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Agrobacterium-mediated transformation of the aquarium plant *Cryptocoryne willisii* with *gus* and *gfp* genes

Sher Ming Wong, Norhanizan Sahidin, and Norzulaani Khalid*

Institute of Biological Sciences, Faculty of Science and Centre for Research in Biotechnology for Agriculture (CEBAR), Level 5, Block B, IPS Building, University of Malaya, Kuala Lumpur, 50603 Malaysia.

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Abstract

The effects of different infection times (2, 4, 6, 8, and 10 min) and co-cultivation periods (1, 2, 3, and 4 days) on transformation of *Cryptocoryne willisii* with *Agrobacterium tumefaciens* strain *LBA4404* harbouring *pCAMBIA1304* were assessed. Fluorometric assay revealed that the highest expression of β-glucuronidase (GUS) enzyme driven by the CaMV 35S promoter can be achieved by 6 min infection time and one day of co-cultivation (582.1 ± 84.3 pmol 4MU/mg/min). Expression of green fluorescent protein (*mgfp5*) gene fused to 5' GUS gene was also observed. Transformants appeared green fluorescent under ultraviolet light (UV) excitation. Plantlets of *C. willisii* showing GFP positive results were subjected to molecular analysis. Polymerase chain reaction (PCR) and Southern blot analysis confirmed the transformation event of individual regenerated plantlets. For direct shoot regeneration, 6 mg·L^{-1} 6-BA supplemented Murashige and Skoog medium was optimal and was then subsequently used for regeneration and propagation of transformed *C. willisii*. Shoot proliferation and elongation were achieved on a single medium without the need for subculturing.

Keywords: ß-glucuronidase, Green fluorescent protein, Shoot regeneration.

Introduction

Cryptocoryne willisii belongs to Araceae family and is called water trumpet because of its' trumpet-like shape inflorescence. It is famous for its beautiful red-brown leaves and exotic shape of the inflorescence, which makes commercial cultivation a viable option. The generation of fluorescent *C. willisii* is also appealing – not only as a novel ornamental aquarium plant but also as a method to detect the release of transgenic plants into the environment (Mercuri et al., 2002). Therefore, an attempt was made to generate fluorescent properties in *C. willisii* via genetic modification through *Agrobacterium*-mediated transformation using a green fluorescent protein gene (*mgfp5)*.

Several factors are known to influence *Agrobacterium*-mediated plant transformation. These include bacterial strain and concentration, infection time, co-cultivation period, and pre-culture duration (Veluthambi et al., 2003). Optimization of these parameters are often done by utilizing reporter genes such as β-glucuronidase (GUS) from *E. coli*, green fluorescent protein (GFP) from jelly fish, and luciferase from firefly as screenable and scorable markers (Jefferson, 1987). These reporter genes of non-plant origin are important to quantify the effects of different parameters on T-DNA transfer to plant cells. In this study, the effects of infection time and co-cultivation period on transient gene expression were investigated. The best conditions were then applied to generate transgenic plants. To date, there is no previous report on the transformation of *C. willisii*.

Materials and methods

Tissue culture of C. willisii

Healthy *in vitro* plantlets of *C. willisii* were obtained from

*Author for correspondence: Tel + 60379677142; Fax +60379674178; Email <lani@um.edu.my>.

the Freshwater Fisheries Research Centre, Malaysia. The plantlets were subcultured on Murashige and Skoog (1962) (MS) medium supplemented with 3% (w/v) sucrose and 0.2% (w/v) Phytagel (System® ChemAR®, US). The pH of the medium was adjusted to 5.8 prior to autoclaving at 121ºC, 15 psi for 20 min. Basal shoot tip explants (5 to 10 mm) of *C. willisii* were cut and cultured on MS supplemented with different concentrations (2, 4, 6, 8, and 10 mg·L^{-1}) of 6-benzylaminopurine (6-BA; Sigma, US) in 60 ml universal disposable containers with 20 ml media. All cultures were prepared under aseptic conditions and grown at 26ºC under 16 h light: 8 h dark photoperiod with a light intensity of 31.4 µmol m^{-2}·s^{-1} provided by cool fluorescent lamps. Each treatment had five replicates and the experiment was repeated twice. The number of normal shoots formed from single node explants were recorded and analysed after four weeks of culture. Data were then analysed statistically using ANOVA (SPSS, Inc, US) with Duncan's multiple range test.

