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ARTICLE

In planta Transformation Strategy to Generate Transgenic Plants in Chickpea: Proof of Concept with a *cry* Gene

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The paper presents a non-tissue culture-based transformation of chickpea using *cry*1AcF gene with 5′UTR. The protocol involves raising of plant transformants (T0 plants) directly from *Agrobacterium*-infected young seedlings. The apical meristem of the seedling axes were targeted for transformation. The resulting chimeric plants were allowed to grow in the greenhouse and the transgenics were analysed in the T1 generation. The T1 generation plants were raised in the greenhouse and initial screening was carried out in 109 plants using ELISA for the expression of the cry1AcF protein. On the basis of this, the plants were grouped as non-transformants and transformants, expressing low and high level of the cry protein. The plants expressing the cry1AcF protein in the range of 2.06–9.70 µg/g fr wt were selected for further analysis. Bioefficacy of these 44 plants against *Helicoverpa armigera* allowed identification of 14 plants that not only accumulated good amount of protein but were also effective against *Helicoverpa*. Molecular analysis by PCR for the amplification of both the *cry*1AcF and *npt*II genes confirmed the transgenic nature of the selected plants. The protocol ensured generation of transgenic chickpea plants with considerable ease in a short time and might be applicable across different genotypes/cultivars of the crop and offers immense potential as a supplemental or an alternate protocol for generating transgenic plants of difficult-to-regenerate crops.

Keywords: Chickpea (*Cicer arietinum*), *in planta* transformation, *Agrobacterium*, *cry* gene, transgenics.

Introduction

Cicer arietinum (chickpea) is a protein-rich, food legume crop grown widely in India and in 40 countries throughout the world. Chickpea ranks third among the world's pulse crops after dry bean and dry pea. Despite its significant nutritional value, the grain productivity is adversely affected and the crop suffers heavy losses primarily due to various biotic factors. Field infestation of Lepidopteron pod borer insects, *Helicoverpa armigera* and *Heliothis virescens* cause 22–35% damage. Cut worms (*Agrotis* sp.), leaf miner (*Liriomyza cicerina*, *Phytomyza lathyri*), aphids (*Aphis* sp.), *Callsobruchus* species (storage pests) are the other pests which cause damage to the crop. Other than these, fungal diseases also lead to reduction in yield. A screening of cultivated genotypes has not identified inherent resistance (Sharma and Ortiz, 2000), breeders are turning to wild annual *Cicer* species as a possible source of desired traits. Even interspecific hybridization with chickpea has been greatly unsuccessful (Ahmad *et al.*, 1988), and the wild species have not responded well to introgression through conventional breeding techniques for yield improvement (Van Rheenen *et al.*, 1993). Therefore emphasis is to find resistance sources from wild annual *Cicer* species. From this context, we need to resort to biotechnological approaches for crop improvement of chickpea against Lepidopteron insects using insecticidal proteins.

Introduction of Lepidopteron-specific toxin encoding genes of *Bacillus thuringiensis* into chickpea by genetic manipulation is a promising option for developing insect resistance. Major pre-requisite for the development of transgenic crops is a successful transformation protocol. Legumes are generally considered recalcitrant. Chickpea being a legume and a recalcitrant species, successful transformation protocol is still a limiting factor (Chakraborti *et al.*, 2009). Nevertheless, a protocol that is devoid of tissue culture would be advantageous in chickpea. The technique that minimizes or avoids plant tissue culture compo-

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nent is called the *in planta* transformation protocol. The feasibility of the *in planta* transformation protocols has been tested in *Arabidopsis thaliana* (Feldman and Marks, 1987), soybean (Chee *et al.*, 1989) *Medicago truncatula* (Trieu *et al.*, 2000), buck wheat (Bratic *et al.*, 2007), wheat (Supartana *et al.*, 2006) and rice (Supartana *et al.*, 2005) which were difficult to regenerate by *in vitro* techniques. Our group has developed a similar meristem-directed *in planta* transformation strategy. It essentially involves *in planta* inoculation of embryo axes of germinating seeds and allowing them to grow into seedlings *ex-vitro*. The success of this methodology has been seen in many species like sunflower (Sankara Rao and Rohini, 1999), groundnut (Rohini and Sankara Rao, 2000a,b, 2001), safflower (Rohini and Sankara Rao, 2001), pigeon pea (Rao *et al.*, 2008), bell pepper (Manoj Kumar *et al.*, 2009) and field bean and cotton (Keshamma *et al.*, 2008a,b). The present paper describes transformation of chickpea cv. JG-11 with 5′utr cry1AcF using the *in planta* transformation strategy and analysis of the T1 generation plants.

