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Cotyledon with Hypocotyl Segment as an Explant for the Production of Transgenic *Citrullus vulgaris* Schrad (Watermelon) Mediated by *Agrobacterium tumefaciens*

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Abstract: In this study, we describe the successful production of transgenic watermelon using *Agrobacterium tumefaciens* after optimizing a couple of transformation parameters using cotyledon with hypocotyl segment from 5 day old *in vitro* seedlings as target tissues. It was found that the best condition for transformation of watermelon was using bacteria at a concentration of OD_{600} 0.6, inoculation for 30 min and 3 day of co-cultivation. Addition of 200 μM acetosyringone in the medium helped to further increase the transformation efficiency. *Agrobacterium tumefaciens* strain EHA101 was found to be more effective to infect this watermelon variety as compared to LBA 4404 strain. Wounding of the explant meristems to induce multiple shoot was best carried out after 3 day. Transformed explants were selected on medium containing 5 mg L^{-1} hygromycin. A total of 31 putative transformed plants were recovered with 25 shoots were found to be GUS positive after transformation of 110 cotyledons. A total of 15 GUS positive shoots amplified the *hpt* gene using PCR. However, only 7 shoots were successfully regenerated into whole plants.

Key words: Transgenic plant, *Agrobacterium tumefaciens*, cotyledons with hypocotyl segment, watermelon, *Citrullus vulgaris*

INTRODUCTION

Watermelon (*Citrullus vulgaris*) is primarily cultivated for its edible fruits. The genus *Citrullus* consists of three diploid species, namely (1) *Citrullus lanatus* (Thunberg) Matsumura and Nakai, the cultivated watermelon which is widely grown in several parts of the world, (2) *Citrullus* var. *citroides*, a wild watermelon found in South Africa and mainly used as animal feed and (3) *Citrullus colocynthis* (L.) Schrad, found in the North and Southwest areas of Africa and Asia (Assis *et al*., 2000). In the last century, plant breeders from around the world have released many varieties of watermelon that are resistant to disease, such as resistance to *Fusarium* wilt of Crimson sweet cultivar which was released in 1963 (Hall, 2004). Nutrition scientists at USDA reported that the red pigment found in watermelon is mainly lycopene which has antioxidant activity to inhibit growth of cancer cells besides as a source of vitamin A, C, B6 and potassium (Hall, 2004).

Being a popular fruit and a good source of antioxidant, continuous improvement of this crop is essential. Improving its resistance to environmental biotic and abiotic stresses and improving the nutritional quality are good targets to be achieved through genetic engineering approach. The use of genetic transformation for production of transgenic plants and somaclonal variation to produce polyploidy plants appear to be the most common biotechnological approaches for cultivar improvement (Compton *et al.*, 2004). Polyploid watermelons, were found to be resistant to watermelon fruit blotch and nematodes (Garret *et al*., 1995). Moreover, in triploid watermelons, orange flesh turns into deeper orange color as it ripens and the flavour can even improve after harvesting. Their tough sunburn-resistant rind also makes them excellent for long-distance shipping (Raza *et al*., 2003).

Efficient methods for regenerating watermelon *in vitro* have been in place for over a decade and have paved the way for genetic engineering studies through *Agrobacterium*-mediated and biolistics-mediated

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transformation (Compton *et al*., 2004). Genetic transformation of *elite* watermelon cultivars (*Citrullus vulgaris*) is a potentially important tool to improve traits such as disease and herbicide resistance without altering the cultivar identity. Recently, a method to regenerate shoots from diploid and triploid *Citrullus vulgaris* cultivars has been reported by Suratman *et al*. (2009).

Stable genetic transformation of *Citrullus* sp., by *Agrobacterium tumefaciens* strain LBA 4404 has been reported using cotyledon explants (Choi *et al*., 1994). Recently, Park *et al.* (2005) have described the successful transformation of a watermelon rootstock (gongdae) by *Agrobacterium tumefaciens*-mediated transformation with the *CGMMV-CP* gene for developing virus resistance watermelon plant. Even though the transformation efficiency was low, this was the first report on the production of transgenic watermelon with virus resistance.

A transformation of wild watermelon plants has also been reported by Akashi *et al.* (2005). The group indicated that the use of kanamycin as a selectable marker resulted in higher number of transgenic shoots produced as compared to selection using hygromycin. However, the selection was not effective as it produced some chimeric shoots as demonstrated by patches of blue staining on the leaves after GUS staining. Recently, a method to transform *Citrullus lanatus* Thumb (cv. Daesan) using *Agrobacterium tumefaciens* was reported by Cho *et al.* (2008).

Transformation efficiency of plants using *Agrobacterium*-mediated is influenced by several factors such as addition of phenolic compounds (e.g., acetosyringone) in the co-cultivation medium and wounding treatment of the target tissue with sonication, glass beads or biolistics gold particles. Different *Agrobacterium* strains have also been evaluated to produce the best transformation system for *Citrullus lanatus* (Cho *et al*., 2008). To date, most of the published methods for transformation of watermelon via *Agrobacterium*-mediated uses cotyledon sections as the target tissues or explants (Choi *et al*., 1994; Ellul *et al*., 2003; Akashi *et al*., 2005; Park *et al*., 2005). However, the limitation of cotyledon section is that it has a low regeneration efficiency. Therefore, in this present study we evaluated cotyledon with hypocotyl segment, besides cotyledon alone, as a possible effective target tissue for watermelon transformation.

In this study, watermelon transformation was carried out using two *Agrobacterium* strains and the explant's meristemic region were wounded by injecting with multi wired points of electrical cord as previously described by Park *et al.* (2005). In addition, other factors that affect transformation rate, such as the *Agrobacterium* culture concentration, inoculation times and inclusion of acetosyringone during co-cultivation, were also tested.

MATERIALS AND METHODS

***Agrobacterium* strains and plasmids:** The explants were separately transformed with two different strains of *Agrobacterium tumefaciens* carrying two different plasmids: (1) The *A. tumefaciens* strain EHA101 harboring a binary plasmid, pCAMBIA 1301 (Fig. 1a). The plasmid contains the $-glucuronidase reporter gene (*gus*) (Jefferson *et al*., 1986) from *Escherichia coli* with an intron, driven by cauliflower mosaic virus (CaMV) 35S promoter and *nos* poly-A terminator sequences together with the selectable marker gene hygromycin phosphotransferase gene (*hpt*) under the control of CaMV 35S promoter and CaMV 35*S* poly-A terminator.

