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Transformation of *Morinda citrifolia* Via Simple Mature Seed Imbibition Method

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Abstract: *Morinda citrifolia*, is a valuable medicinal plant with a wide range of therapeutic properties and extensive transformation study on this plant has yet been known. Present study was conducted to establish a simple and reliable transformation protocol for *M. citrifolia* utilising *Agrobacterium tumefaciens* via direct seed exposure. In this study, the seeds were processed by tips clipping and dried and subsequently incubated in inoculation medium. Four different parameters during the incubation such as incubation period, bacterial density, temperature and binary vectors harbouring β-glucuronidase (GUS) gene (pBI121 and pGSA1131), were tested to examine its effect on transformation efficiency. The leaves from the treated and germinated seedlings were analysed via Polymerase Chain Reaction (PCR), histochemical assay of the GUS gene and reverse transcription-PCR (RT-PCR). Results of the study showed that *Agrobacterium* strain LBA4404 with optical density of 1.0 and 2 h incubation period were optimum for *M. citrifolia* transformation. It was found that various co-cultivation temperatures tested and type of vector used did not affect the transformation efficiency. The highest transformation efficiency for *M. citrifolia* direct seed transformation harbouring pBI121 and pGSA1131 was determined to be 96.8% with 2 h co-cultivation treatment and 80.4% when using bacterial density of 1.0, respectively. The transformation method can be applied for future characterization study of *M. citrifolia*.

Key words: *Morinda citrifolia*, seed transformation, *Agrobacterium tumefaciens*, PCR, histochemical assay, RT-PCR

INTRODUCTION

Plant genetic transformation is an approach used to introduce foreign gene in plants (Chawla, 2002). Techniques in plant transformation have been widely established. Among others, the most frequently utilised methods are the Ti-system of *Agrobacterium tumefaciens* (Mohamed *et al*., 2006; Gonzalez *et al*., 2008; Ogaki *et al*., 2008; Yasmeen *et al*., 2009; Xu *et al*., 2009a, b), microinjection of DNA (Evans, 2006), polyethylene glycol mediated DNA uptake (Mathur and Koncz, 1998), the use of plant virus vectors (Scholthof *et al*., 1996; Nagl *et al*., 2005) and particle bombardment of naked DNA or plasmids into plant organs and protoplasts (Ueki *et al*., 2008). Traditionally, plant transformation have successfully utilised the tissue culture method but the drawback in using tissue culture-based transformation is the possibility of increased contamination in nutrient-rich media and somaclonal variation in transgenic plants due to the long exposure with growth hormones. Feldman and Marks (1987) have successfully used *Arabidopsis thaliana* seeds to generate transgenic plants. Subsequently, Bechtold *et al*. (1993) developed the widely used in planta transformation method to deliver foreign gene into plants. The direct seed transformation utilising *A. tumefaciens* is a method that bypasses the tissue culture method which is tedious, time-consuming and prone to contaminations and variations (Slater *et al*., 2005; Yasmeen *et al*., 2009). *A. tumefaciens* is a natural target of dicotyledonous plants. Since, its introduction, direct seed transformation has been applied to various plants, such as *A. thaliana* (Feldman, 1995), *Zea mays* (Wang *et al*., 2007) and *Brassica napus* (Song *et al*., 2009). Transformation of *A. thaliana* was conducted using pre-imbibed seeds in a co-cultivation medium containing fresh log phase *A. tumefaciens* culture (Feldman, 1995) whereas in maize, the transformation was conducted using germinating seedlings with cut wounds at the apical meristem (Wang *et al*., 2007).

Morinda citrifolia originated from the division Magnoliophyta, class Magnoliopsida, order Rubiales and family Rubiaceae. It is a small evergreen tree, native to the Southeast Asia and Australian region (Nelson, 2006) with the variety bracteata indigenous to Malaysia and Indonesia. The plant is usually 3-10 m tall and has the advantages in that it thrives on non-fertile, acidic or alkaline soils and can withstand adverse environmental conditions such as wind, fire, drought and flooding (Nelson, 2006). There are three main varieties of

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M. citrifolia; citrifolia, bracteata and 'Potteri', depending on the area of distribution (Nelson, 2006). The leaf morphology varies from elliptic, rounded or long and strap-like (McClatchey, 2002) with deep vein while the flowers grow in ovoid to globose head containing 5 stamens and the corolla is white in colour (Nelson, 2006). The fruit is green in colour until it reaches maturity which then turns yellowish and fruiting occurs throughout the year. The seeds are triangular in shape and reddish brown in colour with air chambers in the seed structure (Wang *et al*., 2002). Previous studies have shown that *M. citrifolia* has medicinal properties such as antibacterial (Zaidan *et al*., 2005), antitumor (Bui *et al*., 2006) and antioxidative (Zin *et al*., 2006) from various plant parts such as root, fruit and leaf.

