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Agrobacterium Mediated Transfer of *Bacillus thuringiensis* Strain 6e *Cry* Gene to *Ixora odorata* var. Vulcan: A Comparison of Tissues

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ABSTRACT. *Ixora species are popular among landscape architects and gardeners as hedge plants and potted ornamentals. However, infestations by pests such as Ixora leaf webber, flower webber, green horned caterpillar and looper caterpillar have become a problem to the commercial exporters. Most chemical pest control methods adopted have hazardous effects on the environment and other beneficial insects and therefore, are unpopular among the buyers. As an alternative, Ixora odorata var. Vulcan was transformed with the cry gene of Bacillus thuringiensis (Bt) strain 6e by Agrobacterium mediated gene transfer. Leaf discs, four month old calli and in vitro grown I. odorata shoot tips (2 cm) were co-cultivated with LBA4404, harboring the Bt 6e cry cloned binary vector pLG121Hm. Co-cultivated shoot tips were transferred to the shoot multiplication medium (½ MS with 2 mg/L Benzyl Aminopurine (BAP) and 500 mg/L cefotaxime) and incubated at 2500 lux with a 16 hrs photoperiod. After four weeks, the axillary shoots were screened on ½ MS supplemented with 200 mg/L hygromycin. Hygromycin positive plants were used for the Polymerase Chain Reaction (PCR) analysis and Southern blot analysis. PCR analysis of the putative transformants, carried out with Gus specific primers, produced the expected 1.68 kb fragment in all the hygromycin positive transformants. Southern blot analysis of these with the Dig labeled Bt 6e cry probe produced positive results. The positive results obtained from the PCR analysis and the Southern blot confirmed genomic integration of the Bt 6e cry gene. The transformation efficiency of shoot tips was 10% whereas it was 25% and 20% respectively for callus and leaf disks transformation.*

INTRODUCTION

Ixora is an ever popular landscape plant that is infested by a number of pests such as Ixora leaf webber, flower webber, green horned caterpillar and looper caterpillar creating no win situation. Most chemical pest control methods adopted have hazardous effects on the environment and other beneficial insects and therefore, are unpopular among the buyers. As an alternative, plant transformation with *cry* genes from the entomopathogenic bacterium *Bacillus thuringiensis* provides an exciting approach in which transgenic plants produce their own protective proteins against the pests. Several *cry* genes have been introduced to

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plants, starting with tobacco and now include many major crop species. Among many a transformation technique, *Agrobacterium* mediated gene transfer is considered to be the best due to characteristics such as integration into transcriptionally active regions and the delivery of low copy numbers of a transgene, which lead to stable and efficient gene expression (Sujatha and Sailaja, 2005; Travella *et al*., 2005). This investigation is the first successful attempt to develop a transformation system for *I. odorata*.

MATERIAL AND METHODS

Vector and bacterial strain

Recombinant vector pLG121Hm (*cry* cloned) was maintained in *Agrobacterium* strain Logical Block Addressing (LBA) 4404 and cultured on Yeast Extract Broth (YEB) medium supplemented with 100 µg/mL rifampicin, 50 µg/mL hygromycin and kanamycin.

Effect of kanamycin and hygromycin on untransformed *Ixora* calli

Four-month-old Ixora calli were cultured on ½ MS medium supplemented with four levels of kanamycin (200, 300, 400 and 500 mg/L) and 3 levels of hygromycin (100, 200 and 300 mg/L) separately and incubated under dark conditions. Each treatment had 20 replicates and the experiment was repeated twice. Percentage dead calli was recorded over a period of four weeks.