(2) *Agrobacterium tumefaciens* strain LBA 4404 harboring a binary plasmid, pCambar containing *gus*, *hpt* and *bar* genes (Fig. 1b). The *bar* gene was originally cloned from the bacterium *Streptomycin hygroccopius.* It encodes phosphinothricin acetyltransferase (PPT) (Thompson *et al*., 1987) that detoxifies phosphinothricin or glufosinate, the active ingredient of the herbicides Liberty and Basta (De Block *et al*., 1987). The *gus* reporter gene is driven by CaMV 35S promoter and *nos* poly-A terminator, *hpt* gene is driven by CaMV 35S promoter and CaMV 35*S* poly-A terminator and the *bar* gene is driven by CaMV 35S promoter and *nos* poly-A terminator.

Transformation into *Agrobacterium tumefaciens*: The plasmid (pCambar) was transformed into *Agrobacterium tumefaciens* by electroporation. The competent cells, strain LBA 4404 was prepared according to the transformation protocol provided by the Multiporator® / Electroporator 2510 supplier (Eppendorf, Germany, Protocol No. 4308 915.502-12/2001). The competent cells were thawed on ice and diluted with sterile water to the density of 1×10^{10} cell mL^{-1}. Two µL of plasmid were added into 40 µL of competent cells and homogenized by gently mixing with pipette several times. The mixture was transferred into a pre-cooled 1 mm electroporation cuvette. After an electric pulse, 400 µL LBG (LB medium with 0.5% glucose) medium was immediately added and transferred into sterile Eppendorf tube. The culture was incubated at room temperature for 1 h. The transformed cells were then plated onto LB agar containing 50 mg L^{-1} kanamycin and incubated at 28°C for 12 to 72 h.

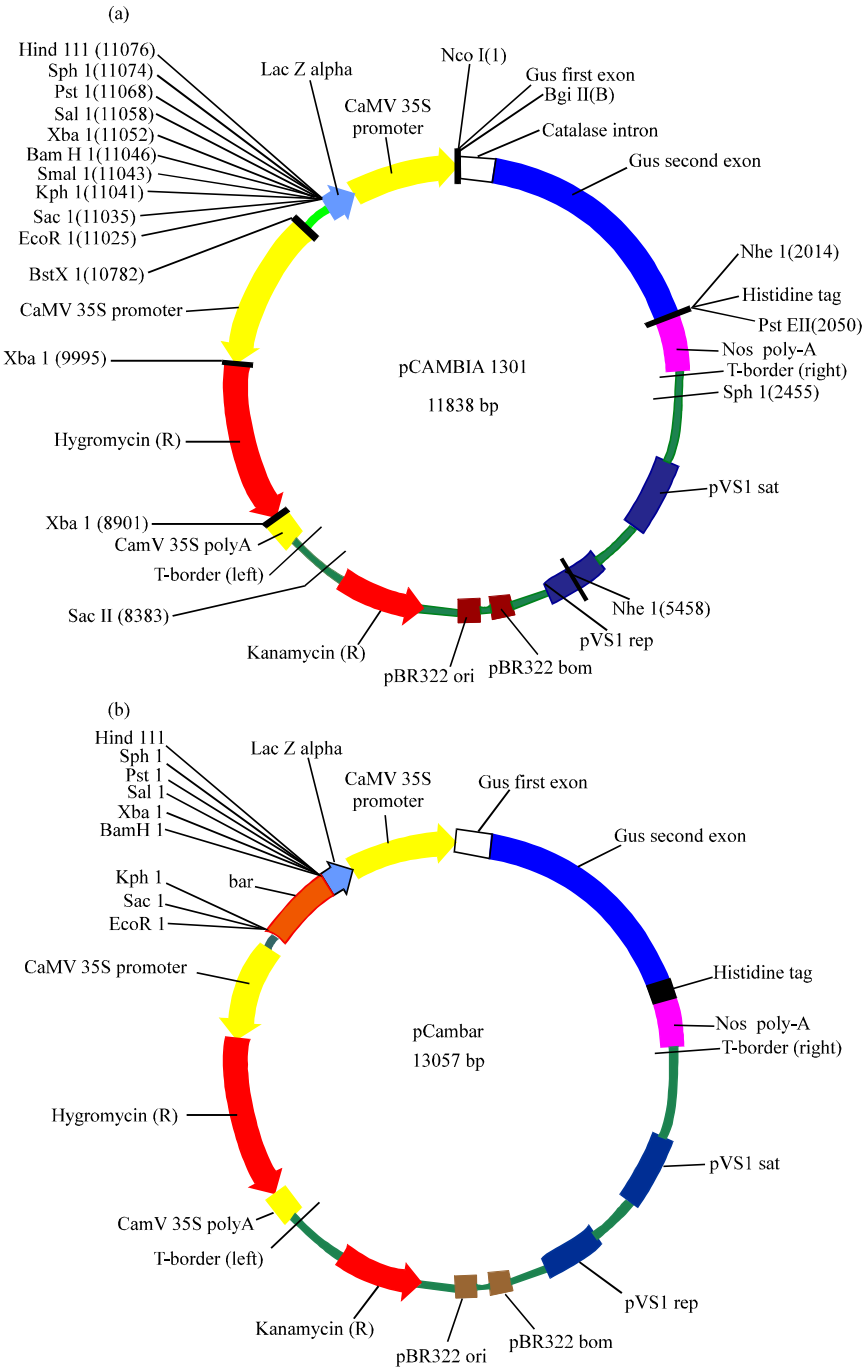

Fig. 1: Restriction map of cloning vector (a) pCAMBIA 1301 and (b) pCambar

Bacterial growth and explant inoculation: *Agrobacterium* strain EHA101 harboring pCAMBIA 1301 from stock culture was streaked onto YEP solid medium (10 g yeast extract, 10 g Bacto Peptone and 5 g NaCl LG^{1}, pH 7.0), containing 50 mg LG^{1} kanamycin. After 2 day incubation at 28°C, a single bacterial colony was inoculated into 10 mL YEP liquid medium containing 50 mg LG^{1} kanamycin. The cultures were incubated in an incubator shaker (Gerhardt, Germany) with shaking at 200 rpm, 28°C for 18 h. Twenty mililiter of bacterial culture were later transferred into a 500 mL sterile conical flask containing 180 mL of YEP broth with 50 mg LG^{1} kanamycin and

further incubated until OD_{600} reached 0.5, 0.6, 0.7 and 0.9. Twenty milliliter cultures from each OD reading were transferred into 50 mL sterile centrifuge tubes and centrifuged at 4°C for 10 min at 10,000 g (Hettich, Germany). The pellet was then resuspended with 20 mL MS liquid medium, incubated at 28°C for 1 h and was ready for co-cultivation.

