ISSN print: 1224-5984
ISSN online: 2248-3942

Rom Biotechnol Lett. 2022; 27(1): 3282-3291
doi: 10.25083/rbl/27.1/3282-3291

**Received for publication, December, 19, 2021**
**Accepted, January, 15, 2022**

*Original paper*

# *An Efficient Agrobacterium-mediated Genetic Transformation Using cry1F gene in Castor (Ricinus communis L.) for protection Against lepidopteran Insects*

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**Abstract**

Castor (*Ricinus communis* L.) is an essential non-edible, pharmaceutical, and industrial oilseed as well as vulnerable to foliage feeders like *Spodoptera litura* which resulted in a loss of production. This report focuses on the development of an optimized protocol for the transformation of castor shoot apices by *Agrobacterium tumefaciens* strain *LBA4404* containing plasmid construct *pBIN1F* harboring neomycin phosphotransferase (*nptII),* as selectable marker gene and *Bacillus thuringiensis var. aizawai (Bt) cry1F* gene controlled by cauliflower mosaic virus 35S promoter. Several parameters like O.D., concentration of kanamycin and acetosyringone were optimized and produced a significant difference in the transformation efficiency. Co-cultivation time and seedling age were factors, with overall transformation efficiency of 2.0% in 15 days-old seedlings and co-cultivated for 3 days. The surviving and actively developing shoot apices were validated for gene integration by molecular analysis after being preliminarily screened on kanamycin. Furthermore, PCR, qRT-PCR and insect bioassay were used to confirm the putative primary transformants. When bioassayed against newly hatched *Spodoptera litura* hatchlings, these putative transgenics with *cry1F* gene caused significant (≤93%) insect mortality. *Cry1F* gene expressing transgenic plants had adequate defence against *Spodoptera litura* when exposed to castor.

**Keywords** *Ricinus communis*, *Agrobacterium*-mediated transformation, *Cry1F* gene, Shoot apex, *Spodoptera litura,* qRT-PCR

To cite this article: KANSARA RV, SANJAY JHA, SOLANKI VH, SUMANKUMAR JHA, SRIVASHTAV VS, MEHTA VV, PATEL HK, MOGAL CS. An Efficient *Agrobacterium*-mediated Genetic Transformation Using *cry1F* gene in Castor (*Ricinus communis* L.) for *p*rotection Against *lepidopteran* Insects. *Rom Biotechnol Lett*. 2022; 27(1): 3282-3291. DOI: 10.25083/rbl/27.1/3282-3291.

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# Introduction

The most significant commercial oilseed crop is castor (*Ricinus communis* L.). Because of the seed's strong oil content (48%-60%), notable oil output (500-1,000L of oil/acre), and this plant's unusual capacity to manufacture oils with exorbitant amounts (80%-90%) of hydroxylated, monosaturated fatty acid, i.e. Ricinoleic acid, it is an excellent candidate for the production of high value, industrial oil feedstocks (BRIGHAM [1]; NAIK & al [2]). Castor oil has unique properties that make it a cost-effective source of raw materials for ultrapure biodiesel, short-chain aviation fuels, fuel lubrication additives, and regular biopolymer processing (GOODRUM & GELLER [3]; PINZI AND PILAR DORADO [4]). India, China, Brazil, and Thailand are the top castor-producing nations, while the United States and Japan are the top importers (M. SUJATHA & al [5]). In 2019, India's provisional castor oil exports to major countries totaled around 0.5 million tonnes (SOLVENT EXTRACTORS' ASSOCIATION OF INDIA [6]). India is a major producer of castor seeds, producing around 1.082 million tonnes in 2018–19, with Gujarat leading the way with 0.889 million tonnes (SOLVENT EXTRACTORS' ASSOCIATION OF INDIA 2019 [6]).

Globally, the cultivation of castor is constrained by the improved cultivars' vulnerability to insect attack. Reliable sources of resistance to major insect pests are rather limited in the available germplasm of this monotypic genus (M. SUJATHA, M. SAILAJA [7]). About 100 species of insect pests are recorded on castor at differing phenological stages of the crop among which castor semi looper (*Achoea janata*), capsule borer (*Conogethes puncitiferalis*), *Spodoptera litura*, red hairy caterpillar (*Amsacta albistriga*), jassids (*Empoasca flavescenes*) and whitefly (*Trialeurodes ricini*) cause considerable damage to castor (M. LAKSHMINARAYANA, M.A. RAOOF [8]). *Spodoptera litura* is the most common insect that causes castor defoliation (A.K. SARMA & al [9]). According to the yield loss calculation, seed yield loss will vary from 35% to 50%, based on crop growth stages and pest attack. Castor has the inherent ability to absorb up to 25% leaf damage without substantial seed yield loss, while damage to spikes and capsules results in significant yield loss (V.D. REDDY & al 2011 [10]).