Agrobacterium *culture conditions*

Agrobacterium tumefaciens strain *LBA4404* harbouring *pCAMBIA1304* was used in the experiments. The *Agrobacterium* culture was grown on yeast extract (YE) medium consisting of 27.0 g·L^{-1} nutrient agar, 1.0 g·L^{-1} yeast extract, 0.5% (w/v) sucrose, and 10 mM magnesium sulphate supplemented with 100 mg·L^{-1} kanamycin and 25 mg·L^{-1} streptomycin. A single bacterial colony was selected and inoculated in 10 ml YE broth (27.0 g·L^{-1} nutrient agar replaced with 18 g·L^{-1} nutrient broth) supplemented with the same antibiotics. Bacterial cultures were then incubated at 28ºC on a rotary shaker (250 rpm) in darkness. Optical density (OD) of *Agrobacterium* cultures were measured at λ=550 nm using a Novaspec II spectrophotometer (Pharmacia Biotech, Cambridge, England). Readings were taken at hourly intervals until a stationary phase of bacterial growth was obtained (40 h). Mid-log phase bacteria were then used for the transformation.

Agrobacterium-*mediated transformation of* C. willisii

Shoot tip explants were used as target tissues in transformation experiments. Explants were submerged in mid-log phase cultures of *Agrobacterium* for infection (2, 4, 6, 8, or 10 min), blotted-dry, and then placed on MS medium without hormones for co-cultivation (1, 2, 3 or 4 days) at 28ºC in darkness. Treatments were done in triplicates and the experiment repeated three times. Explants were soaked in 300 mg·L^{-1} cefotaxime washing solution for 10 min at the end of the co-cultivation period to eliminate *Agrobacterium*. The explants were mixed well by shaking in the washing solution to rinse off all the bacteria and were then transferred to optimized shoot induction medium containing 150 mg·L^{-1} cefotaxime and 200 mg·L^{-1} carbenicilin. This combination of cefotaxime and carbenicilin was identified in our previous experiments as minimal bactericidal concentration (MBC), which completely eliminates growth of *A. tumefaciens* in the transformation experiments. Antibiotics-containing medium was renewed every fortnight and regenerated shoots were selected on medium containing 50 mg·L^{-1} of hygromycin and further selected using GFP visualisation. Plantlets that survived hygromycin selection and expressed GFP were subjected to molecular assessment.

GFP visualisation, GUS histochemical and fluorometric assay

For GFP visualisation, explants were exposed to blue ultraviolet light at excitation bandpass of 400 nm with fluorescent inverted microscope (Olympus IX71, Japan) equipped with narrowband filter of 460 – 490 nm. Pictures were captured with a camera (Olympus, Japan) attached to a computer. Meanwhile, GUS expression in transformed *C. willisii* was determined by fluorometric assays after two weeks of transformation by standard protocol (Jefferson, 1987). Crude extract of each sample was subjected to Bradford protein assay in order to determine total protein concentration with a spectrophotometer (Pharmacia Biotech Novaspec II) at a wavelength of 595 nm (OD_{595}). Data were expressed as *p*moles of 4-methylumbelliferone (4-MU) min^{-1}·mg^{-1}. A fluorescence value of 35 ± 4.30 pmol 4 MU mg^{-1}·min^{-1} obtained from control wild type *C. willisii* was deducted for each sample, which reflected

background fluorescence in this species. Data were generated from three replicate samples for each treatment and normalized with the total protein content in each sample. Analysis of variance ANOVA (SPSS, Inc) was carried out at 5% confidence level of Duncan's multiple comparison tests. For histochemical staining, plantlets were submerged in histochemical reagent [0.1 M phosphate buffer, 0.5 mM ferricyanide, 0.5 mM ferrocyanide, 0.1 % (v/v) Triton X- 100, 20 % (v/v) methanol, 1.0 mM 5-Bromo-3-indolyl-glucuronide (X-gluc, Fermentas), and 10.0 mM EDTA] and incubated at 37ºC in darkness for 4 to 24 h until blue coloration was visible. Stained samples were then transferred and washed in 70% (v/v) ethanol. Finally, stained samples were transferred and fixed in formalin/acetic acid/ alcohol (FAA) solution.

