This chapter describes the effects of acetosyringone, co-cultivation period and density of *Agrobacterium* suspension on transformation in tomato cv. Dhanashri and presents a rapid, reproducible, and an efficient transformation protocol.

*Agrobacterium* mediated transformation is one of the effective and commonly used approach to introduce foreign DNA into dicotyledons plants. Different gene transfer protocols are applicable in various plant species. McCormick *et al.*, (1986) have reported the first successful *Agrobacterium*-mediated transformation attempt in tomato. Since the 1980s several *Agrobacterium*-mediated transformation protocols have been developed in tomato, using cotyledons or leaves (Van Eck *et al.*, 2006; Sharma *et al.*, 2009; Pino *et al.*, 2010).

Several factors seems to be crucial for tomato transformation using *Agrobacterium tumefaciens*, including the use of nurse cells or acetosyringone in the culture or pre-culture media, the type of explants, the *Agrobacterium* strain used and concentration of its suspension, co-cultivation period and the concentration of plant growth regulators, thiamine, 6-benzylamino purine (BAP), zeatin and indole acetic acid (IAA).

The success in tomato transformation has been dependent on genotype, *Agrobacterium* strain used, co-cultivation period, density of *Agrobacterium* culture and antibiotic used. However, there is no report on *Agrobacterium*-mediated transformation of P5CS in tomato cv. Dhanashri.

Therefore, in this chapter we report a simple and reproducible transformation in tomato cv. Dhanashri.
5.1 Material

5.1.1 Plant material

Seeds of tomato variety Dhanashri were obtained from the Department of Horticulture (Vegetable Wing), Mahatma Phule Krushi Vidhyapith, Rahuri, Ahmednagar (MS), India.

5.1.2 Agrobacterium strain and transformation vector

*Agrobacterium tumefaciens* LBA 4404, harboring plasmids pCAMBIA1301-P5CS-F129A gene containing *hptII* hygromycin transferase conferring resistance to hygromycin B (a plant selectable marker), neomycin phosphotransferase (*nptII*) conferring resistance to kanamycin (a selectable marker for bacterial selection) and *gus* reporter gene was used for the transformation in tomato. The culture of the bacterium used for genetic transformation experiments was kindly provided by Prof. Kavi Kishor, Osmania University; Hyderabad.

5.2 Methodology

5.2.1 *In vitro* germination of tomato seeds

Tomato seeds were thoroughly washed under running tap water, and then placed in 5% Tween-20 for 5 min, followed by 3–4 rinses in sterile distilled water. They were surface sterilized with aqueous solution of 0.1% *HgCl*₂ for 3 min, followed by 4–5 rinses in sterile distilled water. The surface sterilized seeds were aseptically inoculated in culture bottles (10 seeds in each) containing 40 ml half-strength MS basal medium and incubated at standard conditions for 15 days.

5.2.2 *Agrobacterium* culture

The *Agrobacterium* strain LBA 4404 containing the binary vector was grown in 100 ml conical flask containing 10 ml YEM medium (*Table 5.1*) supplemented with 50 mg l⁻¹ kanamycin. The cultures were incubated for 24 h on a rotary shaker at 200 rpm in the dark at 28 °C. *Agrobacterium* culture was centrifuged at 4000 rpm for 5 min. The supernatant was discarded and the pellet was suspended in MS basal liquid medium (pH 5.8) supplemented with 100 µM acetylsyringone (AS: 3, 5-Dimethoxy-4-hydroacetophenone, Sigma-Aldrich,
Germany). Cultures were diluted with MS basal medium optical density (OD) of 0.2 to 1.0 at A_{600} nm) and then used for transformation experiment.

### Table 5.1 Composition of YEM medium

<table>
<thead>
<tr>
<th>Constituents</th>
<th>Concentration (g l^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast extract</td>
<td>0.4</td>
</tr>
<tr>
<td>Mannitol</td>
<td>10.0</td>
</tr>
<tr>
<td>MgSO_4.7H_2O</td>
<td>0.2</td>
</tr>
<tr>
<td>K_2HPO_4</td>
<td>0.5</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.1</td>
</tr>
</tbody>
</table>

(pH – 7.0 and medium was gelled with 10 g l^{-1})

#### 5.2.3 Antibiotic sensitivity of cotyledonary leaf explants

To check the lethal dose of antibiotics, cotyledonary leaves from 15 days old in vitro seedlings of tomato cv Dhanashri were subjected to hygromycin (0, 5, 10, 15, 20, 25, 30, 35 and 40 mg l^{-1}) selection media. These media contained MS salts, vitamins, and were supplemented with 6.65 μM BAP, 1.14 μM IAA, 30 g l^{-1} sucrose and were solidified with 8.0 g l^{-1} agar. Similarly, sensitivity of cotyledonary leaf explants was studied against different concentrations of cefotaxime (0, 100, 200, 300, 400 and 500 mg l^{-1}). The cultures were incubated for 15 days, illuminated with white fluorescent light (600 μM m^{-2} s^{-1}; 16 h light /8 h dark) at 25 ± 2 °C and 70% relative humidity and the results were recorded after 15 days.