Insect pest tolerance, disease resistance, seed production, oil content, and ricinoleic acid content are the primary breeding targets for rectification in castor. Traditional breeders, on the other hand, are limited by time, space, and the need to maintain genetic fidelity. Cross-pollinating behaviour makes it much more difficult for traditional breeders to keep paternal lines pure. For traditional breeders and biotechnologists, the effective tissue culture protocol is often a call for further development and upliftment of castor farmer's economies (A.S. SINGH & al [11]). The major bottleneck in biotechnological interventions for castor improvement is the lack of regeneration protocol, which is reproducible and applicable to a broad range of genotypes. Difficulties in tissue culture-related regeneration have compelled researchers to adopt meristem-based transformation methods that have revolutionized plant genetic engineering of major agronomic crops, which were considered recalcitrant to i*n vitro* manipulations (M. SUJATHA, M. SAILAJA [7]; I. POTRYKUS [12]). No published report(s) dealing with the genetic transformation of castor plants, successfully deploying agriculturally useful gene(s), exist especially using *cry1F*.

Different genetic transformation methods were used to confer insect pest resistance to transgenic castors, including *Agrobacterium*-mediated (M. SUJATHA, M. SAILAJA [7]; B. MALATHI & al [13]; A.M. KUMAR & al [14]); Direct gene (M. SAILAJA al [15]); containing the Bt genes *cry1Ab* (B. MALATHI & al [13]), *cry1EC* (M. SUJATHA & al [16]), chimeric *cry1AcF* *Agrobacterium*-mediated genetic manipulation protocols have also been successfully optimised and applied to industrially relevant crops such as *Cajanus cajan* (M.J. PAREKH & al [17]), *Ocimum gratissimumi* (S. KHAN & al [18]), *Gossypium hirsutum* (V.H. SOLANKI & al [19]; V. KHANDELWAL & al [20]), *Trachyspermum ammi* (M. NIAZIAN & al [21]), *Oryza sativa* (V. SRIVASHTAV & al [22]) and *Camellia sinensis* (H.R. SINGH & al [23]) deploying agriculturally useful gene(s) for biotic and abiotic stresses.

As a parental line and cross hybrid, castor plant variety SKP-84 (SardarKrushinagar Pistillate-84), Fusarium wilt resistant, has strong general combiner for seed yield per plot and for one or more characters [D. DUBE & al [24]) as well as yield contributing characters (A.V. PANERA & al [25]). This multiple character inheritances may be used to establish potential breeding viewpoints for variety production. Using the meristem proliferation method evolved in our laboratory (R.V. KANSARA & al [26]) to defend against foliage feeder *Spodoptera litura* by insect bioassay, the current study was conducted to optimize conditions and to develop an efficient for *Agrobacterium*-mediated transformation protocol of castor.

# Materials and methods

### *Plant materials*

Fusarium wilt resistant Variety SKP-84, which had been used as parent line for some high yielding hybrids like GCH 7 (Gujarat Castor Hybrid-7), was procured from the Castor-Mustard Research Station, Sardarkrushinagar Dantiwada Agricultural University, Gujarat, India.

### *Agrobacterium strain and the binary vector*

*Agrobacterium tumefaciens* strain LBA4404 was used for the experiments. The binary vector pBIN1F harboring the construct neomycin phosphotransferase (nptII), which is a kanamycin resistance gene, acts as a selectable marker and cry1F gene (Bacillus thuringiensis var. aizawai), which confers resistance to lepidopteran insects (selectively damages midgut). The chimeric genes were under the control of the CaMV35S promoter and OCS terminator. The Agrobacterium strain harboring given construct (Fig. 1) was provided by Dr. P. AnandKumar, Director, National Institute for Plant Biotechnology, (NIPB), Indian Council of Agricultural Research (ICAR), New Delhi, India for the genetic transformation experiment purpose.

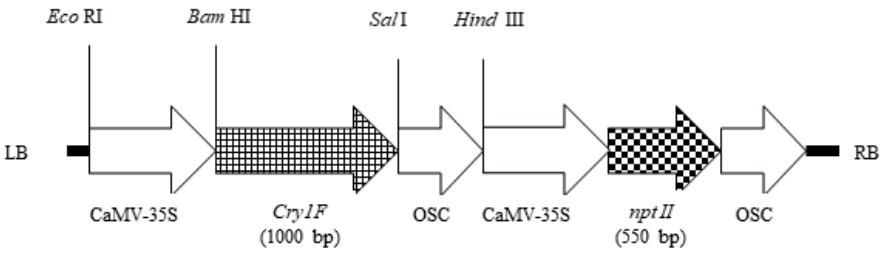


**Figure 1.** The T-DNA construct of binary vector pBIN1F harboring *cry1F* gene and selectable marker *nptII*

***In vitro regeneration process for preparation of shoot apex***

In vitro studies were conducted based on our previous successfully optimized in vitro plant regeneration protocol for fusarium wilt-resistant castor (Ricinus communis L.) parental line SKP-84 through the apical meristem (R.V. KANSARA & al [26]) in the Department of Plant Molecular Biology and Biotechnology at Navsari Agricultural University, Navsari, Gujarat, India. Mature seeds were decoated, rinsed (in running tap water), and surface sterilized with 0.1% (w/v) aqueous mercuric chloride solution for 4 min, subsequently washed (6 times) with sterile distilled water. Then, the material was blotted dry on sterile filter paper. Subsequently, the seeds were carefully dissected from the dorsal side and incubated on basal MS (T. MURASHIGE, F. SKOOG. [27]) medium MS basal salt medium with 3% (w/v) sucrose, pH 5.8, and solidified with 0.8% agar (Hi-media) for germination. The cultures were maintained at 25±2$^0$C under 16/8 h (light/dark) photoperiod with light provided by cool white fluorescent lamps at an intensity of 50 µmol $m^{-2}s^{-1}$. After that germinated 10, 15, and 18 days old seedlings were selected and the shoot apexes were carefully excised for co-cultivation with A. tumefaciens culture.