Independently, the *Agrobacterium* strain LBA 4404 was plated on LBG solid medium containing 50 mg LG^{1} kanamycin. After 2 day of incubation, a single bacterial colony was inoculated into 10 mL LBG broth containing 50 mg LG^{1} kanamycin and incubated for another 24 h. After incubation, 20 mL culture was transferred into a 500 mL sterile conical flask containing 180 mL LBG broth with 50 mg LG^{1} kanamycin and incubated until OD_{600} reached 0.6, 0.8 and 1.0. Twenty milliliter culture was later harvested and centrifuged to pellet the bacteria. The pallets were resuspended in 20 mL MS liquid medium.

Transformation of *Citrullus vulgaris*: All *Agrobacterium*-mediated transformation experiments were carried out using the cotyledons from 5 day old seedlings of yellow watermelon cultivar Hwang Fong Yellow Queen. The seed was sterilized and germinated on basal MS medium (Murashige and Skoog, 1962) using a method we recently published (Suratman *et al*., 2009).

Transformation of *Citrullus vulgaris* cotyledon sections: The cotyledons from 5 day old seedlings were excised 1-2 mm beyond the point of attachment to the hypocotyls. The cotyledon margins (1 mm) were removed and the cotyledons were cut transversely into two halves. The distal portions were discarded and the proximal tissues were cut lengthwise into two and used as explants. The explants were used to produce adventitious shoot as described by Krug *et al.* (2005).

For each experiments, twenty explants were immersely inoculated into 20 mL bacterial suspension for 30 min. Explants were then removed and blotted dry with sterile filter paper and cultured on co-cultivation media (solid MS basal amended with 20 µM BAP and 200 µM acetosyringone) at 25°C for 3 days. Following co-cultivation, explants were washed with sterile distilled water containing 500 mg LG^{1} carbenicillin and 200 mg LG^{1} cefotaxime for 5 min to remove excess *Agrobacterium.* The explants were later blotted dry with sterile filter paper and placed on the selection medium consisting of MS with 20 µM BAP, 200 mg LG^{1} carbenicillin, 100 mg LG^{1} cefotaxime and 5 mg LG^{1} hygromycin or 2 mg LG^{1} PPT. As a control, explants without inoculation with *Agrobacterium* were plated on selective media. All cultures were incubated at room temperature (25°C) under 16 h photoperiod and sub-cultured every 3 weeks. After six weeks, the surviving shoots were transferred to fresh medium without hygromycin to elongate the shoots and induce the roots.

Transformation of wounded cotyledons with hypocotyl segments of *Citrullus vulgaris:* The cotyledons from 5 day old seedlings were excised 1-2 mm including the point of attachment to the hypocotyls. The cotyledon with 1-2 mm hypocotyl was cut into half. The distal parts were discarded and only the proximal parts were used as explant. These explants were later subjected to wounding of the meristemic region using a multi-wire point electrical cord to induce multiple shoots from the meristem. Ten pieces of cotyledon were placed into the 9 cm diameter Petri dishes containing semi-solidified MS medium, supplemented with 20 µM BAP and incubated for 3 day at 25°C with 16 h photoperiod.

After 3 day of incubation, cotyledon explants were injected with multi-wire points of electrical cord in the meristemic region to create wounds (Fig. 2). Then, each set of twenty explants were immersed in bacterial suspension for 1, 10, 20, 30 and 60 min. The explants were then blotted dry on sterile filter paper and then co-cultivated with *A. tumefaciens.* Ten explants were placed on MS medium containing 20 µM BAP and 0, 100, 200 and 400 µM acetosyringone and were incubated at 25°C under 16 h of photoperiod for 3 days. After co-cultivation, explants were washed with 30 mL sterile distilled water containing 500 mg LG^{1} carbenicillin and 200 mg LG^{1} cefotaxime for 5 min. Explants were blotted dry on sterile filter paper and cultured on the selection

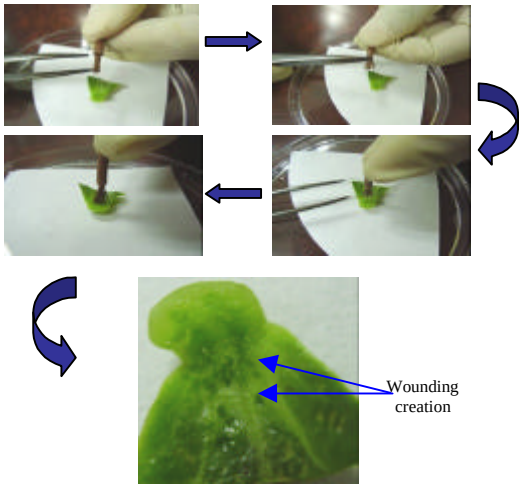

Fig. 2: Diagram of wounded cotyledon explant at meristem region by multi-wire cord points of electric cord

medium consisting of MS medium with 20 µM BAP, 200 mg L^{-1} carbenicillin, 100 mg L^{-1} cefotaxime and 5 mg L^{-1} hygromycin or 2 mg L^{-1} PPT.

Control explants were also plated on selection medium as a negative control. All explants were incubated at 25°C under 16 h of photoperiod and sub-cultured for every 3 weeks. After six weeks, the surviving shoots were transferred to an MS medium without hygromycin to elongate the shoots and enhance rooting of the plants.

Histochemical GUS assay: The histochemical assay for GUS gene expression was performed as previously reported by Jefferson (1987). Histochemical assay was conducted on explants after 3 days co-cultivation with *Agrobacterium*. The explants were incubated in GUS buffer [potassium ferricyanide (8.2 mg mL^{-1}), potassium ferrocyanide (10.6 mg mL^{-1}), 5-bromo-4-chloro-3-indolyl-$-D-glucuronide (X-Gluc) (100 mg mL^{-1}), sodium phosphate buffer (0.2M, pH 7.0), 20% (v/v) methanol and 0.5% (v/v) Triton X-100] at 37°C overnight. The chlorophyll was removed by washing the explants with 100% (v/v) ethanol before GUS detection. The number of explants stained with blue spots was recorded. Young leaves of putative transform plants were also collected for GUS staining for the evaluation the transformation event.