Molecular assessment

Genomic DNA of transformed regenerated plantlets was isolated using CTAB method (Doyle and Doyle, 1987) and the presence of *mgfp5* reporter gene was detected by PCR with forward primer (5' AAG GAG AAG AAC TTT TCA CTG GAG-3'and reverse primer 5' AGT TCA TCC ATG CCA TGT GTA-3') under the following cycling conditions: 95°C for 1 min, 58°C for 1 min and 72°C for 1 min with 30 cycles and a final extension at 72°C for 6 min.

Southern blotting

Genomic DNA digested with the restriction enzyme *HindIII* (Vivantis®, Malaysia) was separated in 1% (w/v) agarose gel and then transferred onto nitrocellulose membrane (Hybond-N, Amersham, US) as described by Sambrook et al. (1983). Blotting paper was changed from time to time to provide better transfer capillary action from the agarose gel to the membrane. Membrane was then briefly air-dried and UV cross-linked at 1.5 $J \cdot cm^{-3}$ for 3 min. Probes were biotin-labelled by random priming with exonuclease activity-free Klenow fragment according to manufacturer's protocol (PureExtreme™, Fermentas, US). Hybridization was then carried out by labelled probes containing hybridization buffer (6X SSC, 1% (w/v) SDS, and 0.01M EDTA) at 42°C in hybridization oven with agitation for 16 h. Membrane was washed twice with 2X SSC, 0.1% (w/v) SDS at room temperature for 10 min followed by 20 min high stringency wash with 0.1X SSC, 0.1% (w/v) SDS at 65°C for 20 min. Membrane was then incubated with BCIP-T (5-bromo-4-chloro-3-indolyl phosphate, p-toluidine salt) solution at room temperature in the dark until blue coloration was obtained. Finally, the reaction was stopped by removing the substrate solution and the membrane was rinsed several times with $d H_2O$ and blotted-dry on tissue paper.

Results and Discussion

Multiple shoot induction

The highest number of shoots (5.0 ± 2.0) were formed when shoot tip explants of *C. willisii* were cultured on MS medium supplemented with 6 $mg \cdot L^{-1}$ of 6-BA (Table 1). Results were significant (p=0.05) when contrasted using ANOVA (SPSS, Inc, US) with

Table 1. Effects of different concentrations of 6-BA on multiple shoot formation from *C. willisii.*

Concentration of 6–BA (mg L^{-1})	Average number of shoots formed (n = 10)
2	2.6 ± 1.1[a]
4	3.2 ± 2.0[a]
6	5.0 ± 2.0[a,b]
8	3.6 ± 1.3[b]
10	3.4 ± 0.9[b]

Different letters indicate values that are significantly different at 95% confidence level using Duncan multiple comparison test. The values following means are standard errors.

Duncan's multiple comparison test. Fewer shoots formed on MS medium supplemented with either lower or higher concentration of 6-BA than 6 mg·L^{-1}. However, no roots were formed when the *C. willisii* plantlets were cultured on 6-BA containing media. Hence, induced shoots with at least three leaves were excised and subcultured onto fresh MS medium without any plant growth regulators for root induction.

Successful plant regeneration is a prerequisite for plant transformation (Rup and Sukanya, 1993). Thus, the effect of the plant growth regulator 6-BA on multiple shoot induction was investigated. This experiment is crucial for bulking up the target tissues required for transformation experiment and also important to ensure that the transformed explants could be regenerated and propagated into a whole individual plant at high frequency. A simple and efficient method using 6-BA alone was developed for direct multiple shoot induction from shoot tip culture of *C. willisii*. Shoot proliferation and elongation were achieved on the same shoot induction medium without the need for subculturing.

