Short communication

Agrobacterium-mediated genetic transformation of tomato (*Solanum lycopersicum* L.) with *Cry1Ac gene* for resistance against fruit borer

Rizwan Rashid* and S.S. Bal

Department of Vegetable Crops, Punjab Agricultural University (PAU), Ludhiana 141004, India.

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Abstract

Agrobacterium-mediated genetic transformation was used to produce transgenic plants of tomato (*Solanum lycopersicum* L.) resistant to fruit borer. Cotyledon and leaf explants from *in vitro* tomato seedlings of variety 'Punjab Upma' were co-cultivated with *A. tumefaciens* strain GV 3101 containing *npt II* (plant selectable marker gene providing resistance to kanamycin) under the control of nopaline marker gene providing promoter (*pNOS*) and coding region containing a plant intron linked to the cauliflower mosaic *35 S (CaMV35S)* promoter. Out of the 376 cotyledons used for infection, 18 got selected on kanamycin (30 mg L^{-1}) showing 4.78% regeneration and of the 336 leaf explants, 11 got selected on kanamycin (30 mg L^{-1}) showing 3.27% regeneration. Histochemical *GUS* assay of the kanamycin selected cotyledon explants revealed 47.82% and in case of leaf explants it was 40%. PCR analysis confirmed presence of the transgene of 1.2 kb in five individual plantlets. These results signify the successful introduction of *Cry1Ac* gene into tomato plants.

Keywords: β-glucuronidase, Cotyledon explants, Fruit borer, *GUS* assay.

Fruit borer (*Helicoverpa armigera* Hubner) is the most devastating insect pest of tomato (*Solanum lycopersicum* L., 2n = 2x = 24). It is a polyphagus insect that is widely distributed. Non-judicious use of pesticides often results in health hazards, environmental pollution, destruction of natural enemies and beneficial insects, development of insecticide resistance, and increased production costs. To avoid such adverse fallouts, tomato plants tolerant to fruit borer is to be developed. A wild species of tomato *viz., Lycopersicon hirsutum* f. *glaburatum* has been reported to be highly resistant to fruit borer (Salinas et al*.,* 1993). However*,* transfer of the resistance gene through conventional breeding is time consuming, resource- and labour intensive, besides being adversely affected by linkage drag (Majid, 2007). Among the alternative strategies, for its control, transgenic plants expressing toxic proteins of *Bacillus thuringiensis* var. *israelensis* offer a sustainable and effective method to prevent crop losses due to *H. armigera* infestation. It is, therefore, important to develop a genetic transformation system for introduction of exogenous genes into plants in order to produce resistant varieties. Reporter genes have been used as convenient markers to visualize gene expression and protein localization *in vivo* in a wide spectrum of prokaryotes and eukaryotes (Jefferson, 1989). This paper describes the transformation of tomato by using *Agrobacterium tumefaciens* and the examination of transgenes in plant genome, especially insect resistant gene *Cry 1Ac*.

The investigation was carried out at the Tissue Culture and Genetic Transformation Laboratory, PAU, Ludhiana during 2006–2009. Seeds of the tomato genotype 'Punjab Upma' were surface sterilized in 2% sodium hypochlorite for 20 min in laminar air flow and rinsed thrice with sterile distilled water. Seeds were then germinated on MS medium supplemented with 100 mg

*Author for correspondence: Tel 09858069517; Email <rizwan60@gmail.com>.

L^{-1} myoinositol and 30 g L^{-1} sucrose. All cultures were maintained at 25 ± 2°C under 16 h light (2500 lux) and 8 h dark periods. Cotyledons were excised from 15 to 20 day-old *in vitro* germinated seedlings and leaves from 25 to 30 day old seedlings. *Gus* expression and mortality of explants were recorded.

