CHAPTER III

3. AGROBACTERIUM–MEDIATED GENETIC TRANSFORMATION AND PLANT REGENERATION FROM COTYLEDONARY NODE EXPLANTS OF CUCUMIS SATIVUS L.

3.1. Introduction

Cucumber (Cucumis sativus L., cv. ‘Green long’) is an important horticultural crop belongs to the family Cucurbitaceae. It is one of the most important vegetable crops, consumed raw as a fresh vegetable and fruit. Global production of cucumber including of gherkins, reached 60.6 million tons in 2009 (http://faostat.fao.org) which was among the top ten vegetables produced globally in 2009. In India, Cucumber consumed by large number of populations mainly in their daily food dishes. In nutrition, cucumbers are rich in fiber and contain a variety of beneficial minerals like silica, potassium, phosphorus, magnesium, molybdenum. It’s also a good source of vitamin C, vitamin A, folic acid (only if it is unpeeled) and oxalic acid.

One of the pre-requisites for successful gene transfer to plants is the availability of a suitable protocol for transformation which is compatible with in vitro plant regeneration method of the targeted plant species. Genetic engineering can improve crops by exploiting useful genes and engineering transgenic plants with desirable properties such as quality improvement, disease resistance, introduction of antibiotic resistant npt II gene, herbicide resistant bar gene, HBV surface antigen gene into cucumber genotypes via Agrobacterium–mediated gene transfer using different explants and cultivars has been documented earlier (Rajagopalan and Perl Treves, 2005; Vengadesan et al., 2005a; Sindhu and Soniya, 2010). Although transformation is influenced by several factors including genotype, explants source and type of Agrobacterium strain, type of plasmid vector and culture conditions, it is necessary to develop a transformation system for each species and sometimes different genotypes within a species. In order to establish a successful strategy for practical plant genetic engineering, it is important to develop systems for recovering large numbers of whole plants from primary explants (Vasudevan et al., 2007). An
efficient plant regeneration system is most essential for transformation of this important cultivar. Though, the plant regeneration and transformation was reported in Cucumis sativus L., cv. ‘Green long’, but there were only few reports in ‘Green long’ cultivar.

Recent advances in insertion of genes through Agrobacterium–mediated transformation would facilitate the development of new genotypes without significantly altering the genetic composition and have made it possible to improve their productivity and quality beyond the limit of traditional breeding. The gus reporter gene has been widely used as a marker for plant transformation (Lee et al., 2006; Chin et al., 2009). Agrobacterium–mediated genetic transformation methods were also documented by some workers. There were few reports on development of transgenic plants in cucumber (Sindhu and Soniya, 2010; Vengadesan et al., 2005a). Both GUS and GFP genes were expressed in cucumber by Soniya and Das (2002), Selvaraj et al. (2010). Plants are also one of the most cost effective and safe systems for large scale production of recombinant proteins for industrial applications (Hunt, 2005). However, production of transgenic cucumber carrying agronomically important traits in cucumber has been infrequently reported (Nanasato et al., 2013).

Tissue culture studies in cucumber has been achieved via organogenesis using different explants such as cotyledons (Selvaraj et al., 2007), shoot tips (Vasudevan et al., 2004), embryonal axis (Vasudevan et al., 2007), hypocotyls (Selvaraj et al., 2006), nodal segments (Ahmad and Anis, 2005; Kontas and Kintzios, 2003). Plant regeneration via somatic embryogenesis was also reported in cucumber by Mashayekhi et al. (2008). But the plant regeneration from one cultivar may be very different from that of another cultivar within the same species (Oridate et al., 1992). Therefore, efficient plant regeneration protocol should be developed for each cultivar (Walden and Wingender, 1995). The morphological or physiological variations were much lower in the shoots produced by the direct regeneration of explants. Therefore, in the present study, an efficient method for high frequency shoots production via direct organogenesis from cotyledonary node explants of cucumber was established. Accordingly the present study was attempted to develop a more efficient and reproducible cucumber transformation via direct organogenesis.
in an important cultivar ‘Green long’ with an important Agrobacterium tumefaciens strain EHA 105.