Materials and methods

Agrobacterium tumefaciens strain and plasmid vector

Binary vector, pBIN AR with Cry1AcF was developed by Dr P. Anand Kumar, NRCPB, IARI, New Delhi. A 525 bp UTR sequence was tagged to the 5′ region of the gene Cry1AcF (Figure 1) at the *Sma*I site. The 5′utr cry1AcF is under the control of 35s CaMV promoter and ocs terminator. The binary vector has *npt*II under the control of Nos promoter and terminator as the selectable marker.

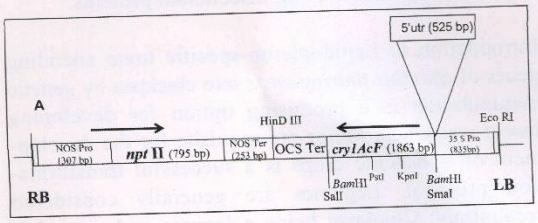

Figure 1. pBinBt8 derivative Ti-plasmid construct *cry1AcF :: npt*II used for chickpea transformation. The *cry1*AcF coding region was inserted as a *BamHI–SalI fragment between the 35S* promoter and the OCS terminator in the pBinBt8 vector. The *npt*II gene conferring resistance to kanamycin was used as selectable marker for chickpea transformation.

Plant material and in planta transformation

Cicer arietinum Desi variety JG-11 seeds were surface sterilized with Bavistin for 20 min, and then rinsed 3–4 times thoroughly in distilled water. The seeds were imbibed overnight with sterile distilled water and were placed on wet blotting paper in petri plates, in dark for germination.

In planta transformation protocol (Keshamma *et al.*, 2008) was followed to develop the primary transformants in chickpea. The meristem of 2-day-old germinating seedlings was pricked with a needle and immersed in the *Agrobacterium* suspension for 1 h. Following infection, the seedlings were transferred to soilrite in bottles and maintained for a week under growth room conditions before being shifted to greenhouse. The chimeric T0 plants thus obtained were maintained in greenhouse and T1 seeds were harvested. T1 plants were screened for expression and efficacy of gene along with molecular confirmation.

Expression analysis by ELISA

Qualitative estimation of Cry1AcF expressed in transformed chickpea plantlets was made using a sandwich ELISA. The pre-coated ELISA plates from Desigen, India, were used for quantitative determination of expressed Cry1AcF protein in transgenic chickpea plants. Absorbance was measured at 450 nm by a microplate ELISA reader. Total soluble protein in plant extracts was assayed by Bradford's method (Bradford, 1976).

Bioefficacy analysis

Entomocidal activity of Cry1AcF expressed in the tissues of the T1 chickpea transformatnts was assayed through leaf feeding bioassay, using neonate larvae of *Helicoverpa armigera*. Larvae of *H. armigera* were initially reared on artificial diet. Young chickpea leaves were kept turgid by wrapping the petiole with wet cotton. Subsequently, the leaves were challenged with 10 neonate larvae of *H. armigera*. Observations were recorded daily for four days on the number of dead and live larvae, percentage of leaf damage and the leaf condition.

Molecular analysis

Genomic DNA was extracted from chickpea leaves following the CTAB method (Dellaporta *et al.*, 1983).

PCR was performed with the genomic DNA using *cry*1AcF gene-specific primers as well as *npt*II gene-specific primers. Nested PCR was performed for the *cry*1AcF gene products. In this reaction, the PCR product generated by gene-specific primers was used as a template and nested PCR carried out. To amplify all the fragments, PCR was initiated by a hot start of 94° for 4 min followed by 31 cycles of 94° for 1 min, 58° for 45 sec, 72° for 1 min with a final extension of 10 min. The amplification was confirmed by 0.8% agarose gel electrophoresis.