The main aim of this study was to develop a simple protocol for the genetic transformation of *M. citrifolia*. To the best of our knowledge and after extensive searches conducted via published article databases, we could not find articles on the transformation of *M. citrifolia*. The plant was selected for this transformation work because of its characteristics such as fruiting all year round and each fruit contains many seeds which would be beneficial for transformation work. Apart from that, the plant could also withstand harsh growing environment which enable easy growing of the plant in tropical countries such as Malaysia. This would be the first report of transformation for the plant which can serve as a model plant to be used for tropical countries. For those reasons, we applied a modified pre-imbibed seed transformation method on *M. citrifolia* and undertaken optimization of the transformation parameters that includes; incubation period, bacterial density and temperature. Two binary vectors were used for the transformation experiments. Putative transformants were analysed to determine the incorporation of the T-DNA region into plant genomic DNA and the expression of transgenes were also characterized.

MATERIALS AND METHODS

Plant material: Mature *M. citrifolia* Linn. var bracteata fruits were obtained from the nursery located at the Faculty of Resource Science and Technology, Universiti Malaysia Sarawak. The fruits were processed to collect the seeds for transformation.

***Agrobacterium* strain, plasmids and culture:** The *Agrobacterium* used for transformation was a nopaline *Agrobacterium* strain, LBA4404 (gift from Caddick, The University of Liverpool) that carries a binary vector either pGSA1131 (Arabidopsis Biological Resource Centre, ABRC) or pBI121 (ABRC). The binary vectors were transformed into LBA4404. Both the pGSA1131 and pBI121 contained β-glucuronidase (GUS) under transcriptional control of a 35S Cauliflower Mosaic Virus (CaMV 35S) promoter. The pGSA1131 harbours a coding region for herbicide resistance (Bar gene) while the pBI121 carry the selectable marker *Neomycin phosphotransferaseII* (NtpII) (Matsumoto and Fukui, 1998). The *Agrobacterium* carrying each of the binary vectors were cultured on Luria Bertani broth media containing rifampicin (50 μg mL^{-1}) (PhytoTechnology Laboratories™, Kansas) supplemented with either the antibiotic chloramphenicol (35 μg mL^{-1}) (DUCHEFA, Europe) for pGSA1131, or kanamycin (100 μg mL^{-1}) (Sigma, United Kingdom) for pBI121. The *Agrobacterium* was grown overnight at 28°C with vigorous shaking at 200 rpm. The cells were then spun down and resuspended in inoculation solution prior to infiltration.

***M. citrifolia* seed imbibition method:** The processed seeds were firstly subjected to surface sterilization treatment using 95% (v/v) ethanol (Scharlau, European Union) and washed several times in sterile distilled water. The seeds were then scarified by clipping the tip end of the seeds and air dried for 1 h in a laminar flow prior to imbibition. Prepared seeds were then subjected to imbibition with inoculation medium [0.5X Murashige and Skoog medium (Sigma, United Kingdom), 0.5 g L^{-1} of 2-N-morpholinoethanesulfonic acid (MES) (J.T. Baker, USA), 5% (v/v) sucrose (Amresco, USA), 0.03% (v/v) Silwet L-77 (Lehle Seeds, USA), adjusted to pH 5.7]. The *Agrobacterium* containing either pGSA1131 or pBI121 was resuspended in the inoculation medium. Bacterial density of the *Agrobacterium* suspension in inoculation medium was determined to be OD_{600} = 0.5-1.0. The seeds were then co-cultivated with *Agrobacterium* suspension with gentle shaking at 130 rpm for 30 min, 1, 2 h and 1 day. Ten seeds were transformed for each parameter tested. The seeds were co-cultivated with *Agrobacterium* at two temperature parameters of 23°C (room temperature) and 28°C. After co-cultivation, the inoculation medium was removed and seeds were left at room temperature. The planted seeds were then placed on germination soil bedding and were allowed to grow under natural conditions for 3 weeks. Forty wild type seeds were also randomly selected to be planted on soil as controls.