***Determination of kanamycin threshold level***

The castor plant's minimum inhibitory concentration of the selective antibiotic kanamycin was determined in the first phase of the experiment. As a result, the shoot apex was excised from developing seedlings and cultured separately on castor shoot proliferation media (MS medium with 0.25 mg/L BAP+ 0.50 mg/L kin) (R.V. KANSARA & al [26]) with kanamycin concentrations of 0, 25, 50, 75 and 100 mg/L. Both cultures were incubated at 25°C under a 16/8 h photoperiod with cool white fluorescent lamps providing an intensity of 50 µmol $m^{-2}s^{-1}$. To determine the optimal kanamycin concentration on a selective medium, the number of explants that survived and produced several shoots was counted.

***Genetic transformation***

A. tumefaciens strain LBA4404 culture was maintained on YEB medium (Beef extract 5 g/L, Yeast extract 1 g/L, Peptone 5 g/L, $MgSO_4.7H_2O$ 0.05 g/L, Sucrose 5 g/L, Agar 15 g/L) containing filter-sterilized 50 mg/L kanamycin and 10 mg/L rifampicin, incubated at 28ºC under dark conditions for 3–4 days. Single, isolated colonies from YEB medium plates were inoculated individually in 50 ml YEP medium (Yeast extract 10 g/L, Peptone 10 g/L, NaCl 5 g/L) containing 50 mg/L kanamycin and 10 mg/L rifampicin, incubated at 28ºC in an incubator-shaker at 200 rpm for 20–24 hours. Bacterial cells corresponding to an optical density (OD) of approximately 0.6 at 600 nm were pelleted by centrifugation (at 6000 rpm for 10 min), followed by washing twice with liquid YEP. The meristem explants excised at different days were injected with A. tumefaciens culture harboring the cry1F gene constructs and co-cultivated in dark for different periods. After co-cultivation, explants were rinsed with sterile distilled water 4 times containing 300 mg/L cefotaxime for 15 min and rinsed with sterile distilled water 4 times for 10 min with constant shaking. Cleaned shoot apices were blotted dry using a sterile paper towel and cultured on the selection medium consisting of castor multiplication medium (MS + 0.25 mg/L + 0.5 mg/L Kin) with 300 mg/L cefotaxime and 50 mg/L kanamycin. The Petri dishes were incubated at a temperature of 28ºC under 16-h photoperiod and kanamycin-resistant shoots were sub-cultured every two weeks. Surviving shoot apices were transferred to a half-MS medium containing 0.5 mg/L NAA without kanamycin for rooting of plantlets. For hardening, the rooted plants were moved to vermicompost-filled containers. Figure 2 shows the method for explant preparing, co-cultivation, collection, and recovery of plantlets.

***Factors influencing Agrobacterium mediated gene transformation***

Various factors influence transformation efficiency viz. Seedling age (10, 15 and 18 days), bacterial growth phase (OD 0.2, 0.4, 0.6, 0.8 and 1.0), co-cultivation duration (3, 5 and 7 days) and acetosyringone concentration (50, 100, 200, 300 and 400 µM) were investigated. Four replicates of thirty explants were used in each experiment. Any of the tests were carried out three times. On the basis of surviving shoots on a selective medium expressing the marker genes and positive by polymerase chain reaction, all of the above parameters were assessed and optimized.

***DNA extraction and polymerase chain reaction (PCR)***

Total genomic DNA was isolated from young leaves of independent putative transformed and untransformed (control)

plants using the CTAB method (J.J. DOYLE [28]) and screening was done by PCR using the nptII and cry1F genes specific primers (Bangalore Genei, India) for amplification of 550 bp and 1000 bp fragments (Table 1). The PCR reaction mixture (20 ml) contained 50 ng template DNA, 1x PCR buffer, 2.5 mM $MgCl_2$, 400 µM dNTPs, 0.3 U Taq DNA polymerase, 1 µl of each forward and reverse primer at a final concentration of 0.25 mM. For the positive control, 50 pg of the pBIN1F plasmid DNA was used. DNA from an untransformed castor plant was used for transformation control and reaction mix without DNA as a negative control. The amplification reactions were conducted on thermocycler of Eppendorf from Germany. The PCR reaction profile included 94ºC for 5 min followed by 30 cycles at 94ºC for 1 min, 53ºC (nptII) and 55 ºC (cry1F) for 1 min, 72$^0$C for 1 min 30 seconds with a final extension at 72$^0$C for 5 min. The amplification products were analyzed on 1.2% agarose-ethidium bromide gels by electrophoresis and documented in the gel documentation system.