Statistical analysis

Data was analysed using MS excel software. Mean values of all the plant parameters were subjected to ANOVA (Sokal and Rohlf, 1969). Correlation and regression analysis was done following the method of Snedecor and Cochran (1967). Scatter plots and frequency distribution graphs were generated where necessary for representing the data.

Results

In planta transformation of chickpea variety JG-11

Approximately 50 seedlings were subjected to *in planta* transformation. Twenty seven plants survived after shifting to the pots in the greenhouse. Under the greenhouse conditions, the plants grew normally, flowered and set pods. These plants were designated as the T0 generation plants. Seeds were harvested, T1 generation plants generated and analysed for the transformants.

Analysis of T1 generation plants

Expression analysis by ELISA: Initial analysis of the T1 generation plants was by ELISA. All the 109 plants obtained from 27 T0 plants were analysed for expression. Approximately 40% of the plants showed perceptible levels of cry protein (Figure 2). These 44 plants with cry protein content in the range of 2.06–9.70 µg/g fr wt were selected as putative transgenic plants and analysed further. Among these plants, only seven showed >6 µg/g fr wt of cry protein. However, all the putative transformants were assessed for bioefficacy by subjecting to leaf bioassay against *H. armigera*.

Bioassay of transgenic chickpea harbouring Cry1AcF against H. armigera: Bioefficacy of the plants against *Helicoverpa* also revealed significant variability in larval mortality and leaf damage (Figure 3A and 3B). The effect of the *cry*1AcF gene was also seen on the larva as there was a considerable difference in the size of the larva that fed on the transgenics and wild type (Figure 3C). The damage varied between 5% and 82.5%, whereas mortality varied between 10% and 100% (Figure 3D). A strong co-relation was seen between these two parameters (Figure 3E). These experimental results gave clear evidence about the gene integration, expression and efficacy of the cry1AcF protein.

Expression and efficacy analysis therefore allowed the identification of putative transformants (Figure 3F). On the basis of analysis, 14 plants were selected that showed high cry protein with good bioefficacy. Further, these plants were confirmed for the presence of transgene at molecular level.

Molecular analysis

PCR analysis was carried out with the genomic DNA of the selected chickpea plants. Amplification of the expected fragments (Figure 4A and B) with both the *cry*1AcF gene and *npt*II gene-specific primers in all the 14 plants confirmed the integration of the transgenes in the chickpea genome. Further, nested PCR for the confirmation of the amplification of the *cry*1AcF gene in some randomly selected plants proved the authenticity of the PCR products (Figure 4C).

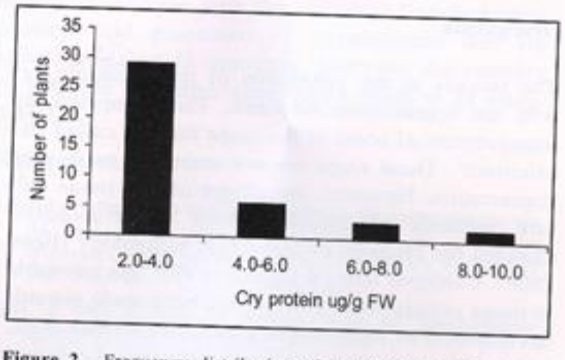

Figure 2. Frequency distribution of putative transgenics transformed with utr-*cry1AcF* gene for the quantity of cry protein in 44 T1 generation plants.