Selection study on *Citrullus vulgaris* by hygromycin and PPT: The sensitivity of cotyledon explants of *Citrullus vulgaris* to hygromycin or PPT (ammonium glufosinate) was tested to determine the natural tolerance of explants to these antibiotics. The antibiotic and herbicide selection were chosen based on the *hpt* or *bar* genes present in the plasmids pCAMBIA 1301 and pCambar, respectively.

The hygromycin-resistance curve for cotyledons of *Citrullus vulgaris* was measured by culturing cotyledon explants on shoot regeneration medium (MS+20 µM BAP) supplemented with hygromycin at concentrations of 0, 2.5, 5, 10, 20 mg L^{-1}, whereas the PPT-resistance curve was supplemented with PPT at 0, 0.2, 1.0, 2.0, 5.0 and 10.0 mg L^{-1} concentrations. The cotyledon explants were obtained from 5 days old seedling. Ten explants were cultured on solidified medium in Petri dishes with 3 replicates for each concentration of the selection agent.

All cotyledon explants were sub-cultured to fresh medium with respective concentration of selection agent every 2 weeks. The number of surviving explants was determined every 2 weeks. All cultures were incubated in growth room at 25°C with a 16 h photoperiod.

Molecular analysis

DNA isolation: The plant genomic DNA was isolated using Wizard® Genomic Purification Kit (Promega) according to the manufacturer's instruction. The DNA was isolated from young leaves with positive GUS and hygromycin-resistant shoot. The DNA solution was stored at -20°C.

Polymerase Chain Reaction (PCR) analysis: The PCR was used to confirm the presence of the transgene in hygromycin-resistant tissues. Total DNA from young leaves of putative transformed and untransformed plants was extracted using Wizard® Genomic Purification Kit. Regenerated plants transformed by *Agrobacterium* strain EHA101 were screened for the presence of the *hpt* gene by PCR using the *hpt* gene specific primers (*hpt*-forward, GGG GGG TCG GTT TCC ACT A; *hpt*-reverse, ATC GTT ATG TTT ATC GGC ACT TTG {produce 821 bp fragment}) and the PCR conditions as follow: one cycle of 94°C, 2 min; followed by 30 cycles of 94°C, 45 sec (Denaturation); 55°C, 30 sec (Annealing) 72°C, 30 sec (Extension) and finally one cycle of 72°C, 5 min. The regenerated plants transformed by *Agrobacterium* strain LBA 4404 were screened by PCR using the *bar* gene specific primers (*bar*-forward, TCA AAT CTC GGT GAC GGG CA; *bar*-reverse, GGT CTG CAC CAT CGT CAA CC {produce 492 bp fragment}) and the PCR conditions are as follow: 35 cycles of 95°C, 30 sec (Denaturation); 62°C, 40 sec (Annealing) 72°C, 60 sec (Extension). The PCR reactions were carried out in 50 µL reaction using MyCyler PCR System 9700 thermal cycle.

RESULTS AND DISCUSSION

The development of an efficient transformation system is important for gene manipulation of any crop species. To optimize the conditions for *Agrobacterium*-mediated DNA transfer to cotyledon segments of watermelon, the effects of different concentrations of acetosyringone during co-cultivation, concentration of *Agrobacterium* and the effect of inoculation times were examined. Disarmed *Agrobacterium tumefaciens* strain EHA101 harboring a binary plasmid pCAMBIA 1301 was used.

Sensitivity of *Citrullus vulgaris* to hygromycin and PPT: This experiment was carried out to determine the sensitivity of *P. vulgaris* cotyledon to hygromycin and PPT. The viability of control cotyledon explants on media containing different concentrations of hygromycin, ranging from 0 to 20 mg L^{-1}, was determined (Fig. 3). Within 4 weeks, all the cotyledons died at concentrations above 10 mg L^{-1} hygromycin. At 5 mg L^{-1} hygromycin, all the cotyledons remained viable up to 4 weeks before they turned yellow and died within 6 weeks. At 2.5 mg L^{-1} all

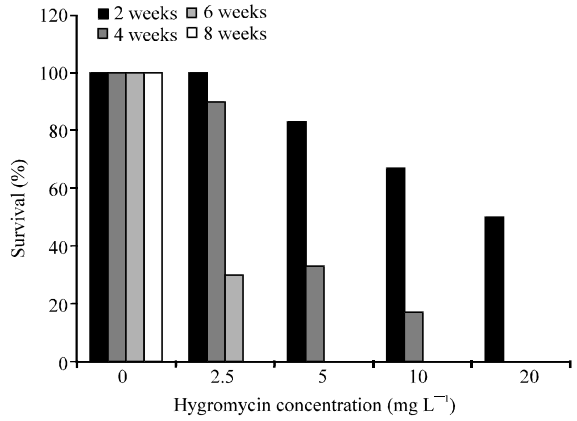

Fig. 3: Survival of non-transformed cotyledons on medium containing hygromycin at various concentrations

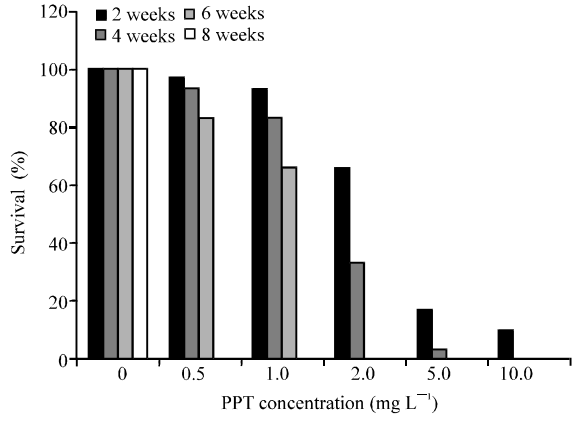

Fig. 4: Survival of non-transformed cotyledons on medium containing PPT at various concentrations

the explants remained healthy up to 6 weeks but they eventually turned yellow and died within 8 weeks. Based on these results, 5 mg L^{-1} of hygromycin was chosen for the selection of transformed tissues.