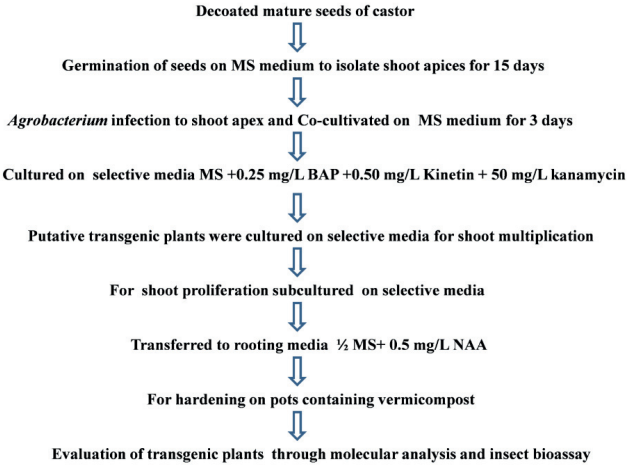


**Figure 2.** The representation of procedure developed for *Agrobacterium*-mediated genetic transformation of castor plants

**Table 1.** Details of used primers for amplification of *nptII* and *cry1F* genes

| Gene name | Primer | Primer sequence | Amplicon length(bp) |
|---|---|---|---|
| ❖ Gene specific for PCR | | | |
| *nptII* | Forward | 5'–AAGAACTCGTCAAGAAGGCGATA–3' | 550[a] |
| | Reverse | 5'–ATGGGGATTGAACAAGATGGATT– 3' | |
| *cry1F* | Forward | 5'–ATG GAG AACAACATCCAGAAT– 3' | 1000[a] |
| | Reverse | 5'–CAGTTTGTTGGAAGGCAACTC– 3' | |
| ❖ Endogenous gene for qRT-PCR | | | |
| *β-ACT* | Forward | 5'–TTCGCAGCAACAAACAT– 3' | 371[b] |
| | Reverse | 5'–TAAGCGGTGCCTCGGTAAGAAG– 3' | |
| ❖ Gene specific for qRT-PCR | | | |
| *cry1F* | Forward | 5'–GATGAAATCCCACCTCAGGA–3' | 247[b] |
| | Reverse | 5'–CGGGTCCTCTAACAACCGTA–3' | |

*β-ACT: Beta-actin; a: Length of the PCR amplified fragment with DNA template; b: Length of the PCR amplified fragment with cDNA template*

***Quantification by qRT-PCR***

A modified TRIzol™ (Invitrogen, USA) method was used to isolate total RNA from adolescent leaf tissues of putatively transformed, untransformed, and control plants (V. SRIVASHTAV & al [22]). The analysis was conducted using QuantiFast SY BR Green PCR Master Mix in real-time PCR (qRT-PCR) (Qiagen, USA). The endogenous control was beta-actin (β-ACT) (Table 1). qRT-PCR (ABI-7300 Applied Biosystem, USA) reactions was performed with an

initial denaturation at 95°C for 5 minutes followed by 30 cycles of 95°C for 10 seconds and 60°C for 20 seconds. The endogenous control and gene specific qRT-PCR primers were used for cry1F are mentioned in Table 1. Gene-specific primers were designed using Primer-BLAST Software.

***Insect bioassays***

Control plants and putative transformants were subjected to insect bioassays. Freshly hatched neonate larvae of the Spodoptera litura [Collected from the Castor Research Station, Department of Plant Breeding, Navsari Agricultural University, Navsari, Gujarat, India (Latitude: 200 57' N and Longitude: 720 54' E; Agro-ecological situation-III)] were used in detached leaf bioassays under controlled environmental conditions, and the experiments were replicated three times. Using a fine camel hairbrush, ten larvae were infested on a leaf per petri plate with moist filter paper. The plates were incubated at 26 ± 2°C with relative humidity (RH) of 65%-70% and a 16 h photoperiod of light and 8 h of darkness for 2 days. According to the insect assay conducted by V.H. SOLANKI & al [19], data on percent mortality were computed after two days of feeding.

***Statistical analysis***

Statistical approaches were used to compare treatment means when optimizing criteria for genetic transformation and Microsoft Excel 2010 software was used to evaluate the results. All graphs were created using the Prism 8.09 programme. The findings are shown as mean ± standard error (SE). The results were subjected to analysis of variance (ANOVA), and the critical difference at 5% was used to compare treatment means (V.G. PANSE & P.V. SUKHATME [29]).

## Results and discussion

This experiment describes results dealing with the establishment of a reproducible Agrobacterium-mediated genetic transformation protocol for insect resistance.

***Optimization of Antibiotic Concentration***

The use of proper concentrations of antibiotics used in the selection medium is essential in transformation experiments, in which the antibiotic serves as the selective agent that allows only transformed cells or plants to survive. Kanamycin has been extensively used as a selective antibiotic in transformation experiments, mainly because several plant transformation vectors include neomycin phosphotransferase II (nptII) gene as a selectable marker (M. NIAZIAN & al [21]; S. MISHRA & al [30]). Only transformed cells can grow in the presence of kanamycin. In our experiment, isolated shoot apices were transferred onto MS medium supplemented with 0.50 mg/L Kin + 0.25 mg/L BAP containing kanamycin at 0.25.50.75 and 100 mg/L after pre-culturing on basal MS medium for 7 days. Ten shoot apices were placed in each Petri plate and replicated four times for each concentration. Over a period of three weeks, the number of elongated shoot apices was counted and recorded each week. The control (without kanamycin) grew very well in MS media. Shoot elongation was significantly decreased on MS media containing kanamycin in a dose-dependent manner. The concentration of kanamycin at and above 50 mg/L was extremely lethal for survival and growth of castor shoot apices killing all the shoots within two weeks of culture with 13.55% of plant survival (Fig. 3a). Hence, a concentration of 50 mg/L kanamycin was decided for selecting putative transgenic apices henceforth in the present study. These results agree with earlier reports on some other medicinal plants like Ocimum gratissimumi (D. AGGARWAL & al [31]), Trachyspermum ammi (S. KHAN & al [18]), Withania somnifera (S. MISHRA & al [30]) and Bacopa monnieri (M. NIAZIAN & al [21]).