Leaf disk transformation

Co-cultivation of Ixora leaf explants was carried out according to Herrera-Estrella and Simpson, (1988). Two hundred leaf disks (1.0 cm^2) were precultured for 2 days on hormone free ½ MS medium supplemented with 0.2 M sucrose to allow for initial growth and to eliminate damaged discs during sterilization. Overnight culture of LBA 4404 grown in minimal A medium (10.5 g/L K_2HPO_4, 4.5 g/L KH_2PO_4, 1.0 g/L $(NH_4)_2SO_4$, 0.5 g/L sodium citrate $2H_2O$, 0.2 g/L $MgSO_4$ $7H_2O$ and 2.0 g/L glucose) was adjusted to 5 x 10^8 cells/mL. Precultured leaf disks were immersed in the LBA 4404 culture for 20 min and blot dried. These were incubated on ½ MS medium supplemented with 0.4 M sucrose for 24 hr at 26^{o}C. Co-cultivated leaf disks were transferred to ½ MS medium supplemented with 500 µg/mL of cefotaxime and 50 µg/mL of hygromycin. Positive transformants were screened with an increasing series of hygromycin (100, 150, 200 and 300 µg/mL) to eliminate escapes. Hygromycin positive transformants (at 200 µg/mL) were cultured on ½ MS supplemented with 3 mg/L 2, 4-D for callus induction.

Transformation of *Ixora* callus

One hundred four-month old Ixora calli were transferred to plasmolysis medium (½ MS supplemented with 4.0% sucrose) an hour prior to co-cultivation. Overnight culture of *Aagrobacterium* strain LBA 4404 (with *cry* inserted pLG121Hm) was pelleted by centrifugation (5,000 g, 10 min) and resuspended to an OD_{600} of 0.2 in liquid plant inoculation medium (½ MS supplemented with 3.0% sucrose with 200 µM acetosyringone). Plasmolyzed calli were directly transferred to bacterial suspension in the inoculation medium. These were incubated for a total of 30 min in two phases, 10 min shaking and 20 min stationary. Inoculated calli were blotted on sterile filter papers and immediately

transferred to co-cultivation medium (½ MS supplemented with 3.0% sucrose, 2.0 g/L gellan gum and 200 μM acetosyringone). After a 2 day co-cultivation period under darkness, calli were transferred to ½ MS supplemented with 500 μg/mL cefotaxime and 100 μg/mL hygromycin.

Confirmation of transformation

Gus analysis

Hygromycin positive calli were dipped in 100 μg/mL solution of XglcA and incubated at 37^{o}C under dark conditions. Number of blue calli was recorded after 24 hrs.

Dot blot analysis

Gus positive Ixora calli DNA was isolated along with untransformed calli DNA according to Walbot (1989). Calli (0.1 g each) were homogenized in a cold mortar by rapid grinding with 0.5 mL of solution 1 [250 mM Na-EDTA, 50 mM Tris Cl (pH 8) and 1.5% sucrose]. Fluid was transferred into ice-cold centrifuge tubes and pelleted at 12,000 g for 5 min. The pellet was re-suspended in solution 2 (20 mM Tris HCl and 10 mM Na-EDTA). Twenty micro liters of 20% Sodium Dodecyl Sulpate (SDS) was added and mixed. This was incubated at 70^{o}C for 15 min and placed on ice. Hundred and fifty micro liters of 7.5 M ammonium acetate was added and kept on ice again. After centrifugation at 12,000 g for 45 min at 4^{o}C, supernatant was treated with 0.7 mL of isopropanol and incubated at room temperature. After centrifugation at 12,000 g for 15 min the pellet was washed twice with 70% ethanol. Precipitated DNA was re-suspended in 50 μL of TE and stored at -20^{o}C. Gus positive calli DNA was dot blot analyzed using Dig labeled Bt 6e *cry* probe according to the manufacturer (Boehringer Mannhiem Biochemica, Germany). Plasmid DNA of Bt 6e and *cry* DNA were used as the positive controls and DNA from untransformed *Ixora* calli was used as the negative control.