Agrobacterium tumefaciens strain GV 3101 carrying plasmid *pPzp200* and *GUS 35S poly A* under the control of cauliflower mosaic *35 S CaMV35S* promoter were used in the genetic transformation study. The *Agrobacterium tumefaciens* strain GV3101 contained *npt II* (plant selectable marker gene providing resistance to kanamycin) under the control of nopaline marker gene providing promoter (*pNOS*) and the *Cry1Ac* coding region containing a plant intron linked to the cauliflower mosaic *35 S CaMV35S* promoter. All the components were dissolved in Milli Q water with constant stirring and the final volume was made up to 1 L. The medium was adjusted to a pH of 7.0 with 1N NaOH solution. For liquid medium, measured volume of 100 ml was dispensed into glass jam jars of 500 ml capacity and autoclaved. For solidification of the medium, agar after boiling on a hot plate was dispensed (100 ml) into jam jars and autoclaved. When the temperature of the medium became lukewarm, Rifampicin, Spectinomycin, and Gentamycin were added at concentrations of 250 µl (20 mg L^{-1}), 50 µl (100 mg L^{-1}), and 40 µl (100 mg L^{-1}) respectively in 100 ml of the medium, which was then used for bacterial inoculations aseptically by adding a colony of *Agrobacterium* containing plasmid gene constructs. *Agrobacterium* strain GV 3101 was maintained at 28°C on solid yeast extracted YEB medium supplemented with selective antibiotics such as Rifampcin, Gentamycin and Streptomycin. YEB with bacterial inoculations (*Cry1Ac* and GUS constructs separately) was left for 36 h in incubator shaker at 28°C with constant shaking at 120 rpm. The cotyledons of 20 day-old seedlings and leaves of 25 to 30 day old seedlings were injured with the sterilized blade under aseptic conditions. Explants were placed in sterile distilled water to avoid desiccation. Bacterial broth at different dilutions (undiluted, 1:15 and 1:20) in liquid MS medium was taken in petri-dishes. Explants were dipped in diluted broth for 10, 20, and 30 min. After that the explants were kept on sterile filter paper in order to drain off excessive bacterial suspension. The tissues were sub-cultured on medium containing cefotaxime 500 mg L^{-1} to kill the adhering bacteria. In the first experiment, 376 cotyledon explants (15 to 20 days old) and 336 leaf explants (25 to 30 days old) were inoculated with 1 ml overnight grown broth diluted in 20 ml of liquid MS medium. After infection for 20 min with *Agrobacterium*, the explants were cultured on MS medium for 2 to 3 days. At the end of co-cultivation, overgrowth of *Agrobacterium* was observed all around the explants. Eighteen cotyledons were cultured on regeneration medium (MS) supplemented with kin 0.5 mg L^{-1} and BAP 0.5 mg L^{-1}. Eleven leaf explants were cultured on regeneration medium (MS) supplemented with kin 0.5 mg L^{-1}, BAP 0.5 mg L^{-1}, kanamycin 30 mg L^{-1}, and cefataxine 500 mg L^{-1}. Tissues were grown on the selection medium containing kanamycin at 20 mg L^{-1} for two cycles of two weeks each and the resistant tissues were regenerated.

GUS assay of explants was carried out by putting them in *X-gluc* solution at 37°C in the dark to know the frequency of *GUS* expression. Histochemical assay was performed to visualize *GUS* activity. Explants from randomly selected kanamycin resistant plantlets growing incubated in *GUS* histochemical buffer [50 mM sodium phosphate, pH 7.0; 50 mM EDTA, pH 8.0; 0.5 mM K3Fe(CN)6; 0.5 mMK4Fe(CN)6; 0.1% Triton X-100; 1 mM *X-gluc* (5-bromo-4-chloro-3-indolyl β-D-glucuronide)] were incubated at 37°C for up to 24 h. Chlorophyll in leaf tissues was extracted in acetone: ethanol (1:3) solution before assessment of *GUS* activity.

Putative transformants were screened by PCR analysis using tomato genomic DNA from non-transformed and putative transgenic plants. The putative transgenic shoots obtained on regeneration medium were excised and transferred to the shoot elongation medium. The elongated shoots were excised and placed in culture jars containing rooting medium. Template *Cry1Ac* forward primer was 5'-TGGAGAACGCATTGAAA-CCG-3 and reverse primer 5'- TGTTGCTGAATCC-GGAACGG-3'. PCR analysis was carried out in the

reaction volume of 25 µl containing the template genomic DNA (100 ng), forward and reverse primers: 70 ng each, dNTPs (20 µM), PCR buffer 1x (1.5 $MgCl_2$, 10 Mm Tris), and Taq polymerase 1 unit. PCR conditions were 95°C for 7 min, 54°C for 1 min, and 72°C for 1 min (one cycle) followed by 95°C for 1 min, 54°C for 1 min, and 72°C for 1 min (30 cycle). Final cycle was carried out at 72°C for 7 min. Aliquot of 10 µl from each reaction was used for electrophoresis on 0.8% agarose gel. In total five experiments were con-ducted, in which 376 cotyledon and 336 leaf explants were used for co-cultivation. For *GUS* expression, 23 and 15 cotyledon and leaf explants were incubated in *X-gluc* in which 11 and 6 expressed. The data on *Gus* expression and mortality of explants were recorded. Statistical analysis was done using CPCS-1 package (Cheema and Singh, 1993).