***Optimization of A. tumefaciens concentration, co-cultivation duration and acetosyringone concentration***

Efficient transformation parameters were analyzed using different A. tumefaciens concentrations (Absorbance of 0.2.0.4. 0.6.0.8 and 1.0 at OD600) and duration of co-cultivation (1.2.3.4 and 5 days). In each treatment combination, twenty shoot apices were placed with four replications. The survival percent of transformed plants after co-cultivation and frequency of transformation were recorded and showed that both A. tumefaciens concentrations had a significant effect on transformation frequency. The highest survival of transformed plants number and frequency of positive plants observed at OD600=0.6.

The transfer of T-DNA from A. tumefaciens to plant cells is a complicated process. The highest observed survival percent transformed plant was 48.16% and frequency of transformed plant were 2.2, which occurred at OD600=0.6 and 3 days co-cultivation (Fig. 3b and c). Co-cultivation with A. tumefaciens for 1 day was not long enough to maximize the transfer event. The data show that transformation frequency was always lower in 1-day co-cultivation than 2 days co-cultivation at different A. tumefaciens concentrations. Increasing the A. tumefaciens concentration did not always increase the transformation frequency. This may be because having A. tumefaciens concentration too high can cause A. tumefaciens overgrowth problems and reduction in plant regeneration (S. JIN & al [32]). Therefore, OD600=0.6 for 3 days co-cultivation was selected as the efficient transformation parameters in the present investigation.

In this step, the effects of five concentrations of acetosyringone, including 50, 100, 200, 300 and 400 µM, were evaluated with bacterial culture at O.D.600 to increase the transformation efficiency (Fig. 3d). The results showed the highest transformation frequency 2.18% on the supplementation of 100 µM acetosyringone in the inoculation time (Fig. 3d). Acetosyringone, a phenolic compound, is secreted on the wounding of plant tissues. It is also vir gene inducer and involved in the relocation of T-DNA to plant cells (S.E. STACHEL & al [33]). Earlier, different concentrations of acetosyringone have been reported to enhance Agrobacterium-mediated genetic transformation efficiency in Gossypium hirsutum (KHANDELWAL & al [20]) and Trachyspermum ammi (M. NIAZIAN & al [21]). Thus, the optimal concentration can be varied from plant to plant and affected by other transformation factors.

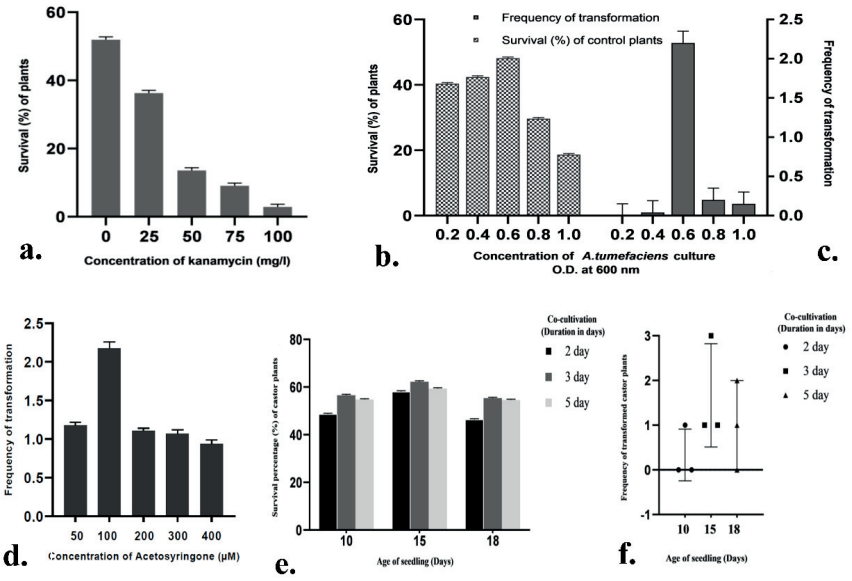


**Figure 3.** Factors influencing genetic transformation efficiency**. a.** effect of different kanamycin concentrations on the shoot apices of control castor plants (n=4); **b.** effect of *A. tumefaciens* (O.D. at 600nm) culture concentrations on survival percentage; **c**. frequency of transformation of castor plants (n=4) **d.** effect of different concentrations of acetosyringone on the frequency of transformation (n=4) **e.** Effect of age of the explants (days after germination of seeds) and co-cultivation duration with *A. tumefaciens* on survival percentage of the isolated shoot apices **f.** Transformation frequency of PCR positive plants after genetic transformation (n=4)**.** All results were represented as mean ± standard error (SE)

***Optimization of seedling age for co-cultivation***

The effect of the age of seedlings on survival percentage was found to be significant. The maximum survival percentage was found for fifteen days old seedlings (59.89%) compared to ten days (53.32%) and eighteen days (52.07%) age of seedlings. The effect of co-cultivation duration was found to be significant. Co-cultivation duration for three days gave the maximum survival percentage (58.06%) of the seedlings. Increasing co-cultivation duration above three days drastically reduced the survival percentage. The interaction effect of the age of the seedlings and co-cultivation duration was also found to be significant (Fig. 3e). The survival percentage for three days old seedlings and the co-cultivation duration for fifteen days gave a higher survival percentage (78.33%) (Fig. 3e). A reduction in the survival percentage (52.33%) was observed in the case of eighteen days old seedlings and two days of co-cultivation duration.