Effects of co-cultivation period and infection time

The highest GUS enzyme activity of 582.1 ± 84.3 *p*mol 4MU mg^{-1}·min^{-1} was achieved with 6 min infection time

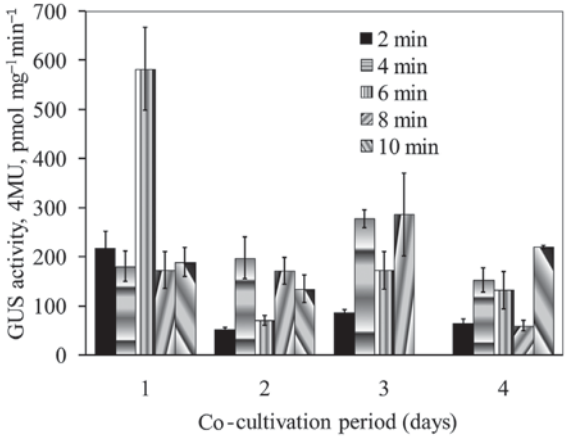

Figure 1. The effect of co-cultivation periods and infection times on transient transformation of *C. willisii*. GUS enzyme activity was expressed in pmol 4 MU min^{-1}· mg^{-1} protein. Each treatment was done in triplicate. Error bars represent standard errors.

and co-cultivation period of one day (Fig. 1). The enzyme activity was approximately 10 fold higher than the lowest activity observed which was only 50.57 ± 5.28 pmol 4MU mg^{-1}·min^{-1} when *C. willisii* was infected for 2 min and co-cultivated for 2 days (Fig. 1). Infection time and co-cultivation period play pivotal roles in determining transformation efficiency (Akbulut et al., 2008). Infection time is important to enhance the attachment of *Agrobacterium* to the plant cells while co-cultivation period is required for the induction of virulence mechanism and gene transfer (Wahlroos et al., 2003). One to three days of co-cultivation period are commonly reported as optimal for *Agrobacterium*-mediated transformation (Men et al., 2003). However, one day of co-cultivation was sufficient to give the highest GUS enzyme activity in *C. willisii*. This is in agreement with Rohini and Sankara (2000) who reported that one day co-cultivation of *Carthamus tinctorius* (safflower) with *Agrobacterium* provided the best transformation efficiency. Although a longer co-cultivation period is generally superior to shorter periods of time, prolonged co-cultivation might be deleterious to the explants and would cause necrosis and cell death. De Bondt et al. (1994) suggested that a co-cultivation period of not more than 4 days was favourable to counter the difficulty in eliminating *Agrobacterium* from plant tissues. Similarly, Suma et al. (2008) reported that longer co-cultivation period associated with overgrowth of *Agrobacterium* had adverse effects on transformation and regeneration of *Zingiber officinale* Rosc. The best transformation condition (6 min infection time followed by 1 day of co-cultivation) was chosen to further generate transgenic *C. willisii*.

Plant transformation, reporter genes, and molecular assessment

Results of GFP visualisation in transformed plant tissues are shown in Fig. 2. Transformed explant showed green fluorescence under UV excitation while untransformed explant appeared red without spots of green fluorescence. Histochemical localisation of GUS enzyme activity in transformed plantlets of *C. willisii* is shown in Fig. 3. GUS and GFP genes were successfully

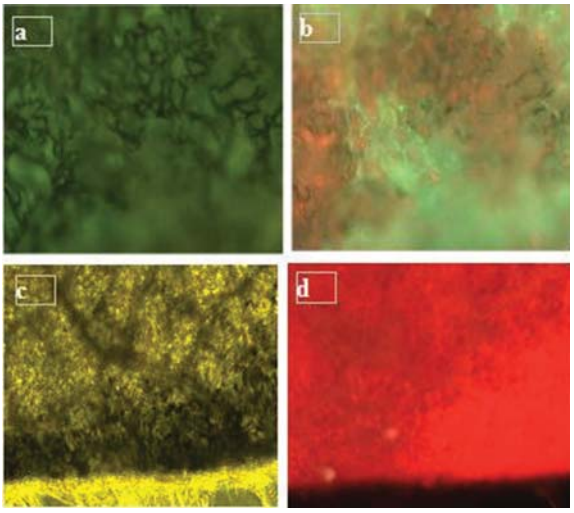

Figure 2. Fluorescence visualization in transformed plant tissues: (a) Transformed plantlet tissue observed under normal light, (b) the same tissue observed under UV excitation (Note: GFP protein was expressed in transformed *C. willisii*), (c) Non- transformed plant tissue viewed under normal light, and (d) Non-transformed plant tissue viewed under UV light appeared red as a result of auto-fluorescence from chlorophyll without green fluorescence. (Scale bar = 0.5 mm).

expressed in the transformed plantlets. DNA from the regenerated plantlets was assessed individually by PCR for the presence of *mgfp5* gene (Fig. 4a). Plantlets that survived hygromycin selection and showed green fluorescence were subjected to PCR analysis. All 13 plantlets showed positive result with a band corresponding to approximately 700 bp in molecular weight (MW). Transgene integration detected in Southern blot analysis (Fig. 4b) suggested that *mgfp5* gene was inserted into the plant genome.