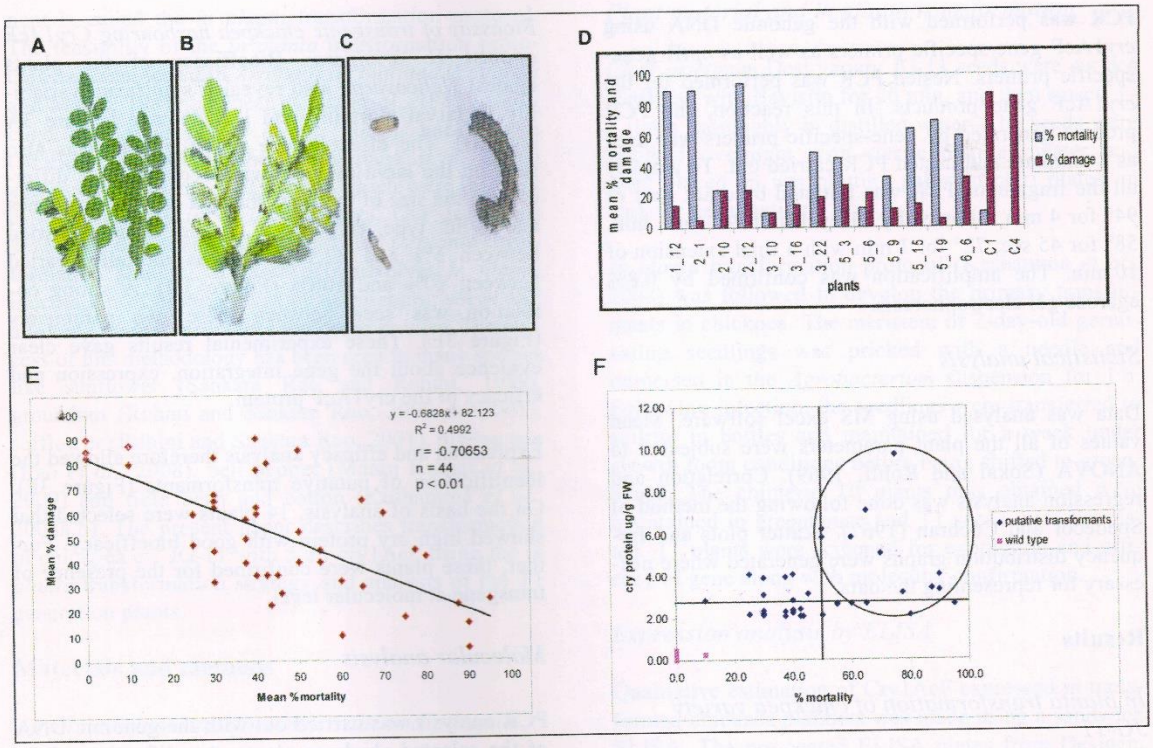

Figure 3. Analysis of T1 generation plants. A–C: Bioefficacy analysis of chickpea plants against *Helicoverpa armigera*; A, Leaf from a transgenic plant; B, Leaf from a wild type plant; C, Difference between the size of the larvae that fed on the wild type and transgenic plant. D, Representative graph showing the variation in the performance of the transgenic plants in the bioassay when compared to the wild type; in each pair of bars, the first bar represents the percentage of mortality and the second percentage of damage. C1 and C4 are wild type. E, Relation between the performance of neonate larvae of *H. armigera* in bioassays of detached leaves of selected putative transgenic plants of T1 generation with respect to percentage of mortalities of the two larvae and percentage of leaf damages by the larvae. The relationship indicated is only for the transgenic plants and the hollow triangles represent the data for control plants. F, Selection of T1 generation plants based on protein accumulation by ELISA and percentage of mortality. The plants within the circle denote the selected plants with high mortality and more protein accumulation.

Discussion

The success in the generation of transformants lies with the regeneration response. This hampered the improvement of some of the crops that are called 'recalcitrant'. These crops are not amenable to *in vitro* regeneration. However, the advent of non-tissue culture methods of transformations in *Arabidopsis* changed the scenario of transgenic technology (Bent, 2006). Chickpea being a legume is also less amenable to tissue culture. Still, efforts have been made towards development of regeneration protocols (Sarmah *et al.*, 2004). However, there are limited reports of efficient rooting and establishment of chickpea plants in the greenhouse. Probably this was the reason for limited progress in genetic transformation of chickpea (Senthil *et al.*, 2004). The few reports published on transgenic chickpea plants were using particle bombardment or *Agrobacterium*-mediated transformation (Kar *et al.*, 1997; Sarmah *et al.*, 2004; Sanyal *et al.*, 2005; Indurker *et al.*, 2007). Further, there are reports of agronomically important traits being introduced into chickpea (Sarmah *et al.*, 2004; Sanyal *et al.*, 2005) and very recently, a lectin gene was stably integrated and evaluated (Chakraborti *et al.*, 2009). Still, serious efforts are needed to explore regeneration and transformation protocols to engineer agronomically desirable genes. In this direction, development of a transformation protocol that avoids or minimizes tissue culture is an alternative.