***Analysis of putative transgenic plants***

Age of the seedlings to isolate shoot apices and co-cultivation duration was found to influence the frequency of transformation. Fifteen days age of the seedlings gave a higher frequency of transformation 2.0%, while ten days and eighteen days age of the seedlings gave 0.66% and 0.33% frequency of transformation, respectively (Fig. 3f). Co-cultivation duration for three days on an average gave a maximum frequency of transformation (1.66%) while the co-cultivation period for the two days and five days gave 0.33% and 1% frequency of transformation. No transformants were obtained with ten days of seedling and two days of co-cultivation and in eighteen days of seedling for two days and five days co-cultivation duration. The interaction effect for the age of seedling and co-cultivation duration was also found to be significant. The maximum frequency of transformation (3%) was obtained for fifteen days of the age of seedling and three days of co-cultivation (Fig. 3f). Ten days and eighteen days age of seedling and co-cultivation for three days gave 1% frequency of transformation. A similar frequency of transformation was also obtained in ten days old seedlings and co-cultivation of five days, for fifteen days old seedling with co-cultivation for two days and for eighteen days old the seedling and co-cultivation for three days.

***Production of putative transgenic plants***

The shoot apices were co-cultivated with A. tumefaciens for 3 days. After co-cultivation, the shoot apices were transferred to MS medium with 50 mg/L kanamycin and 300 mg/L cefotaxime and established multiple shoot regenerative medium (BAP 0.25 mg/L + Kinetin 0.50 mg/L) (Fig. 4 a,b,c and d). Under kanamycin

selection pressure, most of the shoots and leaves appeared to be bleached and some shoots that were initially green bleached out gradually, leaving only a few green shoots (Fig. 4e and f). Even regeneration of bleached plant parts was observed when in vitro regeneration dependent Agrobacterium-mediated transformation approach applied (N. AHMAD, Z. MUKHTAR [34]). At every three weeks' interval, Shoot apices were transferred to fresh media. After four selections, surviving shoots were transferred to MS media without kanamycin to induce rooting. Rooting of the transformed shoot apices occurred when they were transferred from kanamycin selection medium to kanamycin free medium. Further, the hardening of the transformed plants required optimization in the pots. The morphological features of the transgenic plants did not differ from those of non-transgenic plants. Out of 250 explants, A. tumefaciens treated shoot apices placed on kanamycin selection, five (2%) regenerated plants grew (Fig. 5a and b) and were transferred to further culturing while others died.

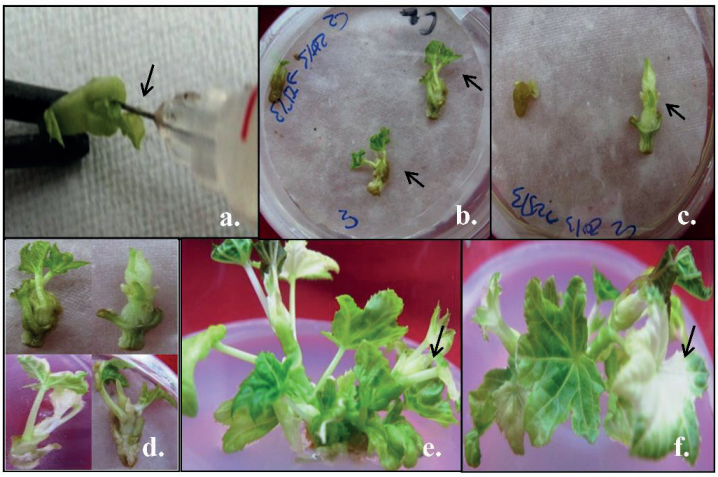


**Figure 4.** *Agrobacterium* infected shoot apices on selective medium containing kanamycin and cefotaxime. **a.** *Agrobacterium* infection to shoot apex; **b and c.** shoot apices on selective medium containing kanamycin and cefotaxime; **d.** putative transformed castor plants; **e.** multiplication and subculturing on selective MS medium contains 0.25 mg/L BAP + 0.5 mg/L Kin + 50 mg/L kanamycin + 300 mg/L cefotaxime after 3 weeks of inoculation and **f.** bleach color leaves