Of the 376 cotyledons, 18 got selected on kanamycin (30 mg L^{-1}) after two complete cycles (15 days each), showing 4.78% regeneration (Table 1). Of the 336 leaf explants, 11 got selected on kanamycin (30 mg L^{-1}) after two complete cycles of selection of two weeks (Fig. 1a) showing 3.27% regeneration (Table 2). Roekel et al. (1993) also reported transformation frequencies averaging 9% for *L. esculentum* cv. 'Moneymaker' from cotyledon explants.

Agrobacterium GV3101 treated and kanamycin resistant tissues were incubated in *X-gluc* solution for 3 to 4 h at 37°C in the dark to know the frequency of *GUS* expression (Fig. 1b and 1c). Fifteen of the 23 randomly selected cotyledon explants exhibited blue colour (48%). *GUS* expression for leaf explants was about 40%. Shahriari et al. (2006) reported a trans-formation rate of 17% for Kal-early and 35% for Kal-G cultivars by *Gus* assay and PCR analysis.

Root formation occurred within 10 to 12 days after culturing onto the medium. Hardening of the rooted plantlets was done on wet cotton containing ordinary tap water. The hardened plantlets were transferred to

Table 1. Genetic transformation of tomato genotype 'Punjab Upma' by co-cultivating cotyledon explants with *Agrobacterium* strain GV 3101.

Experiment	Total number of explants co-cultivated	No. of explants showing regeneration[1]	Per cent regeneration (±SE)
1	87	2	2.29±0.16
2	68	3	4.41±1.17
3	74	4	5.41±1.41
4	71	5	7.05±1.55
5	76	4	5.27±0.05
Total	376	18	4.78

[1]on 30 mg L^{-1} kanamycin; SE =standard eroor.

Table 2. Genetic transformation of tomato genotype 'Punjab Upma' by co-cultivating leaf explants with *Agrobacterium* strain GV 3101.

Experiment	Total number of explants co-cultivated	No. of explants showing regeneration[1]	Per cent regeneration (±SE)
1	75	2	2.27±0.14
2	73	3	4.11±1.32
3	70	2	2.86±0.19
4	60	1	1.66±0.10
5	58	3	5.17±1.42
Total	336	11	3.27

[1]on 30 mg L^{-1} kanamycin

soil in polythene bags in a glasshouse (Fig. 1d). PCR analysis of nine plants of 'Punjab Upma' analyzed with specific *Cry1 Ac* primers showed the presence of *Cry1Ac* gene in five (Fig. 1e). The *Cry1Ac* gene thus has been successfully introduced into the commercial variety 'Punjab Upma' and is useful for producing transgenic tomato resistant to fruit borer.

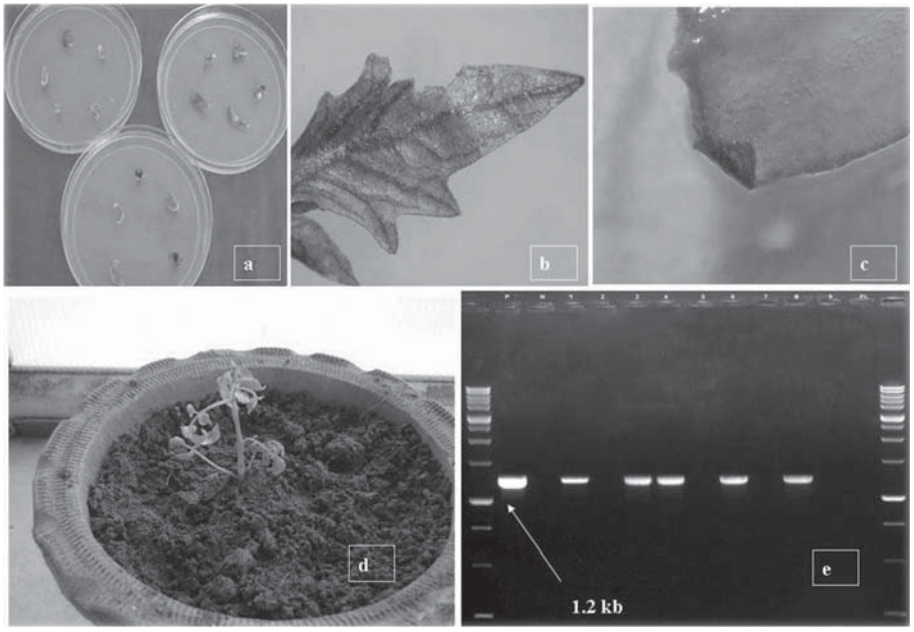

Figure 1. (a) Selection of tissues on kanamycin selectable marker: (b) Kanamycin resistance, *GUS* expression: (c) *GUS* expression under stereomicroscpic: (d) Hardening of putative transgenic plant: (e) PCR analysis.

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