#### 5.2.4 Agrobacterium infection and Co-cultivation

Cotyledonary leaves of 15 days old seedlings of tomato cv. Dhanashri were used as explants. The margins of cotyledonary leaves were injured to allow the bacterium to infect. The explants were dipped for 10 min in the bacterial suspension with gentle shaking and blotted dry on a sterilized paper towel. These explants were placed on co-cultivation medium with the abaxial surface of the leaf in contact with the medium. The co-cultivation medium MST-I contained MS basal medium (pH 5.8) supplemented with 6.65 μM BAP, 1.14 μM IAA, 30 g l^{-1} sucrose, and solidified with 8 g l^{-1} agar. Co-cultivation media were without or with
varying concentration of acetosyringone (0.0-200 µM) and incubated at 25 ± 2 ºC in dark. The co-cultivation period varied from 1 to 5 days for optimization.

To eradicate bacterium after co-cultivation, cotyledonal leaf explants were rinsed thoroughly with sterile distilled water containing 200 mg l⁻¹ cefotaxime, blotted dry on a sterilized paper towel and transferred on MST-II containing MS basal media (pH 5.8) were supplemented with 6.65 µM BAP, 1.14 µM IAA, 30 g l⁻¹ sucrose, solidified with 8 g l⁻¹ agar. These media were also supplemented with 200 mg l⁻¹ cefotaxime and 10 mg l⁻¹ hygromycin B for antibiotic selection. The cultures were maintained on the same media for 4 weeks (two cycles, each consisting of 2 weeks). After 4 weeks the regenerated transformants were subcultured on rooting medium.

5.2.5 Rooting of in vitro grown shoots

Putative transformant microshoots after two selection cycles were subcultured on MSR containing MS basal medium supplemented with 4.92 μM IBA, 200 mg/l cefotaxime and 10 mg/l hygromycin B. The cultures were maintained at 25 ± 2 ºC temperature, 70-80% relative humidity and illuminated with cool white fluorescent light at a 16-h photoperiod for two weeks.

5.2.6 Hardening of putative transgenic plants

Fully developed rooted tomato plantlets were removed from culture vessels and were carefully washed with sterile distilled water. These plants were then planted in plastic cups containing sterilized potting mixture.

The established putative transgenic tomato plants were designated as T₀-generation plants. A set of the untransformed explants were also regenerated and maintained as negative controls.

Table 5.2 Media compositions

<table>
<thead>
<tr>
<th>Media code</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSO</td>
<td>MS basal (without growth regulators) liquid and solid</td>
</tr>
<tr>
<td>MST</td>
<td>MS + 6.65 µM BAP + 1.14 µM IAA</td>
</tr>
<tr>
<td>MST I</td>
<td>MS + 200 mg/l cefotaxime + 100 µM acetosyringone</td>
</tr>
<tr>
<td>MST II</td>
<td>MS + 6.65 µM BAP + 1.14 µM IAA +200 mg/l cefotaxime +10 mg/l hyg B</td>
</tr>
<tr>
<td>MSR</td>
<td>MS + 4.92 µM IBA + 200 mg/l cefotaxime + 10 mg/l hyg B</td>
</tr>
</tbody>
</table>

(30 g l⁻¹ sucrose, 8 g l⁻¹ agar, pH-5.8)
5.2.7 Histological assay for GUS expression

Putative transformants were identified by GUS (β-glucuronidase) expression with the substrate 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc). For histochemical evaluation, callus and leaf tissues were collected after 2 cycles of antibiotic selection on selection media and tested for the histochemical activity of GUS reporter gene. The GUS expression assay was carried out by means of modified method of Jefferson et al. (1987). The tissues were incubated in GUS solution containing 1 mM X-Gluc (Sigma-Aldrich, USA), 100 mM Na-phosphate buffer solution (pH 7.0), 10 mM EDTA, 0.5 mM K₃Fe(CN)₆, 0.5 mM K₃Fe(CN)₆ 3H₂O and 0.1% Triton X-100 and kept in dark overnight (12-18 h) at room temperature. Destaining of tissues was done in a destaining solution [50% (v/v) ethanol, 10% (v/v) formaldehyde, and 5% (v/v) glacial acetic acid] overnight (Zheng et al., 1993). The histochemical GUS expression was also analyzed in tissues of putative antibiotic resistant transformed plantlets.