3.2 Materials and methods

3.2.1 Plant material and growth conditions

Seeds of cucumber (Cucumis sativus L., cv. ‘Green long’) were soaked in tap water for 1 hr and then washed with 10% (v/v) commercial detergent Tween 20 for 10 min followed by three rinses with distilled water. Seeds were further disinfected with 0.1% (w/v) mercuric chloride solution for 5 min and rinsed 5 times with sterile distilled water to remove the traces of mercuric chloride. Finally, the sterilized seeds were blotted dry on sterile Whatmann No.1 filter paper. Disinfected seeds were placed on medium in the culture tubes (25×150 mm) containing sterile moist cotton, plugged tightly with non-absorbent cotton and placed in the dark condition for germination. All media used for the present study were based on MS salts (Murashige and Skoog, 1962) containing 3% (w/v) sucrose. The MS medium were supplemented with 1.5 mg/L BAP for shoot bud regeneration and 1.5 mg/L IBA + 0.5 mg/L KIN for root induction. The medium of the pH was adjusted to 5.7 before solidifying with 0.7% (w/v) agar. The melted medium was autoclaved at 121°C for 20 min with 15 lbs pressure. All the cultures were maintained at 25±2°C under a 16/8-h (light/dark) photoperiod at 60 μ E m⁻² s⁻¹ by cool white fluorescent tubes.

3.2.2 Agrobacterium strain and binary vector

The strain of Agrobacterium tumefaciens used in this study was EHA 105 and LBA4404 harbouring the binary vector pBI121 contains npt II gene for kanamycin resistance and encoding β-glucoridase (uid A or gus) marker gene under the control of CaMV35S promoter. The uid A (gus) gene driven by the CaMV35S promoter and terminator sequences served as reporter gene. The neomycin phosphotransferase II (npt II) gene driven by the nopaline synthase was used as the selectable marker gene. Agrobacterium strain was maintained on LB agar plates containing 50 mg/L kanamycin sulfate and 25 mg/L rifampicin. A single bacterial colony was inoculated into 25 mL of liquid LB (Luria and Bertonii) medium with
antibiotics containing 50 mg/L kanamycin and 25 mg/L rifampicin in an Erlenmeyer flask. The culture was then shaken at 120 rpm overnight in the dark at 28°C until an A600 at 0.6 (late log phase) was reached.

3.2.3 Evaluation of factors influencing plant transformation efficiency

The experiments were designed to evaluate the effect of different parameters on the transformation efficiency of cucumber. The parameters were as follows: Two Agrobacterium strains EHA 105 and LBA 4404, four co-cultivation periods of 2, 3, 4 and 5 day, four pre-cultivation periods of 2, 3, 4 and 5 day and different concentrations (25, 50, 75 and 100 mg/L) of kanamycin. These factors will be helpful for the identification of optimum combination of transformation parameters, which were then employed to produce transgenic plants.

3.2.4 Transformation and plant regeneration

3.2.4.1 Co-culture

The cotyledonary node explants were excised from 5-day-old seedlings and precultured for 3 days on shoot bud regeneration medium prior to infection with bacteria. The precultured explants were infected with 25 mL of bacterial suspension for 10 min, drained the explants in Whatmann No. 1 filter paper to remove the attached excess Agrobacterium tumefaciens cells. Then the explants were cultured on co-cultivation medium and the plates were carefully sealed with parafilm.

3.2.4.2 Selection and plant regeneration

After co-culture, the cotyledonary node explants were washed with sterile liquid half-strength MS medium. Then the explants were blotted dry on sterile filter paper and then transferred onto a selection medium supplemented with 3% sucrose, 1.5 mg/L BAP, 50 mg/L kanamycin and 300 mg/L cefotaxime. Further, the cotyledonary explants were sub-cultured every other week onto fresh selection medium in order to maintain appropriate selective pressure and to avoid over growth of Agrobacterium tumefaciens cells. Kanamycin resistant shoots were regenerated
from the cotyledonary node explants via direct organogenesis were excised and cultured onto fresh medium for further growth.

3.2.4.3 Elongation and rooting development

The adventitious kanamycin-resistant shoots regenerated from the cotyledonary node explants via direct organogenesis were carefully excised and transferred to selection medium along with 1.0 mg/L GA₃ to promote shoot elongation. After the elongation of shoots, well developed elongated kanamycin-resistant shoots were transferred to rooting medium. The rooted plantlets were gently removed from the culture tubes, washed initially to remove adhered agar traces to avoid contamination. Then they were transferred to the plastic cups containing sterile sand and soil in the ratio 1:1 and covered with polythene bags to ensure high humidity and placed in the controlled environment. After 2 weeks, the polybags were removed and the plantlets were shifted to the greenhouse conditions.