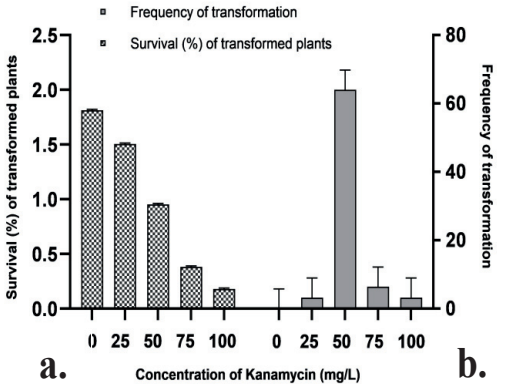


**Figure 5.** Effect of varying kanamycin concentrations on **a.** survival percentage and **b**. frequency of transformation of *Agrobacterium* mediated genetic transformed castor plants (n=4). All results were represented as mean ± standard error (SE)

### *Molecular analysis*

Genomic DNA was isolated from the putatively transformed plants for screening of genetically transformed plants. The presence of transgenes was confirmed by PCR amplification of the gene for nptII and cry1F (Fig. 6a and b) in the kanamycin-resistant T0 plants. The presence of a band at 550 bp and 1000 bp in transformed plants established the integration of the nptII and cry1F genes, respectively in the castor genome. Amplification of 550 bp and 1000 bp fragments were absent in the non-transformed control PCR plants which showed that PCR was free of contamination (Fig. 6a and b). In addition, this negative control revealed the accuracy of the PCR procedure. The dark and strong band with the same size of 550 bp and 1000 bp of nptII and cry1F were observed in the PCR amplification of plasmid as a positive control.

### *Quantitative real-time PCR (qRT-PCR) analysis*

Total RNA was isolated from putative transgenic plants and wild type (Fig. 6c). The PCR positive transformants were evaluated and further subjected to quantitative real-time PCR (RT-PCR) analysis for the presence of

introduced cry1F gene. The primary positive transformants were selected to detect cry1F gene by RT-PCR. The transformed plants showed amplification of cry1F gene in RT-PCR analysis. The realtime PCR indicated amplification with average Ct value. The real-time PCR runs along with the wild type (positive control), one non-transgenic plant (negative control) and four transgenic samples. The amplified RT-PCR DNA was executed and separated on 1.5% (w/v) agarose gel. The RT-PCR results showed that the β-ACT and cry1F gene were amplified to the required band size of 371 bp and 247 bp, respectively from cDNAs of putative transgenic plants (Fig. 6d and e). The higher expression level was observed in plants T1 (2.8 folds), T3 (2.63 folds), and T4 (2.68 folds) plants while the lowest expression level was observed in T2 (1.93 folds) plant (Fig. 6f).

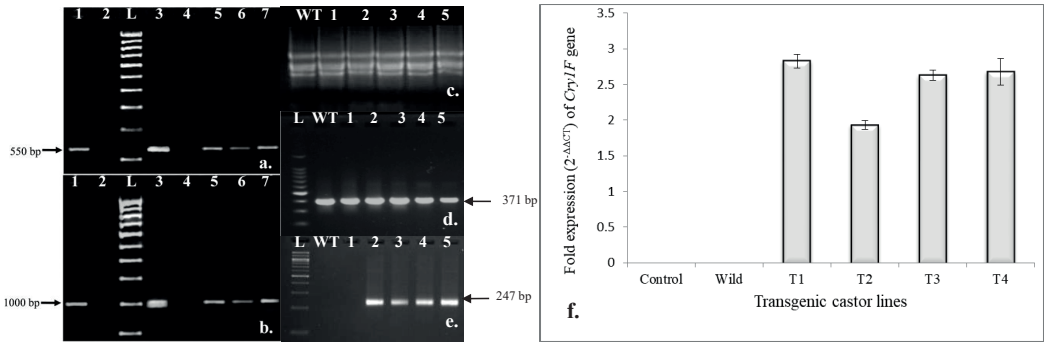


**Figure 6.** Molecular evaluation of putative transgenic castor plants ($T_0$ progeny).
**a.** PCR amplification of specific gene *nptII* and **b.** *cry*1F. Lane 1, 5-7: genomic DNA of transformed plants; Lane 3: positive control pBIN1F plasmid; Lane 2 and 4: negative control (untransformed plants); Lane L: 500 bp DNA marker, purchased from Banglore Genei; **c.** isolated total RNA from castor plants; RT- PCR products showed the amplification of **d.** Beta-actin gene- Internal control and **e.** *cry1*F gene. WT:wild type: Lane 1: untransformed plant; Lane 2-5: transformed plants; Lane L: 100 bp DNA marker, purchased from Banglore Genei **f.** Fold expression levels of *cry*1F gene in different putative transgenic castor plantsby qRT-PCR (T1-T4:Putative transgenic plant leaves; control and wild type)

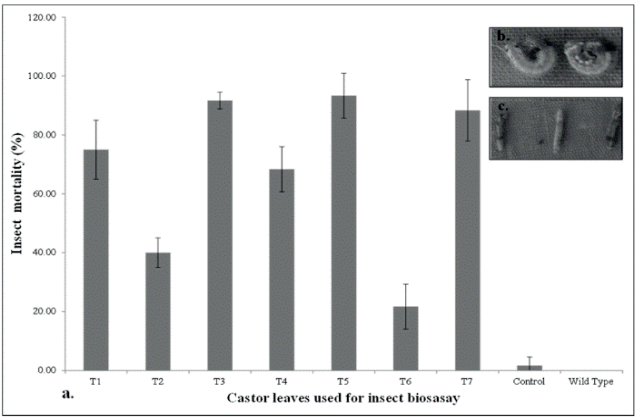


**Figure 7.** Effect of CRY protein on *Spodoptera litura* larva fed on the **a**. untransformed control castor **b.** putative transgenic castor and **c.** percent mortality of larva on the detached leaves of selected putative transgenic plants of $T_0$ generation (T1-T7: Putative transgenic plant leaves; control and wild type) (n=3)