Shoot tip transformation

After slightly puncturing the emerging axillary buds, 100 *in vitro* grown shoot apices (2 cm) were immersed in an overnight culture of *Agrobacterium* suspension (supplemented with 200 μM acetosyringone) for 15 min and washed once with sterile distilled water. These were blotted on filter paper and cultured on co-cultivation medium (½ MS supplemented with 3.0% sucrose, 2 g/L gellan gum and 200 μM acetosyringone) at 26^{o}C in the dark for 3 days. Co-cultivated shoot apices were washed five times with sterile distilled water and blotted dry. These were transferred to regeneration medium (½ MS with 2 mg/L BAP) supplemented with 500 mg/L cefotaxime and incubated at 2500 lux 16 hrs photoperiod. After four weeks, the axillary shoots were screened on selective medium (½ MS supplemented with 200 mg/L hygromycin).

Analysis of transformants

PCR analysis

Genomic DNA was isolated from leaf tissues of the hygromycin positive transformants according to Walbot (1989). Polymerase Chain Reaction (PCR) of the genomic DNA was carried out to confirm the presence of the *gus* gene with gene specific

primers, 5'-GTGGAATTGATCAGCGTTGG-3'and 3'-TGACCGTACTTGAAGCCACG-5' amplifying a product of 1.68 kb (provided by Dr. Nandadeva Yakandawala, Institute for Plant Sciences, Federal Institute of Technology (ETH) Zurich, Switzerland). The reaction mixture used was 25 ng plant genomic DNA, 100 μM of each dNTP, 0.2 μM of each primer and 1unit *Taq* polymerase (Sigma, USA). PCR was performed in a thermal cycler with the following amplification program: 1 cycle of 3 min at 94^{o}C, 30 cycles of 1 min at 94^{o}C, 2 min at 60^{o}C and 1 min at 72^{o}C and a final cycle at 72^{o}C for 7 min. Plasmid DNA was used as the positive control and DNA from untransformed Ixora leaves was used as the negative control.

Southern blot hybridization

DNA from Hygromycin positive transformants, *cry* inserted vector DNA and DNA from untransformed Ixora leaves were electrophoresed on 0.8% agarose gel and transferred to a nylon membrane under alkaline conditions according to Sambrook *et al*. (1989). The gel was soaked for 15 min in denaturation/transfer solution (0.4 N NaOH and 1.0 M NaCl) with constant gentle agitation. The solution was replaced and the gel was soaked for further 20 min. The membrane was floated on deionized water until completely wet and immersed in denaturation/transfer solution for 5 min. DNA was transferred from the gel to the membrane by capillary action. The membrane was peeled out and neutralized in 0.5 M Tris Cl (pH 7.2) and 1 M NaCl for 15 min. The dried membrane was used for Southern blot analysis with Dig labeled Bt 6e *cry* probe according to the manufacturer (Boehringer Mannhiem Biochemica, Germany).

RESULTS AND DISCUSSION

Effect of hygromycin and kanamycin on Ixora calli

Ixora calli were cultured on hygromycin and kanamycin to select the best antibiotic for the screening of the transformants. According to the results, all four levels of kanamycin had no effect on Ixora calli even after four weeks. Thus, it is evident that Ixora shows natural kanamycin resistance to the levels tested. This phenomenon of natural kanamycin resistance, even at higher levels has been reported previously (Xie and Hong, 2002; Damayanthi, 1999) In such a situation, a number of options such as higher levels of the same antibiotic, replacement with an antibiotic of the same group (paramomycin of *npt* group) or the use of another marker could be tested. All three levels of hygromycin were toxic enough to destroy the callus tissues by the end of the first week of culture. Therefore, it was selected as the antibiotic marker for the screening of transformants.