It was also discovered that watermelon explants were highly sensitive to PPT. Figure 4 showed that all the explants died within 4 weeks at concentrations above 5 mg L^{-1}. The cotyledons remained viable at 2 mg L^{-1} of PPT concentration for up to 4 weeks before they died within 6 weeks. At 0.5 and 1 mg L^{-1} all the explants remained healthy up to 7 weeks but they died within 8 weeks. Therefore, from the results, 2 mg L^{-1} of PPT was chosen for selection of transformed plants.

Most watermelon transformation studies have used kanamycin as the selectable marker (Ellul *et al*., 2003; Choi *et al*., 1994). However, Akashi *et al*. (2005) and Park *et al*. (2005) have used hygromycin or kanamycin as the selectable marker for their watermelon transformation studies. Park *et al*. (2005) reported that 7.5 mg L^{-1} hygromycin has been successfully used in transformation system for the watermelon rootstock cultivar gongdae, whereas, Akashi *et al*. (2005) reported that the use of hygromycin resulted in low transformation efficiency. However, in the later study the shoot failed to develop further and eventually died 2 weeks after transplantation. In contrast, shoots were induced at higher efficiency when transformants were selected on medium containing kanamycin. However, the use of kanamycin in the above study resulted in the production of many chimeric plants which may be due to ineffectiveness of kanamycin as selection agent. According to Yang *et al.* (1999), the sensitivity of plant cells to a selectable marker depends on the genotype, physiological condition, size and type of explants and tissue culture conditions. Therefore, the use of proper concentration of antibiotics in the selection medium is essential for the transformation experiments. This is to ensure that the antibiotic used as selective agent allows only transformed cells or plants to survive.

Optimization of *Agrobacterium* transformation using GUS expression: To select the optimal condition for *Agrobacterium* transformation, the transient expression of *gus* gene was examined. The *Agrobacterium tumefaciens* strain EHA101 with plasmid pCAMBIA 1301 was used to optimize transformation parameters. The optimal concentration of *Agrobacterium* transformation were obtained at the concentration of OD_{600nm} 0.6, innoculation for 30 min and 3 days co-cultivation with an addition of 200 µM acetosyringone in co-cultivation medium (Park *et al.*, 2005). Transient transformation frequency was determined 3 days after co-cultivation. The frequency was represented as the percentage of explants showing at least one positive dark blue GUS spot. The GUS expression pattern of watermelon cotyledon explant is shown in Fig. 5a-d.

Co-cultivation of explants with *A. tumefaciens* for an appropriate duration increases the transformation efficiency, but prolonged co-cultivation can result in *A. tumefaciens* overgrowth and subsequent death of explants (Han *et al*., 2005). In the present study, 3 days of co-cultivation were used to investigate the effect of acetosyringone, concentration of *A. tumefaciens* and inoculation times. Results of previous studies also showed that co-cultivation of explants for 3 days either in the dark or in the presence of light was commonly used for *Agrobacterium*-mediated transformation of plant species (Hu *et al.*, 2006; Anuradha *et al.*, 2006; Akashi *et al*., 2005; Park *et al*., 2005). Three days

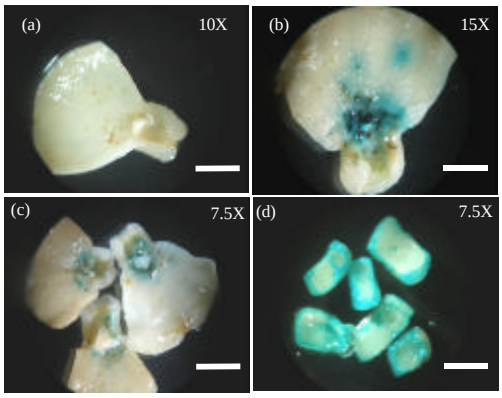

Fig. 5: Transient GUS expression on cotyledon explants and cotyledon sections of watermelon after co-cultivation with *A. tumefaciens* strain EHA101 (a) Control explants (without *A. tumefaciens*), (b, c) GUS expression in wounded explants and (d) GUS expression on cotyledon section (Bar = 2 mm)

of preculture were also found to be best for *Sesbania drummondii* (Padmanabhan and Sahi, 2009). However, 2 days of preculture were found to be best for *Vigna radiata* (L) and *Boehmeria nivea* (Sonia *et al*., 2007; Ma *et al*., 2009).

Effect of inclusion of acetosyringone during co-cultivation: The effect of acetosyringone treatment during co-cultivation using *Agrobacterium*-transformation was investigated. Each set of twenty explants were immersed in bacterial suspension for 10 min. The result showed that the lowest percentage of GUS positive explants (57.50±2.50%) was obtained for explants without acetosyringone treatment (Table 1). As expected, all explants treated with acetosyringone showed strong GUS expressions at the wound sites. The highest percentage of GUS expression was for explants (96.25%) cultured on medium containing 200 or 400 µM acetosyringone (Table 1). As there is no difference in the percentage of GUS positive explants after treatment with 200 and 400 µM of acetosyringone, 200 µM of acetosyringone was further used in the transformation of watermelon.

Acetosyringone is one of the phenolic compounds secreted by wounded plant tissue and is known to be a potent inducer of *Agrobacterium vir* genes. The finding of this study is in agreement with other reports for watermelon transformation (Choi *et al*., 1994; Park *et al*. 2005). In many other plants, such as wheat, cucumber and *Sesbania drummondii*, addition of acetosyringone to the *A. tumefaciens* culture and/or co-cultivation medium proven to be beneficial in improving transformation

Table 1: The effect of different concentrations of acetosyringone on GUS expression

Inclusion of acetosyringone (µM)	Infection explants no.	No. of GUS-positive Explants		Infection frequency (%; Mean±SE)
		Exp 1	Exp 2	
0	40	24	22	57.50±2.50[a]
100	40	32	34	82.50±2.50[b]
200	40	39	38	96.25±1.25[b]
400	40	40	37	96.25±3.75[b]

Infection frequency (%): No. of GUS-positive explants/No. of explants examined. Values with different letter(s) were significantly different at p#0.05. The significance levels (Tukey HSD) between treatments = 0.001

Table 2: The effect of different concentrations of *Agrobacterium* on GUS expression

Concentration of *Agrobacterium* (OD_{600nm})	Infection explants number	No. of GUS-positive explants		Infection frequency (%; Mean±SE)
		Exp 1	Exp 2	
0.5	30	18	17	58.34±1.67[a]
0.6	30	28	30	96.67±3.34[b]
0.7	30	27	26	88.34±1.67[b]
0.9	30	25	27	86.67±3.34[b]

Infection frequency (%): No. of GUS-positive explants/no. of explants examined. Values with different letter(s) were significantly different at p#0.05. The significance levels (Tukey HSD) between treatments = 0.002

efficiency (Wu *et al*., 2003; Nishibayashi *et al*., 1996; Padmanabhan and Sahi, 2009). In *Astragalus racemosus*, addition of acetosyringone in transformation medium showed only a small positive effect on transformation efficiency (Darlington *et al*., 2009). However, acetosyringone was found to have no positive effect on the transformation of *Theobroma cacao* (Silva *et al*., 2009).