5.2.8 Molecular characterization of transformed (T₀) plants

Gene-specific primers were used to detect the transgene in putative transformed plants. The following primer sets were used to amplify the P5CS gene (2.8 Kb).

Forward primer: ACCATATGTGCTCTAAAGGCTATTGC

Reverse primer: GCGTCGACGAATTCCCGATCTAGTAA

These primers were procured from Bioresources Pvt. Ltd. Pune (MS), India. The genomic DNA of tomato was extracted from young leaves of control and putative transgenic plants (T₀), by using GeNie™ Ultrapure plant genomic DNA prep kit (Bangalore Genie, India) as per manufacturer’s instructions and was used as template DNA for PCR analysis. The DNA quantification, pellets obtained in the eppendorf tubes (Tarsons, India) were diluted in 50 µl of TE buffer and electrophoresed for 3 h at 60 V in 0.8% agarose gel prepared in 1x TAE buffer. The PCR reaction was carried out by using purified genomic DNA by using the method described by Sambrook and Russel (2001) and reaction was run in a
thermal cycler (Perkin Elmer – 2400). The total 25 µl PCR mixture contained 1 µl DNA (25-50 ng), 10 pmol of each primer, 200 µM each dNTP, 2 mM MgCl₂, 1x PCR buffer and 1U Taq DNA polymerase (Bangalore Genie, India). PCR amplifications were carried out starting with an initial denaturation at 94 °C for 5 min, denaturation at 94 °C for 1 min, annealing at 48 °C for P5CS primers for 45 sec and extension at 72 °C for 2 min. These steps were repeated for 40 cycles followed by a final extension for 5 min at 72 °C. The reaction mixture devoid of template was run as a negative control. Positive controls for P5CS were also included. Amplified DNA fragments (10 µl of post amplification products) were loaded in to 1.2 % agarose gel and the gel was stained with ethidium bromide. The image analysis was performed on a gel documentation system.

5.2.9 Proline content in T₀ plants:

Free proline content was analysed from leaves of 5 PCR positive transformed (T₀) plants independently along with the in vitro raised non-transformed plants of tomato cv Dhanashri, as described earlier in section 3.2.5. The proline content was estimated from fresh leaves of each plant independently and was expressed as pmol g⁻¹ dry weight.

During this investigation, all biosafety measures were taken into consideration as per guideline of IBSC, DBT, India.

5.3 Results

5.3.1 Antibiotic sensitivity of cotyledonary leaf explants

To find out the appropriate concentration of antibiotics for Agrobacterium-mediated transformation in tomato cv Dhanashri, kill-curve for hygromycin B and cefotaxime were evaluated separately for cotyledonary leaf explants. The results on effect of different concentration of hygromycin B on sensitivity of cotyledonary leaves of tomato cv Dhanashri are presented in Table 5.3. The explants were cultured on MST supplemented with various concentrations of hygromycin B (0-40 mg l⁻¹). The control explants showed 100% survival on MST (without antibiotics). Hygromycin B showed significant effect on percent survival of explants. With increase in hygromycin B concentration, the percent survival of
the explants also decreased. The explants turned brown within 15 days and finally
died on media containing 10 mg l\(^{-1}\) or more hygromycin B. These observations
indicated that 10 mg l\(^{-1}\) hygromycin B was the threshold concentration for being
utilized for transformation experiments and therefore was used for selection of
transformants in tomato cv Dhanashri.

**Table 5.3 Effects of different concentrations of hygromycin B on sensitivity of
cotyledonary leaf explants of tomato cv Dhanashri.**

<table>
<thead>
<tr>
<th>Hygromycin mg l(^{-1})</th>
<th>Percent of survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>100 ± 1.5(^{c})</td>
</tr>
<tr>
<td>5.0</td>
<td>100 ± 1.8(^{c})</td>
</tr>
<tr>
<td>10.0</td>
<td>100 ± 2.0(^{c})</td>
</tr>
<tr>
<td>15.0</td>
<td>85 ± 2.5(^{bc})</td>
</tr>
<tr>
<td>20.0</td>
<td>65 ± 2.8(^{b})</td>
</tr>
<tr>
<td>25.0</td>
<td>50 ± 2.6(^{b})</td>
</tr>
<tr>
<td>30.0</td>
<td>10 ± 1.5(^{a})</td>
</tr>
<tr>
<td>35.0</td>
<td>0.0</td>
</tr>
<tr>
<td>40.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Results are mean of three replicates (20 X 3) ± SE
Means followed by the same letter are not significantly different as per DMRT at \(p \leq 0.05\)