Leaf disk transformation

The results show that two thirds of the initial transformants were transient (Table1). These belong to both nuclear escapes and other unstable genomic transformants. The minimum hygromycin concentration level required to select the stable transformants was 200 mg/L since the number remained static at 300 mg/L. Although this was a reasonable transformation frequency, only 10% of these produced calli on callus induction medium even after 12 weeks. The rest remained green throughout the period of the study. The amount of callus produced was also very low (<0.5 cm^3) and turned brown after a week. Although leaf disk transformation has been successful in a number of occasions (Endo *et al*.,

2002; Miguel and Olivera, 1999; Arcilletti *et al*., 1995; Horsch *et al*., 1985) the problem of low regeneration frequency of infected tissue is common (Ueno *et al*., 1996; Godwin *et al*., 1991). The incorporation of antibiotics is also known to reduce the regeneration potential (Xie and Hong, 2000; Zahang *et al*., 2000). However, this could hardly be the reason because the transfer of co-cultivated leaf disks of first screening stage to the callus induction medium still did not produce viable calli.

Table 1. Screening of positive transformants on hygromycin.

Treatment (hygromycin mg/L)	% of positive transformants
50	60
100	45
150	25
200	20
300	20

Callus transformation

GUS analysis

Only 25% of the cocultivated calli was hygromycin positive at 200 mg/L. However, when compared with the positive transformant percentage of leaf disk method (20%) it is an improvement. This suggests that the callus tissue is more sensitive to *Agrobacterium* infection than leaf tissue. The reason could be due to the presence of a wax layer on the leaves. In fact Xie and Hong, (2002) reported unsuccessful transformation of shoot apices of *Acacia magnum* because of the same reason. The GUS expression was observed in all the hygromycin positive calli after 24 hrs incubation. The color development remained costant throughout the period of study suggesting stable transformation. However, slight tissue browning was observed in the initial stage of this experiment due to phenolic compounds and this made it difficult to observe the true color until it was rectified with the addition of 200 mg/L ascorbic acid. In the cross sections of transformed calli, some cells showed color change while some did not (Plate 1A). This could be due to either somaclonal variation or different levels of exposure to XglcA. The controls (untransformed Ixora calli) did not produce any color development (Plate 1B) suggesting the absence of endogenic Gus expression in *Ixora odorata*.

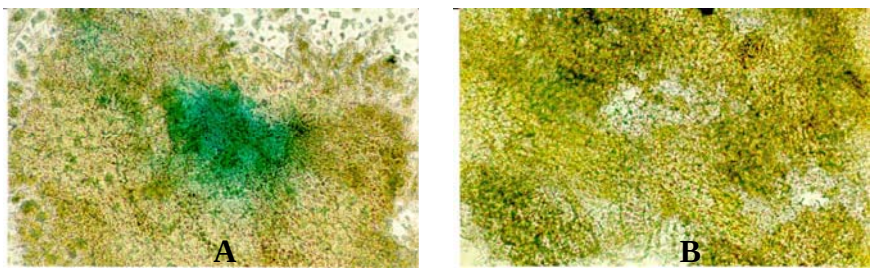

Plate 1. Cross sections of Ixora calli after an overnight dip in X-glcA at 37^{o}C
A- Transformed calli B- Control (untransformed calli)

Dot blot analysis

All of the Gus positive transformants gave positive hybridization in the Dot blot analysis (Plate 2). The positive results in the Gus together with the positive results of the Dot blot analysis suggest that these transformants were stable genomic transformants. This further confirms the ability of hygromycin to screen out nuclear escapes and unstable transformants at 200 mg/L. Dot blot analysis is inferior to Southern blot as an analysis technique and it also does not give an idea about the number of copies inserted. However, positive results of all three screening tests build up a strong point for the transformants' authenticity.

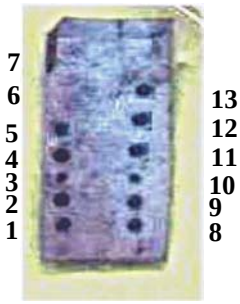

Plate 2. Dot blot analysis of GUS transformants with Dig labeled Bt 6e *cry* probe.
Note: 1-Bt plasmid DNA, 8- *cry* DNA 2- *cry* DNA, 6-7- DNA from untransformed calli, 3-5 and 9-13- DNA from Gus positive transformants.