Genomic DNA isolation: Leaves from the four-leaf stage seedlings were surface sterilized and used to extract genomic DNA individually via., either a rapid DNA extraction method (Roslan, 1999) or using commercial kit, GF-1 Plant DNA Extraction kit (Vivantis, Malaysia).

Polymerase chain reaction (PCR): All PCR was conducted using GoTaq® Green Master Mix (Promega, USA) (400 μm dNTPs, 3 mM $MgCl_2$ and 2X reaction buffer at pH 8.5) following standard protocol recommended by the manufacturer. PCR was performed on plant DNA extracted from a four-leaf seedling using primers specific to GUS reporter gene and selectable marker for each binary vector. The DNA of putative seedlings transformed with *Agrobacterium* containing pGSA 1131 was analysed using GUS- and Bar-specific primers namely GUSe (F:5'-CCCCAGATGAAC ATGGCATCG-3',R:5'-GATCCCCATCAAAGAGATCGCT-3') and Bar-3 (F:5'-ATGAGCCCAGAACGACGCC-3', R:5'-ATCTCGGTGACGGGCAGG-3'), respectively. Meanwhile, for putative transformants generated from *Agrobacterium* containing pBI121, the GUS- and NptII-specific primers, GUS (F:5'-CATGTCGCGCAAAC TGTAAC-3', R:5'-AATCGCCTGTAAGTGCGCTTG-3') and NptII (F:5'-GCATACGCTTGATCCGGCTAC-3', R:5'-TGATATTCGGCAAGCAGGCAT-3'), were used in the PCR analysis. For the detection of *Agrobacterium* contaminants, the primers specifically target for the virulence gene was used, VirA-F (5'-TCTACGGTCATG GTCCACTAGACG-3') and VirA-R (5'-TGCTGCTCAACT GCTACGCCAGCT-3') (Mohamed *et al*., 2004). PCR was performed using a Biorad MyCycler PCR machine (Biorad, USA) with thermal cycling conditions of one cycle of 94°C for 4 min followed by 35 cycles at 94°C for 1 min, 59°C for 1 min, 72°C for 2 min and a 5 min final extension at 72°C. The amplified PCR products were separated in 1.5% agarose gel stained with ethidium bromide.

GUS histochemical assay: GUS histochemical assay was performed to detect the expression of reporter gene according to the method described by Jefferson *et al*. (1987) with modification in the incubation time. The thick cuticle layer underneath the leaves samples were removed via multiple treatments with chloroform before immersing the samples in X-Gluc solution [0.1 M sodium phosphate (Chemika, Switzerland; J.T. Baker, Malaysia), 2 mM of 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (Fermentas, USA), 1 mM potassium ferricyanide (Chemical Reagents, China), 1 mM potassium ferrocyanide (Farco, Hong Kong), 0.1% (v/v) Triton X-100 (Sigma, USA), 10 mM ethylenediaminetetraacetic acid (EDTA) (J.T. Baker, Mexico) and 20% (v/v) methanol (HmbG Chemicals, Hong Kong)]. The sample mix was incubated overnight at 37°C. On the next day, the sample was soaked several times in 70% ethanol with gentle agitation and left overnight at room temperature. The blue staining was visually detected using light microscope.

GUS fluorometric assay: The quantitative measurement of GUS activity was conducted via fluorometric assay (Jefferson *et al*., 1987). The leaf of a putative seedling was surface sterilized using 70% ethanol and ground in liquid nitrogen. GUS extraction buffer [50 mM sodium phosphate at pH 7.0, 10 mM EDTA, 0.1% (v/v) Triton X-100 (Sigma, USA) and 1 g L^{-1} sodium lauryl sarcosine) (ICN Biomedicals Incorporated, Germany)] was then added, mixed and centrifuged to collect the supernatant. The total protein content of the crude extract was measured using the method described by Bradford (1976). The crude protein was mixed with equal volume of MUG (4-methyl-umbelliferyl-β-D-glucuronide) (Sigma, United Kingdom) solution prepared by adding 1 mM MUG to GUS extraction buffer. The reaction mixture was incubated for two h at 37°C under dark condition and the reaction was stopped by adding 0.2 M sodium carbonate solution (HmbG Chemicals, Hong Kong). The assay was then analysed using Ultrospec® 1100 pro spectrophotometer (Amersham Pharmacia Biotech, USA) at two wavelengths; 365 nm excitation and 455 nm emission. The concentration of the fluorescence emission was determined by comparing with a standard concentration of MU (4-methylumbelliferone). The GUS activity was expressed in mmol of MU/min/μg of total protein.