3.2.5 Confirmation of transformants

3.2.5.1 Histochemical GUS assay

Histochemical assay of β-glucoronidase (GUS) activity was performed according to the method described by Jefferson et al. (1987). The transformed leaf obtained from the selective medium were tested for histochemical GUS expression in a X-Gluc solution (5-Bromo-4-chloro-3-indolyl-β-D-glucuronic acid) solution and incubated at 37°C overnight. After incubation, the cultures were bleached after staining by immersion in 80% (v/v) acetone for few hrs followed by several washes with 70% ethanol to remove the chlorophyll content. To detect GUS expression in putative transgenic plants, leaf segments were cut into small sections observed visually or microscopically.

3.2.5.2 Polymerase Chain Reaction (PCR) analysis of transformants

Genomic DNA was subjected to confirm the presence of a 530 bp fragment of the 35S promoter of the uid A (gus) gene and 800 bp of the npt II gene integrated into the plant genome. Plant DNA for PCR analysis was prepared as described
previously by Edward et al. (1991). PCR reactions were carried out in a 20 µL volume containing 2 µL 1× PCR buffer, 1 µL each of forward and reverse primer, 0.5 units of Taq DNA polymerase, 2 µL 1.5 mM dNTPs, 1 µL plant DNA (15 ng) and finally 13 µL of sterile water was added. PCR was performed with 30 cycles for npt II gene amplifications, denaturation at 94°C for 4 min, followed by 28 cycles of denaturation at 94°C for 1 min, annealing at 37°C for 1.30 seconds and extension at 72°C for 2 min, with a final extension at 72°C for 7 min in a thermal cycler (Cyber Lab, USA). Amplified PCR products were analyzed by electrophoresis on 1.5% agarose gel containing 0.5 µg/mL ethidium bromide in 1×TAE buffer. Electrophoresis was performed at 50V for 2 hrs until the bromophenol blue dye front migrated to the bottom of the gel. The gel was visualized under ultraviolet light and photographed with Alfa Image System USA.

3.3 Results and discussion

3.3.1 Transformation and selection

3.3.1.2 Direct Shoot regeneration from cotyledonary node explants

Cotyledonary node explants obtained from 5-day-old seedlings and cultured on MS medium supplemented with different concentrations of BAP (0.5–2.5 mg/L). The highest frequency of shoot bud multiplication (88.4%) and the highest number of shoots (19.85 shoots/culture) occurred on MS medium containing 1.5 mg/L BAP. Elongated shoots were transferred to half-strength MS medium containing different concentrations of IBA (0.5–2.0 mg/L) in combination with 0.5 mg/L KIN for root induction. The highest percent of rooting (96.2%) was noticed on a medium containing the combination of IBA (1.5 mg/L) and KIN (0.5 mg/L). In previous reports, cotyledonary node explants was found to be best for shoot bud multiplication in bottle guard (Saha and Kazumi, 2007), Okra (Rajan and Markose, 2007), Castor (Alam et al., 2010), and Leguminosae (Dang and Wei, 2009).

3.3.1.2 Agrobacterium–mediated genetic transformation

3.3.1.2.1 Optimization of the different parameters influencing the transformation and regeneration of transgenic plants
In cucumber cultivar ‘Green long’, there were very few reports on Agrobacterium–mediated genetic transformation using cotyledonary node as explants. In this study, four main parameters of influencing Agrobacterium–mediated genetic transformation were optimized including Agrobacterium strain, precultivation periods, co-cultivation periods, and different concentrations of kanamycin (Table 18). The cotyledonary node explant was selected as a suitable explant for direct shoot organogenesis and Agrobacterium tumefaciens mediated genetic transformation. The age of the explants was also most important in the genetic transformation and regeneration of transgenic plants. However, using explants in the good condition contributes in improving the efficiency of T-DNA insertion into the targeted cells and the regeneration of transgenic plants.

