8: apical, middle and basal leaf samples of NT plant; 9 to 11: *cry1Ab*-positive apical, middle and basal leaf samples of AL201-9]**

(0.05% v/v, i.e. 500 µl/L) to obtain *Agrobacterium* broth.

The floral buds were infected following Clough and Bent (1998) method with minor modifications. The fully opened, yellow colored flowers were removed, whereas green unopened floral buds (139 in number) were tagged, opened with a sterile needle and immersed in bacterial broth (Fig 1b) for various durations (5 s, 7 s, 9 s) with continuous gentle shaking of buds; these were re-infected either after 24, 25, 26 or 27 h of first agro-infection (Table 1). This was practiced for optimization of infection timings, maximizing frequency of transformation and minimizing chances of *Agrobacterium* overgrowth and tissue necrosis. The flowering stage containing a large number of unopened buds is considered optimal for genetic transformation as it results in high frequency of transgenic plants (Bechtold *et al.*, 1993; Clough and Bent, 1998). Sucrose helps *Agrobacterium* to remain in contact with plant tissues for a longer period and has a role in *vir* gene induction (Cangelosi *et al.*, 1990) that increases transformation efficiency. Silwet L-77 reduces surface tension and facilitates bacterial entry into plant's intercellular spaces (Christou and McCabe, 1992).

Post each treatment, the plants were kept overnight under low light conditions (3000 lux) that prolongs the availability of surface water through which *Agrobacterium* cells swim and reach the target tissues (Clough and Bent, 1998). The plants were grown in transgenic screenhouse till pod formation that occurred after 14 to 21 days of treatment (Fig 1b), and then shifted to transgenic glasshouse for further development. A total of 454 seeds were harvested after 45 days of treatment and sown in seedling trays (containing mixture of soil, cocopeat

Table 1. Genetic transformation of AL201 through agro-infection of floral buds

Exp. no.	Agro-infection duration (s)	Re-infection after (h)	No. of floral buds treated	No. of T_1 seeds harvested	No. of T_1 plants analyzed by PCR	No. of *cry1Ab*-positive plants
1	5	24	25	80	80	0
2	7	25	43	143	143	3
3	7	26	45	155	155	7
4	9	27	26	76	76	0
Total			**139**	**454**	**454**	**10**

and field manure) in transgenic glasshouse to obtain putative T_1 plants (Table 1). The non-transgenic (NT) plant of AL201 was raised as control.

For PCR, genomic DNA was isolated from young leaves of 2 month-old putative T_1 plants and NT plant using CTAB method (Murray and Thompson, 1980), quantified through agarose gel (0.8% w/v) electrophoresis and screened using *cry1Ab* specific internal forward 5′-CTATCCCATTGTTCGCAGTCCA-3′ and reverse 5′- GTGTCCAGACCAGTAATACTCTCC-3′ primers. The reaction was performed in 20 µL containing 20 ng/µL of genomic DNA (2 µL), 5 µM of each primer (0.6 µL), 10 mM dNTP mix (0.3 µL), 25 mM $MgCl_2$ solution (0.8 µL), 5X Green GoTaq buffer (2.0 µL), 5 units/µL GoTaq polymerase (0.2 µL) [Promega, USA] and sterile water (3.5 µL) in an Eppendorf thermal cycler. The plasmid DNA was used as positive control. PCR was carried out by initial denaturation at 95 ºC for 5 min, followed by another denaturation at 95 ºC for 1 min, primer annealing at 60 ºC for 1 min, DNA synthesis at 72 ºC for 2 min (35 cycles), final extension at 72 ºC for 7 min and held at 4 ºC. The amplified products were electrophoresed in agarose gel (1.5% w/v) at 5 V/cm for 1.5 h, visualized under UV light and photographed using gel documentation system (Analytik Jena, Germany). The product sizes were determined with the help of 100 bp DNA ladder (GeneRuler, USA). The per cent transformation efficiency was calculated as: (Number of T_1 plants showing presence of *cry1Ab*/ Number of floral buds agro-infected) × 100.

The results revealed that from 454 putative T_1 plants, 10 showed amplification of 526 bp corresponding to *cry1Ab* (Fig 1c) representing an overall transformation efficiency of 7.19 per cent. Agro-infection for 7 s and re-infection after 26 h gave maximum number of seven *cry1Ab*-positive plants, followed by three plants obtained from agro-infection for 7 s and re-infection after 25 h, whereas treatment for 5 or 9 s and re-treatment after 24 h or 27 h did not result in any *cry1Ab*-positive plant (Table 1). Clough and Bent (1998) reported transformation frequency in the range of 0.23 to 0.47 per cent (based on kanamycin selection) in *Arabidopsis* through dipping of primary and secondary bolts (with a few open flowers) in *Agrobacterium* culture containing sucrose and Silwet L-77. Likewise, transformation efficiency of 0.44 per cent (based on paromomycin selection) in wheat by dipping spikes, 3.3 per cent (based on