Effect of *Agrobacterium* concentration on transformation: The effects of different concentrations of *Agrobacterium* strain EHA101 (absorbance at OD_{600} 0.5, 0.6, 0.7 and 0.9) on watermelon transformation were evaluated by immersing explants in the bacterial inoculum for 10 min. The results obtained are presented in Table 2. The result showed that the highest GUS expression was observed after co-cultivation into *Agrobacterium* strain EHA101 at OD_{600} 0.6 (96.67±3.34%). Higher concentration of *Agrobacterium*, OD_{600} 0.7 and 0.9 showed lower transformation efficiency than 0.6. The high concentration of *Agrobacterium* could contaminate the explants and thus reduced the efficiency. Therefore, *Agrobacterium* strain EHA101 at a concentration of OD_{600} 0.6 was selected for subsequent experiment. The concentration of *A. tumefaciens* has also been suggested to be critical in *Citrullus vulgaris* transformation. The use of high concentrations of *A. tumefaciens* may cause overgrowth of the bacteria and may lead to lower transformation efficiency. In this study, it was found that the

Table 3: The effect of different inoculation times on GUS expression

Inoculation time (min)	No. of explants examined	No. of GUS-positive explants		Infection frequency (%; Mean±SE)
		Exp 1	Exp 2	
1	30	9	10	31.67±1.67[a]
10	30	16	15	51.67±1.67[b]
20	30	23	22	75.00±1.67[c]
30	30	29	30	98.34±1.67[d]
60	30	29	29	96.67±0.00[d]

Infection frequency (%): No. of GUS-positive explants/ no. of explants examined. Values with different letter(s) were significantly different at p#0.05. The significantly levels (Tukey HSD) between treatments = 0.000

Agrobacterium concentration of OD_{600} 0.6 is more effective as compared to the higher concentration of OD_{600} at 0.7 and 0.9. Previous report of *A. tumefaciens* transformation of watermelon also showed that the best concentration of *A. tumefaciens* was at OD_{600} 0.6 (Baogong, 2004; Mahalakshmi *et al*., 2006). Park *et al.* (2005) and Akashi *et al.* (2005) used *A. tumefaciens* concentration at OD_{600} 0.5 to 0.9 on watermelon cotyledon transformation. The concentration of *Agrobacterium* at OD_{600} 1.0 was also found optimum for *Theobroma cacao* (Silva *et al.*, 2009). Increasing the concentration caused *A. tumefaciens* overgrowth and the explants became brown. Similar observation was made by Hu *et al.* (2006), who reported that for *Lycium barbarum* transformation, high bacterial density could lead to bacterial overgrowth and tissue browning and consequently, reduced the transformation efficiency.

The effect of inoculation times: The effect of inoculation time was further investigated. The overall results obtained for this experiment are summarized in Table 3. Generally, the frequency of transient GUS expression increased with the increase of inoculation time. The highest percentage was obtained after 30 min (98.34±1.67%). Inoculation longer then 30 min did not increase the transformation efficiently. Therefore, 30 min inoculation was selected for subsequent experiment.

Inoculation time of the *Agrobacterium* with the explants plays an important role in gene transfer. The time will determine the most effective condition for maximum gene transfer could takes place. In the present study, the highest percentage of GUS positive explant was obtained after 30 min of inoculation. This finding is in agreement to results reported by Park *et al*. (2005). It was also reported that 30-45 min inoculation time was the best for *Agrobacterium* transformation of *Sesbania drummondii* cotyledonary nodes (Padmanabhan and Sahi, 2009). Twenty minutes inoculation was found to be optimum for transformation of *Theobroma cacao* (Silva *et al*., 2009). Rajagopalan and Perl-Treves (2005) reported that the frequency of transient GUS expression on cucumber transformation increased with the increase of inoculation time.

Table 4: Effect of *Agrobacterium tumefaciens* strains concentration on transient GUS expression

A. tumefaciens strain	Concentration of *A. tumefaciens* (OD_{600})	No. of explants examined	Frequency of GUS + explants (%; Mean±SE)
EHA101	0.6	40	97.50±2.50[a]
LBA 4404	0.6	40	38.75±1.25[b]
	0.8	40	48.75±1.25[b]
	1.0	40	77.50±2.50[c]

Frequency of GUS + explants (%): No. of GUS-positive explants/ no. of explants examined. Values with different letter(s) were significantly different at p#0.05. The significance levels (Tukey HSD) between treatments = 0.000

The effect of different *Agrobacterium tumefaciens* strains: The optimal condition for *A. tumefaciens* transformation using strain EHA101 was tested against strain LBA 4404. The results are shown in Table 4 and the highest percentage of GUS positive was observed at OD_{600nm} 0.6 for *A. tumefaciens* strain EHA101 (97.50±2.50%) and the lowest for *A. tumefaciens* strain LBA 4404 at a concentration of 0.6 µM (38.75±1.25%). Therefore, *A. tumefaciens* strain EHA101 was used in subsequent experiments.