**Table 5.4 Effect of different concentrations of cefotaxime on cotyledonary leaf
explants cultures.**

<table>
<thead>
<tr>
<th>Cefotaxime mg l(^{-1})</th>
<th>Percentage survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>100 ± 2.5(^{a})</td>
</tr>
<tr>
<td>100</td>
<td>100 ± 3.8(^{d})</td>
</tr>
<tr>
<td>200</td>
<td>100 ± 2.4(^{d})</td>
</tr>
<tr>
<td>300</td>
<td>78 ± 1.9(^{c})</td>
</tr>
<tr>
<td>400</td>
<td>48 ± 2.6(^{b})</td>
</tr>
<tr>
<td>500</td>
<td>22 ± 1.5(^{a})</td>
</tr>
</tbody>
</table>

Results are mean of three replicates (20 X 3) ± SE
Means followed by the same letter are not significantly different as per DMRT at \(p \leq 0.05\)

The results on effect of different concentration of cefotaxime on sensitivity of
cotyledonary leaves of tomato cv Dhanashri are presented in **Table 5.4.** The
cefotaxime was utilized to control the growth of *Agrobacterium* during post
cultivation and subsequent plant regeneration. Different concentrations of
cefotaxime i.e., 0, 100, 200, 300, 400 and 500 mg l\(^{-1}\) were tested to find out the
sensitivity of explants to cefotaxime. Cefotaxime at 0 to 200 mg l\(^{-1}\) in media
showed 100% survival (Table 5.4). However, at 300, 400 and 500 mg l\(^{-1}\) cefotaxime the cotyledonary leaf explants’ survival percentage decreased with increasing concentration of cefotaxime. The 100% explant survival was observed upto 200 mg l\(^{-1}\) cefotaxime, therefore this concentration was used to control growth of \textit{Agrobacterium} during selection of putative transformants.

### 5.3.2 Effect of acetosyringone (AS, 3’, 5’-dimethoxy-4’-hydroxyacetophenone) on transformation in tomato cv Dhanashri

<table>
<thead>
<tr>
<th>Concentration of acetosyringone (µM)</th>
<th>Percentage of calli showing GUS expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>50</td>
<td>13.0 ± 0.75(^a)</td>
</tr>
<tr>
<td>100</td>
<td>29.0 ± 1.50(^b)</td>
</tr>
<tr>
<td>150</td>
<td>23.0 ± 1.20(^b)</td>
</tr>
<tr>
<td>200</td>
<td>18.0 ± 0.80(^{ab})</td>
</tr>
</tbody>
</table>

Results are mean of three replicates (20 X 3) ± SE
Measures followed by the same letter are not significantly different as per DMRT at p= 0.05

Table 5.5 Effect of AS on histochemical GUS activity in cotyledonary leaf explants

The effects of various acetosyringone concentrations used (0, 50, 100, 150 and 200 µM) on \textit{Agrobacterium}-mediated transformation using cotyledonary leaves are summarized in Table 5.5. Acetosyringone was proved to be essential for any transformation and 100 µM concentration was found to be optimal as suggested by the GUS activities in calli following \textit{Agrobacterium} mediated transformation with pCAMBIA 1301. Therefore, in the present study, 100 µM acetosyringone was used in co-cultivation media.

### 5.3.3 Effect of optical density (OD\(_{600}\)) of \textit{Agrobacterium} culture on transformation

<table>
<thead>
<tr>
<th>OD at 600 nm of bacterial suspension</th>
<th>Percentage of calli showing Gus expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>0.2</td>
<td>0.0</td>
</tr>
<tr>
<td>0.4</td>
<td>12.0 ± 0.60(^a)</td>
</tr>
<tr>
<td>0.6</td>
<td>32.0 ± 1.30(^b)</td>
</tr>
<tr>
<td>0.8</td>
<td>24.0 ± 1.15(^{ab})</td>
</tr>
<tr>
<td>1.0</td>
<td>18.0 ± 1.20(^{ab})</td>
</tr>
</tbody>
</table>

Results are mean of three replicates (20 X 3) ± SE
Means followed by the same letter are not significantly different as per DMRT at p= 0.05

Table 5.6 Effect of bacterial growth and optical density on histochemical GUS activity in *Agrobacterium*-mediated transformed tissues in presence of 100 µM AS

The results presented in the Table 5.6, clearly indicate that the concentration of suspension (OD at 600 nm) affects the frequency of genetic transformation in cotyledonary leaves explants. Bacterium was used at densities ranging from 0.0 to 1.0 to infect the plant tissues. The infection and co-cultivation media were supplemented with 100 µM AS. Cell densities below 0.4 did not show any GUS expression in the tissues whereas highest (32.0 ± 1.30) GUS expression was observed at OD 0.6. Thus, concentration equivalent to OD 0.6 was optimum for *Agrobacterium* mediated transformation in tomato cv Dhanashri.