3.3.1.2.2 Effect of A. tumefaciens strain on cucumber transformation

There are several A. tumefaciens strains have been used for transformation of cucumber. In the present experiment, the efficiency of two A. tumefaciens strains, the agropine type EHA 105 and the octopine type LBA 4404 were used. The transformation efficiency obtained with the strain EHA 105 (28.5%) was higher than that of strain LBA 4404 (12.2%). Similarly Nguyen et al. (2013) reported that EHA 105 strain was found to be effective for Agrobacterium-mediated genetic transformation. Therefore, the present study reveals that A. tumefaciens strain EHA 105 is most suitable strain for transformation of cucumber using cotyledonary node as explants.

3.3.1.2.3 Effect of pre-cultivation duration on cucumber transformation

One of the important parameters that contributed to the high transformation efficiency of cucumber was the precultivation period of explants. Preconditioning of explants on shoot regeneration medium prior to infection with Agrobacterium strain leads to an increase in genetic transformation frequency. During the precultivation period, the explants are undergoing a physiological and developmental change to become competence for shoot regeneration and transformation (Ghorbel et al., 2000). A preculture of explants in the regeneration medium for 3 days was found to increase the transformation frequency (15.4%) in cotyledonary node explants.
Venkatachalam et al. (1998) reported that an important factor which enhanced the transformation efficiency was the 3 days preculture of the explants, which probably served to reduce wound stress and increased the number of competent cells at the wound site.

3.3.1.2.4 Effect of co-cultivation period

Co-cultivation period is another key factor influencing the frequency of Agrobacterium–mediated transformation in plants (Yang et al., 2013; Li et al., 2006; Nookaraju et al., 2012). Therefore, to evaluate the effect of duration of the co-cultivation period on cucumber transformation, cotyledonary node explants were co-cultivated with A. tumefaciens strain for 2-5 days. The explants co-cultivated with A. tumefaciens for 3-day had the highest transformation frequency value (24.6%). In contrast, the transformation efficiency obtained with 2-day co-cultivation period was found to be the lower integration of the strain into the plants.

And also, the transformation efficiency obtained with a 4 and 5-day co-cultivation time results in Agrobacterium contamination and necrosis of the infected tissues. In many plant species, a co-cultivation period of 3-day is optimum for transformation (Lee et al., 2006; Aileni et al., 2011). Shorter or longer co-cultivation period leads to lower transformation efficiencies (Lee et al., 2006; Aileni et al., 2011). In the present experiment, a 3-day co-cultivation period gave the highest transformation efficiency and so it was chosen for transformation.

3.3.1.2.5 Effect of kanamycin concentration on cucumber transformation

Kanamycin concentrations also affect the plant transformation efficiency. In this study, four levels of kanamycin sulfate selection were used in the co-cultivation media at concentrations of 25, 50, 75 and 100 mg/L. At 50 mg/L kanamycin sulfate level produced the highest transformation frequency (22.6%). Accordingly, kanamycin sulfate at 50 mg/L was used for selecting transformants in subsequent shoot multiplication, elongation and rooting stages to prevent possible escapes. Similar findings were also reported by Venkatachalam et al. (1998).
3.3.1.3 Production of transgenic plants

With the optimized parameters, a 5-day old cotyledonary node explants (3 days precultured explants) were infected with Agrobacterium tumefaciens strain EHA 105 for 10 min, followed by 3-day co-culture (Fig 8A). Co-cultivated explants were began to develop into greenish shoot buds on selective shoot regeneration medium containing 1.5 mg/L BAP, 50 mg/L kanamycin and 300 mg/L cefotaxime (Fig 8B and 8C). After 4 weeks of culture, some explants were failed to regenerate in the selection medium, the green shoot buds were transferred to fresh medium with reduced concentration of cefotaxime. These elongated shoots were rooted on MS medium containing IBA and KIN (Fig. 8D). Kanamycin selection of transformed cucumber shoots has been adopted previously (Kose and Kos, 2003; Wang et al.,