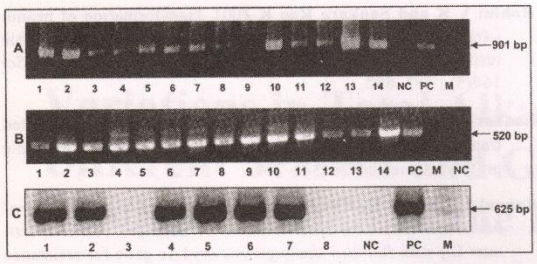

Figure 4. PCR analysis of the T1 generation plants. A, PCR analysis for the amplification of the *cry*1AcF gene. Lanes 1–14: DNA from the transgenic plants. Lane NC: DNA from wild type plant; Lane PC: DNA from the vector. Lane M: marker. B, Nested PCR for the amplification of *npt*II gene. Lanes 1–14: DNA from the transgenic plants. Lane NC: DNA from wild type plant. Lane PC: DNA from the vector. Lane M: marker. C, Nested PCR for the amplification of *cry*1AcF gene. Lanes 1–8: DNA from the transgenic plants. Lane NC: DNA from wild type plant; Lane PC: DNA from the vector. Lane M: marker.

The transformation protocol presented in the paper is an *in planta* protocol which directs the *Agrobacterium* to the differentiated cells of the meristem. This protocol therefore results in the chimeric plants with some cells transformed. However, the generation of stable transformants depends on the type of cells transformed because the cells giving rise to reproductive structures if transformed will develop into stable transformants. Therefore, this requires generation of a large number of T0 plants which would eventually give rise to a larger number of T1 plants. This requires a stringent screening of the T1 generation plants for the selection of not only putative transformants but also high expressing plants. Different methods of screening can be done for the evaluation of T1 generation plants. In these *in planta* transformation strategies, often, the screenable marker gene is made use wherein the seedlings are germinated in the presence of the selectable marker at levels that are lethal to the wild type (Feldmann and Marks, 1987). This strategy is followed in most of the *in planta* strategies by several other groups and also in our earlier studies (Keshamma *et al.*, 2008a, b). Zhao Shuand *et al.* (2008) standardized kanamycin at 200 mg l^{-1} as optimum concentration for screening of transgenic mustard (*Brassica juncea*) seeds. Kojima *et al.* (2000) screened T_1 seeds of buck wheat on geneticin at a concentration of 20 µg ml^{-1} for five days. However, the target gene can also be used for screening of the T1 generation plants at both molecular level and expression level. Earlier, transgenic groundnut harbouring the *cry*1AcF gene (Keshamma *et al.*, 2008a, b) was screened using PCR as a strategy. But this kind of screening would not be rigorous enough to select high expressing transgenics. Therefore screening transformants based on the expression of target gene may not only provide information on integration but also the transgene protein expression. This feature has been exploited in the present study and screening was carried out in the T1 generation plants by ELISA. Analysis by ELISA allowed identification of both transformants and high expressing lines. Among the 109 analysed plants, 44 showed significant expression of the cry protein indicating the stable integration of the gene in the plants. Although the expression to some extent is determined by integration site, as expected, significant variation was seen in the levels of protein expressed. Besides, their efficacy against the target pest is required to judge the performance of the plants. In this direction, the efficacy of the 5′utr *cry*1AcF gene was checked against *H. armigera.* The selected T1 generation plants showed a range in both percentage of damage and percentage of mortality. Some of the plants showed up to 100% mortality with less than 20% damage. These plants when corroborated with the cry protein accumulation showed more protein, confirming that the efficacy of these plants against *H. armigera* is because of the expression of the 5′utr cry1AcF. These results allowed the identification of 14 plants with corroborating expression and efficacy. PCR analysis further confirmed the integration of both the *npt*II and the 5′utr *cry*1Acf gene substantiating the transgenic nature of these chickpea transgenic plants.

The present study thus demonstrates the transformability of chickpea with the *in planta* transformation protocol and generation of transformants that are effective against *H. armigera.* However, the stability of the gene in further generations needs to be elucidated.

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