Higher bacterial densities resulted into overgrowth of bacteria and it adversely affected the growth of explants and subsequent plant regeneration. In the present study, it was observed that cell density equivalent to 0.6 OD reduced the chances of browning of explants after co-cultivation possibly due to reduced damage of explants during infection. Therefore, the most optimum concentration of *Agrobacterium* cell suspension for transformation in tomato cv was equivalent to 0.6 OD.

5.3.4 Effect of co-cultivation period on transformation efficiency:

<table>
<thead>
<tr>
<th>Duration of co-cultivation (days)</th>
<th>Percentage of calli showing GUS expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8.0 ± 0.76\textsuperscript{a}</td>
</tr>
<tr>
<td>2</td>
<td>29.0 ± 1.45\textsuperscript{b}</td>
</tr>
<tr>
<td>3</td>
<td>38.0 ± 1.62\textsuperscript{bc}</td>
</tr>
<tr>
<td>4</td>
<td>32.0 ± 1.55\textsuperscript{b}</td>
</tr>
<tr>
<td>5</td>
<td>13.0 ± 0.85\textsuperscript{a}</td>
</tr>
</tbody>
</table>

Results are mean of three replicates (20 X 3) ± SE
Means followed by the same letter are not significantly different as per DMRT at p= 0.05

Table 5.7 Effect of co-cultivation period on histochemical GUS expression in *Agrobacterium*-mediated transformed cotyledonary leaf explants in presence of 100 µM AS

90
Co-cultivation period of 1, 2, 3, 4 and 5 days was tested and GUS activities were observed in the tissues co-cultivated for 2 or more days results (Table 5.7). It was evident that the expression was less in the beginning but the tissues were adversely affected by prolonged co-cultivation with *Agrobacterium* and resulted over-growth of bacterial cells. Highest GUS expression was observed after 3 days of co-cultivation in cotyledonary leaf explant tissues suggesting the most appropriate duration required for *Agrobacterium* transformation in tomato cv Dhanashri.

### 5.3.5 Frequency of shoot regeneration, rooting and survival of plants after *Agrobacterium*-mediated gene transfer

In the present investigation, the experiments on transformation in tomato cv Dhanashri were designed as described in the section 5.2 and evaluated some parameters for efficient transformation. Based on the results obtained, a standardized reproducible protocol for transformation using cotyledonary leaf explants in tomato cv Dhanashri has been devised and presented here.

Fifteen days old *in vitro*-grown-seedling of tomato cv Dhanashri were used as source of cotyledonary leaf explants. The margins of cotyledonary leaves were injured to allow the bacterium to infect. These explants were dipped in the MS basal liquid medium supplemented with 100 µM AS containing *Agrobacterium* construct pCAMBIA 1301-*P5CS-F129A* for 2-3 min with gentle shaking and blotted dry on a sterilized paper towel. The explants were inoculated on co-cultivation medium MST-I supplemented with 100 µM AS for 3 days at 25 ± 2 ºC in dark. After 3 days of co-cultivation, the explants were transferred on MST-II media fortified with 200 mg l⁻¹ cefotaxime and 10 mg l⁻¹ hygromycin B for selection and growth for two cycles of 14 days each. The integration and expression of transgene into host genome was confirmed using histochemical GUS expression in survived tissues of explants after two selection cycles. In the course of selection cycle, the transformed cells formed callus and direct organogenesis was also observed. The regenerated *in vitro* shoots were transferred to the rooting media (MSR) supplemented with 200 mg l⁻¹ cefotaxime and 10 mg l⁻¹ hygromycin B for 2 weeks. *In vitro* rooted plantlets were taken out from culture vessels and
washed gently under running tap water to remove traces of agar attached to the roots before transferring to plastic pots. The leaves and excess roots were trimmed before planting. Fully developed rooted tomato plantlets were planted in sterilized vermiculite: garden soil (1:1) mixture in plastic cups with drainage holes. The plastic pots were covered with polythene bags to maintain high (70 to 80%) humidity. The cups were kept in the growth room illuminated with 650 lux light intensity and 25 ± 2 °C temperature. The pots were then transferred to shade and subsequently were shifted to the closed polyhouse. All the 5 PCR positive lines were fertile and their seeds were collected. Seeds of in vitro grown non transgenic plants were also collected.