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Treatments</th>
<th>Frequency of Km resistant shoots (%) (Mean±SE)*</th>
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<tbody>
<tr>
<td>Agrobacterium strain</td>
<td>EHA 104</td>
<td>28.5±0.33&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>LBA4404</td>
<td>12.22±2.20&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Precultivation period</td>
<td>2-day</td>
<td>12.64±1.20&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>3-day</td>
<td>15.40±1.22&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>4-day</td>
<td>8.80±0.40&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>5-day</td>
<td>5.67±1.20&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Co-cultivation time</td>
<td>2-day</td>
<td>15.6±0.20&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>3-day</td>
<td>24.66±1.8&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>4-day</td>
<td>18.5±2.20&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>5-day</td>
<td>10.4±0.33&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Kanamycin concentrations</td>
<td>25 mg/L</td>
<td>18.3±2.85&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>50 mg/L</td>
<td>22.6±0.90&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>75 mg/L</td>
<td>16.5±0.64&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>100 mg/L</td>
<td>11.10±1.33&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

*Values represent mean ± standard error. Mean followed by same letter within a column are not significantly different at (P < 0.05).
The production of transgenic plants using cotyledonary node explants was summarized in Table 19.

Table 19. Summary of production of transgenic plants from cotyledonary node explants in this study.

<table>
<thead>
<tr>
<th>No. of incubated explants</th>
<th>Frequency of Km resistant shoots (%)</th>
<th>Transformation efficiency (%)</th>
</tr>
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<tbody>
<tr>
<td>350</td>
<td>24.66</td>
<td>25.54</td>
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</table>

3.3.2 Confirmation of transformants

3.3.2.1 Histochemical GUS assay

In this study, histochemical GUS assay was carried out as described by Jefferson et al. (1987) and GUS assay was used to screen and isolate putative transgenic plants from non-transformed plants. A piece of tissue from transgenic plants were excised and subjected to GUS staining. Most of the tissues from kanamycin sulfate selection medium exhibited a GUS positive response (Fig. 8F and 8G) whereas all the tissues from untransformed plants exhibited a GUS-negative response. GUS-positive tissues with dark blue colouration were observed under light microscope and photographed. The use of the gus gene as a marker for transformation is effective and is widely applied in many plant species such as common bean (Mukeshimana et al., 2013) and alfalfa (Duque et al., 2007).

3.3.2.2 Molecular analysis of putative transformants

To confirm the incorporation and expression of foreign genes into the genome of transgenic plants, GUS positive plants were analyzed by PCR. Total DNA was extracted from putative transgenic plants and untransformed plants (control plant) following the procedure described by Edwards et al. (1991). GUS positive and kanamycin resistant transformants were selected and used for PCR amplification to detect the presence of npt II genes. In the transformants with GUS activity, npt II PCR amplification yielded a DNA fragment of the expected size (0.8 kb) in all GUS expressing shoots, but not in non-GUS expressing shoots (Fig. 8H).
Figure 8. Agrobacterium-mediated genetic transformation in cucumber using cotyledonary node as explants infected with EHA 105 harbouring pBI121. (A) Cotyledonary node explants were cultured on MS medium containing 1.5 mg/L BAP after agro infection; (B) Multiple shoots from cotyledonary node were growing on MS medium containing 1.5 mg/L BAP + 300 mg/L Cefotaxime + 50 mg/L Kanamycin after 2 weeks of agro infection; (C) Transformed plants were cultured on MS medium containing 1.5 mg/L BAP + 250 mg/L Cefotaxime + 50 mg/L Kanamycin for shoot elongation; D) Transgenic plants rooted in MS medium containing 1.5 mg/L IBA + 0.5 mg/L KIN; (E) Transgenic plantlets were transplanted to small plastic pots; (F and G) Histochemical GUS staining detection which shows blue colouration in the transgenic leaf and (H) PCR analysis of the transgenic plants with primers specific to the npt II gene.
3.4 Summary

An efficient system for Agrobacterium mediated genetic transformation of cucumber using cotyledonary node was developed, based on the evaluation of important factors influencing the transformation efficiency. Many factors affected the transformation efficiency including the A. tumefaciens strain, precultivation period, duration of the co-cultivation period, and also the concentration of the antibiotics used. Histochemical GUS activity shows the transgenic leaf showing characteristic blue color of GUS expression visually and microscopically and the blue colouration was absent in the control tissues which confirms the presence of gus gene. Molecular analysis confirms the presence of npt II gene in the transgenic plants. Optimization of protocols for the development of transgenic plants with appropriate vectors and promoters should enable the production of transgenic in cucumber with improved nutritional quality for crop improvement. The transformation technique described in the present study will contribute not only to the basic study but also to the molecular breeding of cucumber using various genetic resources.