Uneven GUS histochemical staining was observed in transformed plantlet (Fig. 3c) suggesting that the transformant was chimeric in nature. Chimeric plants often recovered in organogenesis involved more than one cell in the shoot initiation process (Domínguez et al., 2004). The result is expected as shoot tip explants were used as transformation target tissue in the experiment. Nonetheless, other route of plant regeneration from single cell origin such as somatic embryogenesis of *C. willisii* is under investigation. In

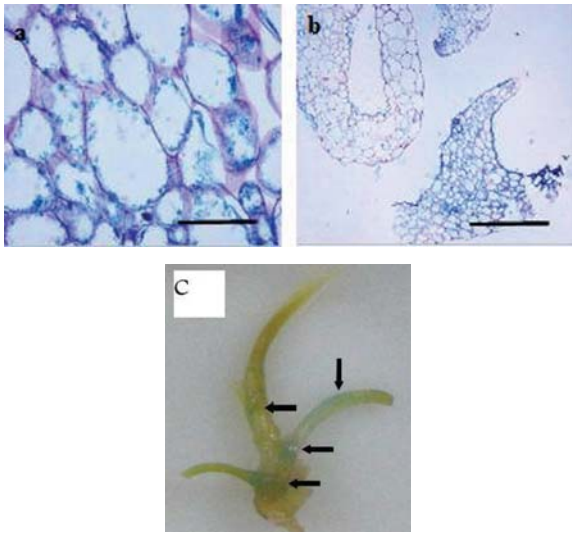

Figure 3. Histochemical staining of GUS enzyme activities in transformed *C. willisii*. (a) and (b) Histology sectioning of FAA-fixed tissue expressing GUS enzyme with blue precipitates (bar = 0.025 mm, bar = 0.2 mm). (c). Parts of the plantlet stained blue (as indicated by arrows) (bar = 1 cm).

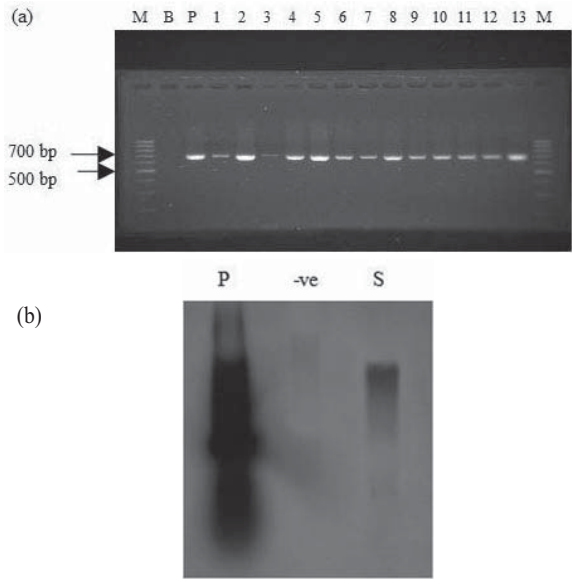

Figure 4. Molecular analysis of transgenic *C. wiliisii*. (a) PCR analysis of the presence of mgfp5 gene in transformed *C. willisii* and non- transformed control plant using primers targeted to *mgfp5* gene. M = 100 bp DNA Ladder, C = Negative control, 1 – 13 = Samples of transformed plantlets, P = pCAMBIA1304 plasmid as positive control, B = Blank. (b) Southern Blot analysis of *mgfp5* in transgenic *C. willisii*. P = Positive control, –ve = negative control, S = Sample.

conclusion, 6 min of infection time followed by one day of co-cultivation gave the highest transformation efficiency in *C. willisii* among all combinations tested. The optimized system for *Agrobacterium*-mediated transformation of *C. willisii* could also be applied to generate other novel fluorescent aquarium plants.

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