Shoot tip transformation

Selection of transformed tissues was delayed as a strategy to recover transgenic shoots. This strategy allows cell division to occur along with the formation of transformed cell clusters or shoot initials depending on the time period. Delayed selection has been successful in apple (Yao *et al*., 1995; Yepes and Aldwinckle, 1994) and in apricot (Machado *et al*., 1992). If the selection pressure is applied straight away the exposure of the few transformed cells to the toxic products resulting from necrosis of adjacent non-transformed cells may prove lethal. In fact, Miguel and Olivera (1999) reported the total failure of transformed almond shoots to survive on regeneration medium supplemented with 30 mg/L kanamycin. When the elongated axillary shoots were finally screened for hygromycin, 10% of the shoots survived after three subcultures (3 weeks). PCR multiplication of genomic DNA from hygromycin positive plants with specific primers for the 1.68 *gus* fragment produced positive results (Plate 3) and no amplification was observed for the wild type control.

Southern blot analysis of all *gus* positive plants showed hybridizations with the Dig labeled Bt 6e *cry* probe, in *Hin*d III digested DNA samples while no hybridization was observed in the negative control (Plate 4). A single sample (Lane 4) showed two distinct hybridizations, one with the same position as the other positive transformants and another at a lower molecular weight. Due to this result, quite evident that the sample in lane 4 should have two copies of the transgene. The results from both PCR analysis and the Southern analysis confirm the stable integration of the foreign DNA (*gus* gene and *cry* fragment) in all of the hygromycin positive transformants. The transformation frequency was 10% for shoot

tips and this was the lowest when compared to the other two transformation techniques (leaf disk-20% and callus-25%). However, lack of a proper regeneration system makes both leaf disk and callus transformations inferior to shoot tip transformation. Moreover, the ability to multiply the transgenic shoots through micropropagation more than compensates for the lower transformation frequency.

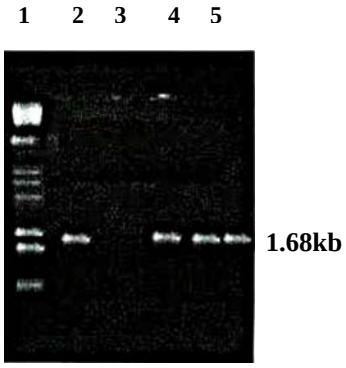

Plate 3. Electrophoretic analysis of the PCR products of putative transgenic Ixora shoots with specific primers for *gus* gene.

Note: Lane 1- 1kb DNA ladder, 2- Positive control (*cry* cloned pLG121Hm), 3-Negative control (untransformed *Ixora* DNA) and 4-6- putative *cry* transformants.

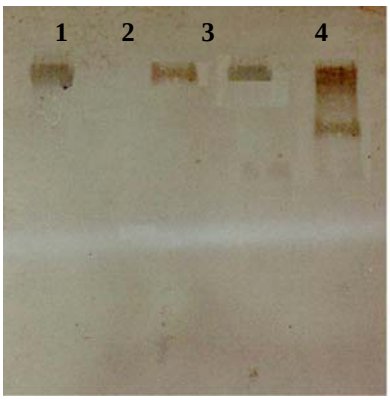

Plate 4. Southern blot analysis of putative transgenic Ixora shoots with Dig labeled *cry* probe.

Note: 1-*cry* cloned pLG121Hm, 2-Negative control (untransformed *Ixora* DNA) 3-5- Putative *cry* transformants.

CONCLUSIONS

Based on positive results obtained from all transformation attempts (leaf disk, calli and shoot tip) the *Agrobacterium* mediated gene transfer system used is successful for *I. odorata* var. Vulcan. However, the absence of an efficient protocol to regenerate Ixora calli

and the failure of many an attempt in this study makes shoot tip transformation the only currently available option for *I. odorata* var. Vulcan.

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REFERENCES

Arcilletti, T., Lauri, P. and Dominico, P. (1995). *Agrobacterium*-mediated transformation of almond leaf pieces. Plant Cell Rep. 14: 267 - 272.