A. tumefaciens strain is another important factor affecting infectivity and efficiency of gene transfer (Akashi *et al*., 2005; Baogong 2004). This experimental finding is agreeable with the finding by Baogong (2004), who reported that *A. tumefaciens* strain EHA105 was more effective than LBA 4404 for the transformation of cotton. Akashi *et al.* (2005) also reported that transformation of wild watermelon using *A. tumefaciens* strain EHA101 led to much higher percentage of GUS-positive explants as compared to C58C1 strain when selected on hygromycin. Recently, it was again reported that EHA101 was more efficient than GV3101 and LBA4404 in *Citrullus lanatus* Thumb (cv. Daesan) (Cho *et al*., 2008). This suggests that EHA101 strain is the most suitable for genetic transformation of watermelon. EHA101 was also reported to be the effective vector for *Agrobacterium* transformation of *Sesbania drummondii* cotyledonary nodes and *Petunia hybrida* leaves (Padmanabhan and Sahi, 2009: Thirukkumaran *et al*., 2009). However in oat, *Boehmeria nivea* and *Astragalus racemosus*, it was found that LBA4404 was a preferred strain (Gasparis *et al*., 2008; Ma *et al*., 2009; Darlington *et al*., 2009).

Transformation of *Citrullus vulgaris* and regeneration of cotyledons: The transient GUS expression was detected on cotyledon sections after 3 day co-cultivation with *Agrobacterium* (Fig. 5). The transformants were selected

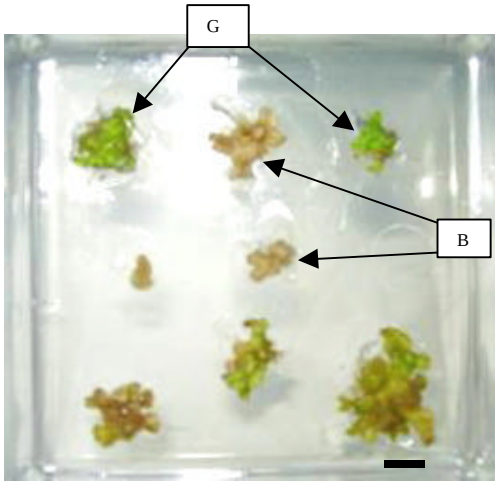

Fig. 6: Multiple shoots from cotyledon segment after 6 weeks on selection media. (G) Shows the survival shoots with the green segment and (B) dead explants were brown in color (Bar = 5 mm)

on MS medium containing 5 mg LG^{1} hygromycin, 200 mg LG^{1} carbenicillin and 100 mg LG^{1} cefotaxime. Cotyledon with hypocotyl segment used in this study successfully resulted in the production of transgenic watermelon plants. At present, there were only few reports available on the watermelon transformation using cotyledon (Choi *et al*., 1994; Ellul *et al*., 2003; Akashi *et al*., 2005). Park *et al*. (2005) managed to produce transgenic watermelon rootstock gongdae using cotyledon as explants. The differences in the results may be due to different cultivars used.

Transformation of GUS gene into wounded *Citrullus vulgaris* cotyledons with hypocotyl segments: The wounded cotyledon segments were co-cultivated with *A. tumefaciens* strain EHA101 for 3 days. After co-cultivation, the cotyledon segments were transferred to selection medium (MS containing 5 mg LG^{1} hygromycin, 200 mg LG^{1} carbenicillin and 100 mg LG^{1} cefotaxime) for selection of transformed plants. It was observed that under hygromycin selection pressure, most of the explants with multiple shoots appeared to be bleached (Fig. 6). Multiple shoots were transferred onto fresh media every 3 weeks. Only a few green multiple shoots were survived. After 6 weeks of selection, surviving multiple shoots were transferred to MS media without hygromycin for further elongation.

The yellow watermelon cotyledon and hypocotyl segments were precultured for 3 days and followed by wounding with multi-wire point electrical cord prior to co-cultivation with *Agrobacterium tumefaciens*. Transformation of *C. lanatus* using wounded cotyledon with hypocotyl segment as explants has not been previously tested (Choi *et al*., 1994; Ellul *et al*., 2003; Akashi *et al*., 2005). The idea of wounding below the meristem region of watermelon cotyledon explants (using multi-wire point electric cord) was pioneered by Park *et al.* (2005). Although, the mechanism of *Agrobacterium* transformation after tissue wounding is unclear, it is thought that multi-wounding may allow more efficient foreign DNA transfer due to more wounded area for the *Agrobacterium* to infect and transfer its T-DNA region (Park *et al*., 2005; Opabode, 2006). The beneficial effect of preculture treatment before wounding the explants on transient expression observed in present study agreed with study by Choi *et al.* (1994).

Histochemical staining of surviving shoots after 6 weeks in selection medium was strongly positive for GUS activity (Fig. 7 b-h). Samples from non-co-cultivated plants did not show any GUS staining (Fig. 7a). From the GUS assay, the stain was observed as strong blue. Some of the shoots were chimeric (Fig. 7g, h). Chimeras are more likely to occur because the starting explants were cotyledon with meristematic region. The results indicated that the percentage of surviving shoots and positive GUS was 28 and 23%, respectively (Table 5).

Evaluation of GUS activity on multiple shoots revealed that 25 out of 31 survived showed positive GUS activity. However, some shoots exhibited only partial GUS-staining (some region of the leaf tissues were stained blue while other parts of the same tissues were unstained). This observation was also reported previously when wild watermelon was transformed with *A. tumefaciens* and selected on kanamycin (Akashi *et al*., 2005). These results indicated that some sectors of these tissues failed to integrate the foreign gene and became chimeric.

PCR analysis of transformed tissues: This experiment was carried out to evaluate the presence of transgene in hygromycin-resistant tissues. Multiple shoots were cultured on the medium without antibiotic for more than 2 months. After 4 months, total DNA from small pieces of shoots was isolated for PCR analysis. The isolated DNA from putative transformed plants; a non-transformed control plant and plasmid pCAMBIA 1301 (isolated from *Agrobacterium* strain EHA101) was used as template DNA for PCR amplification using gene specific primers (Fig. 8). The presence of band pattern at 821 bp in samples from transformed plants (lane 1-15) suggested the presence of the *hpt* gene. Amplification of this fragment (821 bp) was not observed in untransformed plants (lane G). From seventeen shoots tested in this analysis,