Supplementary Table S1: Determination of Cry1Ab content in leaf tissues of T_1 transgenic and non-transgenic (control) pigeonpea plants through ELISA

Step number 1: Optical density values			
Cry1Ab calibrator	**OD values**		
	R_1	**R_2**	**R_3**
NC	0.05	0.05	0.05
C1	0.45	0.45	0.44
C2	1.17	1.17	1.16
C3	2.66	2.66	2.65
Leaf tissue sample			
T_1 transgenic plant			
AL201-1	1.64	1.62	1.55
AL201-2	1.66	1.64	1.62
AL201-3	1.44	1.45	1.45
AL201-4	2.06	2.13	2.08
AL201-5	2.05	2.06	2.04
AL201-6	1.42	1.44	1.40
AL201-7	1.54	1.55	1.53
AL201-8	1.70	1.64	1.67
AL201-9	1.72	1.74	1.70
AL201-10	1.44	1.45	1.45
AL201 (Non-transgenic plant)	0.05	0.06	0.05

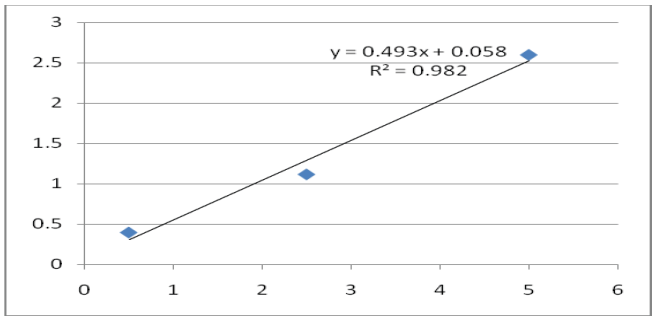
NC is Negative control, C1 is 0.5 ppb Cry1Ab calibrator, C2 is 2.5 ppb Cry1Ab calibrator, C3 is 5 ppb Cry1Ab calibrator

Step number 2: Calculate subtracted OD values¥			
Cry1Ab calibrator	Subtracted OD values		
	R_1	R_2	R_3
NC	0.00	0.00	0.00
C1	0.40	0.40	0.39
C2	1.12	1.12	1.11
C3	2.60	2.60	2.59
Leaf tissue sample			
T_1 transgenic plant			
AL201-1	1.59	1.57	1.50
AL201-2	1.61	1.59	1.57
AL201-3	1.39	1.40	1.40
AL201-4	2.01	2.08	2.03
AL201-5	1.67	1.62	1.63
AL201-6	1.37	1.39	1.35
AL201-7	1.49	1.5	1.48
AL201-8	1.65	1.59	1.62
AL201-9	2.00	2.01	1.99
AL201-10	1.39	1.40	1.40
AL201 (Non-transgenic plant)	0.00	0.00	0.00

¥The subtracted OD values are calculated as OD of each calibrator - mean OD of NC, and OD of each leaf tissue sample - mean OD of NC

Step number 3: Generate linear scale graph of mean OD of each calibrator against its Cry1Ab content	
Cry1Ab content in ppb (x value)	Mean OD (y value)
C1	0.40
C2	1.12
C3	2.60

Use above 'x' and 'y' values to generate the regression equation y = mx + b, where m is slope and b is y intercept. Then generate linear scale graph by inserting scatter chart in Microsoft Excel sheet.



Cry1Ab content in ppb (x axis)

Step number 4: Determination of Cry1Ab content in ppb from the graph			
Cry1Ab calibrator	Cry1Ab content (ppb)		
	R_1	R_2	R_3
NC	0.00	0.00	0.00
C1	0.69	0.69	0.67
C2	2.25	2.25	2.23
C3	5.15	5.15	5.13
Leaf tissue sample			
T_1 transgenic plant			
AL201-1	3.10	3.06	2.92
AL201-2	3.14	3.10	3.06
AL201-3	2.70	2.72	2.72
AL201-4	3.95	4.10	4.00
AL201-5	3.26	3.16	3.18
AL201-6	2.66	2.70	2.62
AL201-7	2.90	2.92	2.88
AL201-8	3.22	3.10	3.16
AL201-9	3.93	3.95	3.91
AL201-10	2.70	2.72	2.72
AL201 (Non-transgenic plant)	0.00	0.00	0.00

Step number 5: Determination of Cry1Ab content in ppm€			
Cry1Ab calibrator	Cry1Ab content (ppm)		
	R_1	R_2	R_3
NC	0.00	0.00	0.00
C1	0.15	0.15	0.14
C2	0.49	0.49	0.49
C3	1.13	1.13	1.12
Leaf tissue sample			
T_1 transgenic plant			
AL201-1	0.68	0.67	0.64
AL201-2	0.69	0.68	0.67
AL201-3	0.59	0.59	0.59
AL201-4	0.87	0.90	0.88
AL201-5	0.71	0.69	0.70
AL201-6	0.58	0.59	0.57
AL201-7	0.63	0.64	0.63
AL201-8	0.70	0.68	0.69
AL201-9	0.86	0.87	0.86
AL201-10	0.59	0.59	0.59
AL201 (Non-transgenic plant)	0.00	0.00	0.00