The results obtained from the experiments on Agrobacterium-mediated transformation of cotyledonary leaf explants by following the standard protocol developed in the present investigation (described in above sections) are presented in Table 5.8

<table>
<thead>
<tr>
<th>No. of explants infected</th>
<th>Number of explants survived after two selection cycle</th>
<th>Number of regenerated shoots</th>
<th>Number of shoots developed roots</th>
<th>Number of regenerated plantlets transferred to pots for hardening</th>
<th>Number of survived plants after hardening and transferred to green house</th>
<th>Transformation efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>200</td>
<td>45</td>
<td>23</td>
<td>18</td>
<td>15</td>
<td>5</td>
<td>2.5</td>
</tr>
</tbody>
</table>

Table 5.8 Frequency of shoot regeneration, rooting and survival of plants after Agrobacterium-mediated gene transfer

After optimization of transformation parameters, about 200 cotyledonary explants were infected with Agrobacterium tumefaciens strain LBA 4404 harboring plasmid pCAMBIA1301-P5CS-F129A gene. Forty five explants survived after 2nd selection cycle, out of which only 5 plants survived after hardening and transferred to green house. Hence, the overall plant transformation frequency (number of independently transformed plants over number of cotyledonary explants infected) was 2.5%. Transformation efficiency was
expressed as the number of putative transgenics (T₀) plants per 100 explants selection and survival upto harvesting stage.

Thus, an efficient and reproducible method for transformation using cotyledonary leaf explants was standardized with around 2.5% transformation efficiency.

5.3.5 Histochemical and Molecular characterization of transformed (T₀) plants

Transgene P5CS-F129A was stably maintained in T₀ lines of transgenic tomato cv Dhanashri plants. PCR analysis proved the stability with genomic insertion of transgene (Plate V). P5CS-F129A stable insertion was also confirmed by following the histochemical GUS expression in T₁ generation plants tissues. Histochemical GUS expression and PCR analysis are commonly used techniques for confirmation of transgene integration into host genome.

3.5.6 Proline content in T₀ plants:

<table>
<thead>
<tr>
<th>Tomato line</th>
<th>Proline content (µg/g dry weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NT</td>
<td>1678 ± 122</td>
</tr>
<tr>
<td>T₀-1</td>
<td>4421 ± 153</td>
</tr>
<tr>
<td>T₀-2</td>
<td>5448 ± 163</td>
</tr>
<tr>
<td>T₀-3</td>
<td>4826 ± 123</td>
</tr>
<tr>
<td>T₀-4</td>
<td>6121 ± 197</td>
</tr>
<tr>
<td>T₀-5</td>
<td>4196 ± 145</td>
</tr>
</tbody>
</table>

Results are mean of three replicates (20 X 3) ± SE.

Table 5.9 Comparison between proline content in leaves of non-transgenic (NT) and T₀ transgenic lines of tomato cv Dhanashri

The results presented in the Table 5.9 clearly show significant difference in proline content in non-transgenic and transgenic (T₀) lines. Proline content varied among the transgenic lines. In general, all the T₀ lines showed higher proline content than the non-transgenic plants. The maximum proline content (6121 µg g⁻¹ dry wt.) was observed in T₀-4 line among all 5 PCR positive lines. Proline content was little over three times more than non-transgenic plants (1678 µg g⁻¹ dry wt.).
These results confirm the functional expression of *P5CS-F129A* gene in tomato cv Dhanashri.

### 5.4 Discussion

#### 5.4.1 Antibiotic sensitivity of cotyledonary leaf explants

From the results are presented in Table 5.3 the observations indicated that 10 mg l\(^{-1}\) hygromycin B was the threshold concentration for being utilized for transformation experiments.

To increase the incidence of transgenic plant production and to efficiently inhibit the growth of non-transformed plants, optimization of concentration of selection agent is one of the key factors in plant transformation. Hygromycin phosphotransferase (*hpt*) is commonly used as the most efficient selection marker gene in post-co-cultivation for antibiotic selection (Gritz *et al*., 1983; Staben *et al*., 1989; Ortiz *et al*., 1996). Hygromycin B is an aminoglycoside antibiotic biosynthesized by *Streptomyces hygroscopicus*, which inhibit protein synthesis in bacteria, fungi and higher eukaryotic cells. It is the most widely used antibiotic selection agent, as it kills the untransformed cells more quickly as compared to kanamycin. This type of selection is referred as negative selection (Shrawat and Lorz, 2006). Therefore, investing the lethal dose of hygromycin B for survival of the cotyledonary leaf explants will of great help before transformation work, as this concentration of selection agent is required to avoid growth of unwanted numbers of escape, post- transformations.