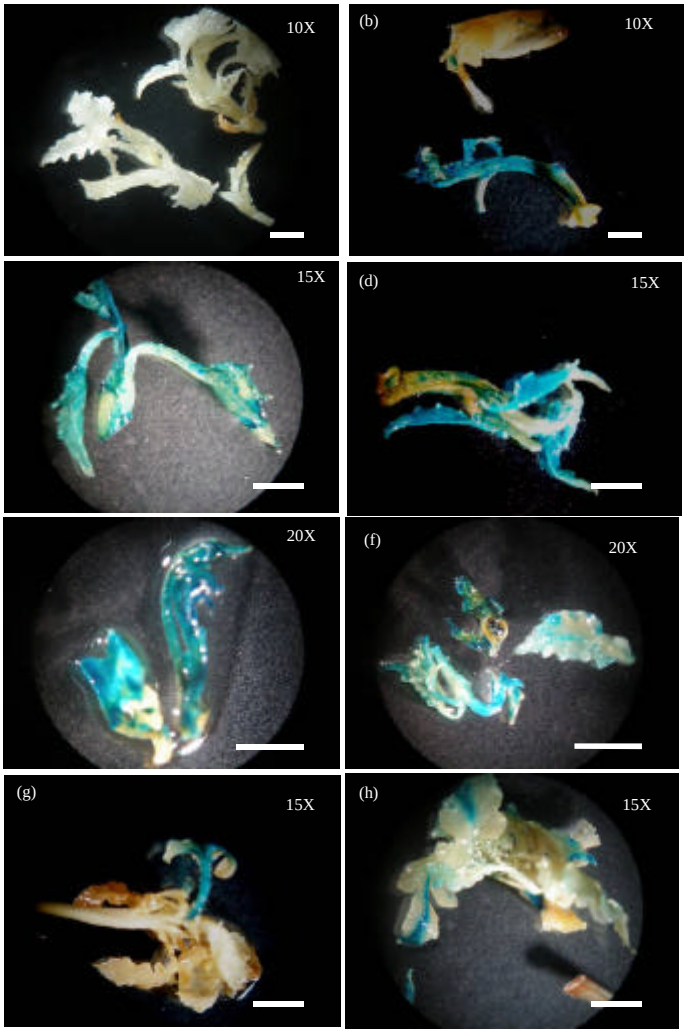

Fig. 7: The GUS expression observed in plant part of different T_0 plants after 6 weeks in selection medium. (a) Control shoots (Bar = 3 mm), (b) A section of the shoot showing untransformed and transformed (blue region) sectors (Bar = 3 mm) (c-f). The pattern of GUS expressions (Bar = 3 mm) (g, h). The chimeric patterns of GUS expression (only some part of shoots successfully transformed). (Bar = 3 mm)

Table 5: The effect of the optimized parameters on GUS expression and regeneration in *Citrullus vulgaris* cotyledons with hypocotyl segments

				Explants survival on hygromycin (6 weeks)		GUS positive (6 weeks)	
Items	No. of explants examined	Explants tested for GUS expression	Infection frequency (%)	No.	%	No.	%
Control	30	15	0	0	0	0	0
Co-cultivation with EHA 101	140	30	93	31	28	25	23

15 shoots (13.6%) showed amplification of the predicted 821 bp fragment for *hpt* gene (Fig. 8 lane 1-15). Out of a total of 110 explants treated with *Agrobacterium* transformation selected on hygromycin, seven (6.4%) regenerated plants (T_0) grew and were transferred to rooting medium (Table 6). Low percentage was possibly due to necrosis of some of the plants during subculture or escapes of regenerated plants.

After 2 months on regeneration medium, 15 multiple shoots were tested positive for PCR using *hpt* gene primers and only 7 of them successfully regenerated into whole plant. Similar observation on the difficulty of multiple shoots regeneration after transformation was reported when wild watermelon was transformed using *A. tumefaciens*. The difficulty to regenerate complete plants from multiple shoot may be due to the use of

Table 6: Survival of yellow watermelon cotyledon with hypocotyl segment after co-cultivation with *A. tumefaciens* EHA101 and selection with 5 mg LG^{1} hygromycin

Items	EHA101	No. of explant examined	PCR positive (%) (after 4 month cultured)	Surviving shoots	Surviving shoots (%)
Co-cultivation	+	110	13.6 (15/17)[a]	7	6.4
Control	-	30	0	0	0

[a] is number of shoots positive GUS per shoot survive after 4 months on culture

Fig. 8: PCR analyses of T_0 plants. Lane C is negative control, PCR cocktail without DNA template, Lane G is untransformed watermelon plant, Lane P is pCAMBIA 1301 plasmid with the *hpt* gene, Lanes 1-15 are transformed plants showing 821 bp PCR product with *hpt* primer

antibiotic hygromycin which is more toxic than kanamycin. Park *et al*. (2005) reported that the growth patterns of transformed watermelon rootstock gongdae selected using kanamycin or hygromycin were nearly identical, but acclimation recovery was more difficult for hygromycin-selected plants.

Therefore, due to the difficulty associated with selection using hygromycin and kanamycin, it is proposed that an alternative selection system, such as the use of herbicide Basta (active ingredient PPT or phosphinothricin acetyl transferase), be evaluated for this yellow watermelon in the future. Another alternative to the difficulty in regenerating transformed multiple shoots is to transform the plant without using selectable marker gene as reported for potato (De Vetten *et al.*, 2003). Potato variety Karnico were transformed with *Agrobacterium* strain AGL0 and resulted in approximately 5000 regenerated shoots. The PCR analysis was carried out to screen for the transformed shoots. Around 225 (4.5%) of the plants showed positive data results using PCR analysis. It is laborious and time consuming to screen for 5000 multiple shoots. Furthermore the 4.5% positive PCR does not assure that they are truly positive transgenic as chimeric shoots could also show positive results in PCR.

Furthermore the transformed yellow watermelon was difficult to develop further after subculture. In every situation, explants suffered from intense enzymatic browning followed by necrosis and eventully die. Similar observation on the browning and dying of multiple shoots was also reported on *Impatiens walleriana* when multiples shoots were selected on kanamycin (Baxter, 2005). Ezura *et al.* (2000) also reported that ethylene production was promoted by *A. tumefaciens* inoculation and the increased levels of ethylene in the culture vessel or flasks of transformed melon cotyledon resulted in a reduction in the efficiency of gene transfer and growth.

CONCLUSION

In this study, we have successfully produced transgenic watermelon using *Agrobacterium tumefaciens* after optimizing a couple of transformation parameters using transient GUS expression. Cotyledon with hypocotyl segment from 5 days old *in vitro* seedlings was transformed with *A. tumefaciens* LBA4404 using a concentration of bacteria at OD_{600} 0.6, inoculation for 30 min and 3 days of co-cultivation in the presence of 200 µM acetosyringone. Transformed cells were selected on medium containing 5 mg LG^{1} of hygromycin.

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