€(Protein content in ppb x dilution factor x weight of leaf tissue)/1000

hygromycin selection and PCR) in maize by treating corn silk, 50 to 60 per cent (based on PCR) in false flax through dipping of inflorescence and 0.8 per cent (based on herbicide selection) in *Camelina sativa* by plunging buds and flowers in *Agrobacterium* solution has been documented (Zale *et al.*, 2009; Liu *et al.*, 2012; Mu *et al.*, 2012; Bastaki and Cullis, 2014). The agro-infection of floral buds is a relatively fast method of genetic transformation as T_1 seeds are obtained after about 45 days of agro-infecting flower buds. The agro-infection of axillary bud region of *in vitro* germinated pigeonpea seedlings resulted in T_0 plants in 120 days (Sharma *et al.*, 2006).

The protein content in leaf tissues of 10 *cry1Ab*-positive T_1 and NT plants was determined through ELISA using QuantiPlate kit™ (EnviroLogix, USA) as described by Singh *et al.* (2021), calculated in ppm as: (Protein content in ppb x dilution factor x weight of leaf tissue)/1000 (Supplementary Table S1). The data in triplicate was analyzed for mean ± SD using Microsoft Excel 2010 software revealing Cry1Ab content as: AL201-1: 0.66 ± 0.02, AL201-2: 0.68 ± 0.01, AL201-3: 0.59 ± 0.0, AL201-4: 0.88 ± 0.015, AL201-5: 0.70 ± 0.01, AL201-6: 0.58 ± 0.01, AL201-7: 0.63 ± 0.005, AL201-8: 0.69 ± 0.01, AL201-9: 0.86 ± 0.005, AL201-10: 0.59 ± 0.0 ppm, whereas non-transgenic plant did not exhibit any Cry1Ab content. Manohar (2014) developed *cry1Ac* transgenic pigeonpea by *Agrobacterium*-mediated *in planta* transformation of flowers expressing protein content in the range of 0.22 to 0.67 ppm in T_2 plants.

The genomic DNA from apical, middle and basal leaves of T_1 transgenic plants AL201-4 and AL201-9 (exhibiting high protein content) was analyzed for presence of *cry1Ab* through PCR using transgene specific internal primers. The leaves from non-transgenic AL201 plant were used as control. The results revealed amplification of 526 bp corresponding to *cry1Ab* in different leaf tissues of the two T_1 plants (Fig 1d). The agro-infection of floral buds has been reported to give rise to T_1 plants that are non-chimeric or genetically uniform in nature as transformation usually occurs in gametophytes or freshly formed embryos or developing ovules that exclusively contain meristematic tissues (Clough and Bent, 1998; Desfeux *et al.*, 2000). On the other hand, the primary pigeonpea transformants developed through *Agrobacterium*-mediated *in planta* transformation of seed embryo axes lead to generation of chimeric T_0 plants (Rao *et al.*, 2008; Kaur *et al.*, 2016). The T_1 plants showing *cry1Ab* presence in different leaf samples were selfed, and the pods were plucked at maturity.

Five seeds each of AL201-4 and AL201-9 T_1 plants were sown; one month-old T_2 progeny plants were examined through qualitative ELISA kit (Immuno Farm Sciences, Hyderabad) following method outlined by Singh *et al.* (2023). A leaf sample was noted as negative for Cry1Ab, if absorbance was less than cut off value (0.2 + absorbance of negative control), whereas, a sample having absorbance more than cut off value was regarded as positive for the protein. All T_2 plants expressed the transgene as was evident from color development; the absorbance values were: 1.43, 2.18, 2.26, 2.47, 2.88 for AL201-4 samples, and 1.28, 1.94, 2.10, 2.80, overflow for AL201-9 samples with a cut off value ranging from 0.59 to 0.85, implying that the T_2 plants were positive for *cry1Ab* expression, however with varied level of transgene expression. This may be due to the effect of protected conditions under which plants are grown (Benfey *et al.*, 1990) or interaction of endogenous plant genes with the transgene (Jadhav *et al.*, 2020).

In conclusion, *cry1Ab* was integrated in pigeonpea through agro-infection of floral buds as confirmed by the presence and expression of transgene in T_1 and T_2 plants by PCR and ELISA, respectively. The *in vitro* bioassay on pods of transgenic pigeonpea plants would help in determining transgene effect on *M. vitrata* larvae. Further, this study provides a pathway for the researchers to explore the possibility of increasing the transformation efficiency using a strong promoter, such as CaMV 35S following this simple transformation method.

Authors' contribution

Conceptualization of research work and designing of experiments (AK, JSS); Execution of field / lab experiments and data collection (MS, IS); Analysis of data and interpretation (MS, AK, GKT, SS, KK); Preparation of manuscript (MS, AK,JSS).

Conflicts of interest

The authors declare that there are no conflicts of interest. The necessary IBSC permission was taken (approval number DR 192-222 dated 03.01.2017) for carrying out this research.

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