In the present study, hygromycin B showed significant effect on percentage survival of cotyledonary leaf explants. On control medium (without antibiotic) 100% explants survived. With increasing hygromycin B concentration, the percent survival of the explants was found to decrease. The explants started turning brown after 2-3 days at 10 mg l\(^{-1}\) hygromycin B. Subsequently, all explants turned completely dark brown and 100% of these explants died. These observations suggested that 10 mg l\(^{-1}\) hygromycin B was the optimum concentration suitable for selecting the putative transformants in further transformation experiments.
The data obtained on the effects of different concentrations of hygromycin-B on explants survival suggested that 10 mg l⁻¹ concentration was optimal for selecting transformants in subsequent experiments. The hygromycin B concentration used for tomato cv Dhanashri in these study was rather low as compared to used in other systems earlier. Roy et al. (2006) used 40 mg l⁻¹ of hygromycin as a selective marker for drought tolerance in tomato cv. Pusa Ruby. This lower hygromycin-B concentration required for transformation in tomato cv Dhanashri is suggestive of the plant’s lower internal resistance to hygromycin-B as compared to other cultivars used by other researchers.

The results presented in the Table 5.4, revealed that the 100% explant survival was observed up to 200 mg l⁻¹ cefotaxime, therefore this concentration was used to control growth of Agrobacterium during selection of putative transformants.

Cefotaxime has been commonly used as an effective antibiotic for elimination or suppression of Agrobacterium cells (Mathias and Boyd, 1986; Tang et al., 2000; Alsheikh et al., 2002). Cefotaxime shows a broad spectrum of activity against bacteria and a low toxicity to eukaryotes. In the previous studies (Roy et al., 2006; Ahsan et al., 2007), 500 mg l⁻¹ cefotaxime was used to control bacteria in tomato cv. Pusa Ruby for drought tolerance.

5.4.2 Effect of acetosyringone (AS, 3’, 5’-dimethoxy-4’-hydroxyacetophenone) on transformation in tomato cv Dhanashri

The effects of various acetosyringone concentrations used (0, 50, 100, 150 and 200 µM) on Agrobacterium-mediated transformation using cotyledonary leaves are summarized in Table 5.5. Acetosyringone was proved to be essential for any transformation and 100 µM concentrations was found to be most favorable.

High necrosis and poor survival rate of target plant tissues (Greenberg and Yao, 2004) are some of the major factors that affect the efficiency of Agrobacterium-mediated T-DNA transfer into plant cells. These factors may be the result of or linked to hypersensitive defense reaction in plants to
**Agrobacterium** infection, which may involve the recognition of specific signals from the *Agrobacterium* that triggers the burst of reactive oxygen species (ROS) at the infection site (Wojtaszek, 1997). Application of antioxidants, addition of acetosyringone and optimization of pre-culture conditions suppress the *Agrobacterium* induced hypersensitive necrotic response in target plant tissues, and induce virulence genes in Ti plasmids thereby enhancing stable transformation (Lippinocott and Lippinocott, 1975; Stachel et al., 1985; Kuta and Tripathi, 2005). Transformation of dicotyledonous plants is enhanced by the addition of acetosyringone to co-cultures or bacterial cultures (Wordragen and Dons, 1992) by multiple insertion of T-DNA (Winans, 1992; Lipp Joao and Brown, 1993). Utilization of acetosyringone not only enhances the transformation efficiency but also activates the differentiation process.

In the present investigation addition of 100 µM AS during pre culture and co-cultivation was found to be the most optimum concentration for transformation ([Table 5.5](#)) and this observation was similar to the previous reports. Acetosyringone has been used to enhance the transformation efficiency in different plants including *Arabidopsis* (Sheikholeslam and Weeks, 1987), sugar beet (Jacq et al., 1993), onion (Eady et al., 2000), buffel grass (Batra and Kumar, 2003), tea (Lopez et al., 2004), and tomato (Davis et al., 1991; Lipp Joao and Brown, 1993; Mathews et al., 2003; Cortina and Culianez-Macia, 2004; Reda et al., 2004; Raj et al., 2005, Chaudhry and Rashid, 2010, Afroz et al., 2010). The results of the present studies also showed improved transformation using acetosyringone. This confirms that chemicals such as acetosyringone for virulence induction are recommended in transformation protocols.

### 5.4.3 Effect of optical density (OD\textsubscript{600}) of *Agrobacterium* culture on transformation

The results presented in the [Table 5.6](#), clearly indicate that the concentration of *Agrobacterium* cell suspension (OD at 600 nm) affects the incidence of genetic transformation in cotyledonary leaves explants.
Co-cultivation duration is one of the important factors affecting *Agrobacterium*-mediated gene transformation. Fifteen days old *in vitro* grown seedlings derived cotyledonary leaves were co-cultivated with *Agrobacterium* for 1, 2, 3, 4 and 5 days in different experiments. In general, co-cultivation period for tomato transformation varied from 2-5 days. However, in the present investigation it was observed that 3 days was optimal duration for tomato transformation ([Table 5.6](#)). These results are in line with (Reda *et al*., 2004; Abu-El-Heba *et al*., 2008) in which transient GUS expression was detected after three days of co-cultivation, while differing from reports of Roy *et al.* (2006) and Paramesh *et al.* (2010), where 2 days duration was used for co-cultivation. The reason for this difference might be attributed to the difference in the tomato cultivars and *Agrobacterium* strain used.