Thus, fifteen day age of the seedling, three days of co-cultivation with O.D.600=0.6 of Agrobacterium at 50 mg/L of kanamycin gave a higher frequency of transformation 2.0%. It can be concluded that the transformation efficiency does depend upon genetic transformation factors as well as other parameters such as the age of an explant and plant growth regulators optimized for different in vitro regeneration protocols (R. BARANSKI [35]).

Insect bioassay of transformed castor plants with S. litura under in vitro conditions

To confirm the effect of cry1F gene, the insect bioassay of neonate larvae of Spodoptera litura was performed using the

leaf of T0 putative transgenic castor plants. Different transformants depicted diversification with insect resistance. During our insect bioassay study, it was observed that the larvae found dead after two days of infestation on the transgenic leaf disclosed different levels of insect mortality range from 21.67% to 93.33%, while on control plant was 1.67% (Fig. 7a). Moreover, the larva fed on putative transformed castor plant observed reduced growth of larva growth compared to the normal larval growth on the susceptible untransformed castor plant (Fig. 7b and c). Likewise, transgenic cotton lines were evaluated and showed higher resistance against chewing insect pests (V.H. SOLANKI & al [19]; KHANDELWAL & al [20]); H.A. SIDDIQUI & al [36]. Consequently, it was defined that the cry1F protein produced in the putative transgenic castors was sufficient toxic to Spodoptera litura and confer resistance in the castor plant. Previously, it was already reported the mode of action of Bt cry toxins to an insect that inserts into the cell membrane of the insect midgut, form pores, disturb the osmotic balance, induce swelling and lysis of the cells. Finally, the insect larvae stop feeding and die (J. GONZÁLEZ-CABRERA & al [37]; L. PARDO-LOPEZ & al [38]). Earlier, transgenic castors depicted higher resistance to neonate larvae castor semilooper on transformed plant with integration of the cry1Ab and bar genes through Agrobacterium genetic transformation (B. MALATHI & al [13]). Similarly, bioassay of castor semilooper and S. litura larvae showed varying levels of larval mortality and slow growth in larvae feed on transgenic plants leaf tissues then control plants which were transformed with Bt chimeric gene cry1EC, cry1AcF, and cry1Aa by Agrobacterium-mediated genetic transformation methods in the laboratory (M. SUJATHA & al [16]; A.M. KUMAR & al [14]; T. MUDDANURU & al [39]).

## Conclusion

To conclude, this study represents an improved *Agrobacterium*-mediated castor genetic transformation that incorporates the binary Ti vector as well as the function of central point impacting. Using shoot apices as explants in the parental line SKP-84, the established *in vitro* genetic transformation protocol proved viable for the Bt cry gene *cry1F*. In the insect testing facility, these putative transgenics demonstrated resistance to *Spodoptera litura*. Furthermore, the standardized protocol has the potential to facilitate the genetic modification in castor inserting agriculturally useful genes (s) and render a healthier approach for further insect resistance plant development studies through genetic engineering which accelerates its immense economic and industrial significance of castor plants.

### Abbreviations

A. tumefaciens – Agrobacterium tumefaciens; Bt – Bacillus thuringiensis; SKP-84 – Sardarkrushinagar pistillate-84; S. litura – Spodoptera litura; GCH 7 – Gujarat castor hybrid-7; nptII – Neomycin phosphotransferase; Bt cry – Bt crystal proteins; CaMV35S – Cauliflower mosaic virus 35S; OCS – Octopine synthase gene; LB – Left border; RB – Right border; YEB – yeast extract beef broth; YEP – yeast extract peptone; MS – Murashige and Skoog medium; BAP – 6-Benzylaminopurine; Kin – kinetin; OD – optical density; CTAB – Hexadecyltrimethylammonium bromide; PCR – polymerase chain reaction; RH – relative humidity; ANOVA – analysis of variance; T-DNA – transfer-DNA; vir gene – virulence gene; T – transgenic; qRT-PCR – quantitative real time PCR

### Acknowledgements

Authors are thankful to Castor–Mustard Research Station, Sardarkrushinagar Dantiwada Agricultural University, Gujarat, India for providing seed material to carry out the experiment. We are also thankful to Dr. P. Anandkumar, NIPB-ICAR, New Delhi, India for providing gene construct.

### Compliance with ethical standards

This article does not contain any studies involving animals or human participants as objects of research. All authors declare that they have no conflict of interest.

### Author contributions

RK, SJ and VHS performed the research work. RK, SJ, VHS, SKJ and VS were contributed to all data collection and transgenic analysis. SJ, RK, SKJ and VHS analyzed data and prepared primary draft of manuscript. SJ supervised the all research activities. RK, VS, HP and VM wrote marked errors and confirmed the final draft of the manuscript. All authors reviewed and approved the final version of the manuscript.

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