Damayanthi, K.P.N. (1999). Studies on transfer of *cry* 2 gene from *Bacillus thuringiensis* to indica rice (*Oryza sativa* L.) using *Agrobacterium* binary vector system and electroporation, M.Phil. thesis, Postgraduate Institute of Agriculture, University of Peradeniya, Sri Lanka.

Endo, S., Kasahara, T., Sugita, K. and Ebinuma, H. (2002). A new GST-MAT vector containing both *ipt* and *iaaM/H* genes can produce marker-free transgenic plants with high frequency. Plant Cell Rep. 20: 923 - 928.

Godwin, I., Todd, G., Ford-Loyd, B. and Newbury, H. J. (1991). The effects of acetosyringone and pH on *Agrobacterium*-mediated transformation vary according to plant species. Plant Cell Rep. 9: 671 - 675.

Herrera-Estrella, L. and Simpson, J. (1988). Foreign gene expression in plants. In: Shaw, C. H. (Ed.), Plant Molecular Biology: A Practical Approach. Oxford, Washington DC. pp.131 - 160.

Machado, M., Camara M., A. da., Hanzer, V., Weiss, H., Regner, F., Steinkellner, H., Mattanovich, D., Plail, R., Knapp, E., Kalthoff, B. and Katinger, H. (1992). Regeneration of transgenic plants of *Prunus armeniaca* containing the coat protein gene of plum poxvirus. Plant Cell Rep. 11: 25 - 29.

Horsch, R.B., Fry, J.E., Hoffmann, N.L., Eichholtz, D., Rogers, S.G. and Fraley, R.T. (1985). A simple method for transferring genes into plants. Science, 227: 1229 - 1231.

Miguel, C. M. and Oliveira, M. M. (1999). Transgenic almond (*Prunus dulcis* Mill.) plants obtained by *Agrobacterium*-mediated transformation of leaf explants. Plant Cell Rep. 18: 387 - 393.

Sambrook, J., Fritch, E.F. and Maniatis, T. (1989). Molecular cloning: A laboratory manual. 2nd ed. vol. 3 New York: Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Sujatha, M. and Sailaja, M. (2005). Stable genomic transformation of castor (*Ricinus communis* L.) via *Agrobacterium tumefaciens*-mediated gene transfer using embryo axes from mature seeds. Plant Cell Rep. 23: 803 - 810.

Travella, S., Ross, S.M., Harden, J., Everett, C., Snape, J.W. and Harwood, W. A. (2005). A comparison of transgenic barley lines produced by particle bombardment and *Agrobacterium* mediated techniques. Plant Cell Rep. 23: 780 - 789.

Ueno, K., Fukunaga, Y. and Arisumi, K. (1996). Genetic transformation of *Rhododendron* by *Agrobacterium tumefaciens*. Plant Cell Rep. 16: 38 - 41.

Walbot, V. (1989). Preparations of DNA from single rice seedling (1996). Plant transformation, Rice Genetics Newsletters. International Center for Genetic Engineering and Biotechnology (ICGEB) Workshop, New Delhi. pp. 149 - 151.

Xie, D. Y. and Hong, Y. (2002). *Agrobacterium*-mediated genetic transformation of *Acacia mangium*. Plant Cell Reports 20: 917 - 922.

Yao, J.L., Cohen, D., Atkinson, R., Richardson, K. and Morris, B. (1995). Regeneration of transgenic plants from the commercial apple cultivar Royal Gala. Plant Cell Rep. 14: 407 - 412.

Yepes, L.M. and Aldwinckle, H.S. (1994). Factors that affect leaf regeneration efficiency in apple and effect of antibiotics in morphogenesis. Plant Cell Tissue and Organ Culture 37: 257 - 269.

Zahang, F. L., Takahata, Y., Watanabe, M. and Xu, J. B. (2000). *Agrobacterium* mediated transformation of cotyledonary explants of Chinese cabbage (*Brassica campestris* L. ssp. *pekinensis*). Plant Cell Rep. 19: 569 - 575.