### 5.4.4 Frequency of shoot regeneration, rooting and survival of plants after *Agrobacterium*-mediated gene transfer

The results obtained from the experiments on *Agrobacterium*-mediated transformation of cotyledonary leaf explants by following the standard protocol developed in the present investigation are presented in [Table 5.8](#). The results in turn shows 2.5% plant transformation frequency in tomato cv Dhanashri.

Several workers have reported varying transformation frequencies in tomato. It has been expressed as percent explants regenerating on selection medium (Madhulatha *et al.* 2007), percent co-cultivated explants showing shoot regeneration on selection medium (Patil *et al.* 2002) or percent co-cultivated explants producing transgenic plants representing independent transformation events (Frary and Earle 1996; Park *et al.* 2003; Sun *et al.* 2006). Transformation efficiency values varies with genotype as reported by several researchers in the literature are 8% (Vidya *et al.* 2000), 7–37% (Ling *et al.* 1998), 9% (Roekel *et al.* 1993), 11% (Frary and Earle 1996), 14% (Hamza and Chupeau 1993), 20% (Park *et al.* 2003), 20% (Qiu *et al.* 2007), 25% (Hu and Phillips 2001) and 28–48% (Sun *et al.* 2006).
5.4.5 Histochemical and Molecular characterization of transformed (T₀) plants

Transgene *P5CS-F129A* was stably maintained in T₀ lines of transgenic tomato cv Dhanashri plants. PCR analysis proved the stability with genomic insertion of transgene (**Plate V**). *P5CS-F129A* stable insertion was also confirmed by following the histochemical GUS expression in T₁ generation plants tissues. Histochemical GUS expression and PCR analysis are commonly used techniques for confirmation of transgene integration into host genome. Several researcher have used these analyses for confirmation of *P5CS* gene in various crop plants like rice (Zhu *et al.*, 1998; Anoop and Gupta, 2003; Su and Wu, 2004); citrus rootstock (Molinari *et al.*, 2004); hybrid larch (Deirdre *et al.*, 2005); pea (Najafi *et al.*, 2005); potato (Hmida-Sayari *et al.*, 2005), wheat (Vendruscolo *et al.*, 2007), chickpea (Bhatnagar-Mathur *et al.*, 2009), *Eucalyptus saligna* (Dibax *et al.*, 2010).

3.4.6 Proline content in T₀ plants

The results presented in the **Table 5.9** clearly show significant difference in proline content in non-transgenic and transgenic (T₀) lines. Proline content varied among the transgenic lines. In general, all the T₀ lines showed higher proline content than the non-transgenic plants. The maximum proline content (6121 µg g⁻¹ dry wt.) was observed in T₀-4 line among all 5 PCR positive lines. Proline content was little over three times more than non-transgenic plants (1678 µg g⁻¹ dry wt.). These results confirm the functional expression of *P5CS-F129A* gene in tomato cv Dhanashri.

The *P5CS*-transgenic wheat plants were shown to exhibit much higher (more than 10 folds higher) proline content than their wild types under non stress conditions (Sawahel and Hassan, 2002). Han and Hwang (2003) reported up to 6 fold higher proline content in callus and leaves of *P5CS*-transgenic carrot. Citrus rootstock Carrizo citrange transgenic plants over-expressing *P5CS* gene showed 120 µmol proline g⁻¹ FW as compared to 17.7 µmol proline g⁻¹ FW in non-transgenic plants. Similar results have been reported in case of P5CS-transgenic
potato plants by Hmida-Sayari et al. (2005). Najafi et al. (2005) observed about 4 times increase in proline content in transgenic pea plants carrying P5CS gene against the non transgenic plants. Yamchi et al. (2007) observed 26 times more proline accumulation in tobacco plants transformed with P5CS gene as compared to non-transgenic tobacco plants. Bhatnagar-Mathur et al. (2009) reported high proline (2–6 folds) accumulation in chickpea produced with the $P5CS-F129A$ gene through Agrobacterium tumefaciens-mediated gene transfer by using of axillary meristem explants. Dibax et al. (2010) also reported four times higher proline content in transformants than untransformed plants in Eucalyptus saligna transformed with Agrobacterium tumefaciens-mediated transformation with $P5CS$ gene.

The results reported in the present investigation are similar to these reports and confirmed the integration of $P5CS$ gene into the genome of tomato cv Dhanashri and its functional expression.

Thus, the present investigation provides a useful transformation and regeneration protocol for elite cultivar allowing for transferred genes to be inherited stably and in predictable manner.