GUS expression study: RNA was extracted via CTAB (cetyltrimethylammonium bromide) (Amresco, USA) method (Zeng and Yang, 2002) and the first strand cDNA was synthesized using RevertAid™ Moloney Murine Leukimia Virus Reverse Transcriptase (M-MuLV RT) (Fermentas, USA) and an oligo(dt)-anchor primer (5'-GACTCGAGTCGACACGATTTTTTTTTTTTTTTT-3'). The first strand cDNA was then used as template in PCR analysis to detect the presence of GUS transcripts and selectable markers.

Calculation of transformation efficiency: The transformation efficiency was determined using diagnosis DNA PCR result and sample size was based on the number of germinated seedlings from particular parameter tested and successfully transformed seedlings. The effect of each variable was statistically calculated using One-way Analysis of Variance (ANOVA) to determine the significance of each variable of a parameter on the transformation efficiency.

RESULTS

Direct seed transformation: Two bacterial densities, 0.5 and 1.0, were tested in the co-cultivation that resulted

in 34.0 and 80.3% transformation efficiency using pBI121 for bacterial density 0.5 and 1.0, respectively. Meanwhile, transformation using *Agrobacterium* containing pGSA 1131 with bacterial density 0.5 and 1.0 gave a result of 76.2 and 80.4%, respectively. Statistical calculation using One-way ANOVA showed that there was no significant difference of the transformation efficiency resulted from varied parameter based on 5% significance level.

The transformation efficiency of varying temperature for pBI121 was 71.9% (room temperature) and 82.0% (28°C) and for pGSA 1131, a transformation efficiency of 79.4% (RT) and 78.0% (28°C) were calculated. The results showed that the transformation of *M. citrifolia* using *Agrobacterium* containing either pBI121 or pGSA1131 did not vary much and no significant difference was detected based on 5% significance level.

Transformation of *M. citrifolia* utilizing *Agrobacterium* harbouring pBI121 with co-cultivation periods of 0.5, 1, 2 and 24 h resulted in 84.3, 66.7, 96.8 and 79.1% transformation rate. Meanwhile transformation using *Agrobacterium* containing pGSA1131 gave a transformation frequency of 77.7, 78.4, 72.2 and 75.3%, respectively. One-way ANOVA analysis result showed there was no significance difference on the transformation efficiency when the incubation period was varied when based on 5% significance level.

Analysis of T-DNA integration: The PCR method was carried out to detect the incorporation of T-DNA region via., amplification of the GUS and Bar from pGSA1131 and GUS and NptII from pBI121. The PCR products were then subjected to agarose gel electrophoresis analysis and the expected size for amplification product were determined to be of the expected sizes of 400, 600 bps (GUS and Bar in pGSA 1131), 600 and 200 bps (GUS and NptII in pBI121), respectively (Fig. 1, 2). Additionally, the transformants were also screened for *Agrobacterium* virulence gene to eliminate the possibility of *Agrobacterium* contamination in the transformed seeds (Fig. 3).

GUS histochemical and fluorometric assay: A total of 182 putative seedlings were analysed with GUS histochemical staining. The seedlings were firstly screened to harbour the T-DNA via., PCR using primers specific to GUS and selectable marker genes. Results of the stained leaf sample showed a non-uniform distribution of GUS activity detected by the blue colour deposition on the vein system primarily along the base of the petiole (Fig. 4). Two putatively transformed seedlings were found to produce blue coloration. A subsequent fluorometric assay was conducted to measure GUS activity in the two leaves samples that showed results on GUS histochemical assay. The quantitative measurements of the leaves samples via fluorometric assay are presented in Table 1. The measured specific expressed GUS activities of both leaves were approximately the same which were 9.92×10^{-10} and 6.44×10^{-10} mmol/min/μg, respectively.

Reporter gene expression study: The gene expression study of GUS was conducted on several positive putative transformants samples from the initial diagnostic PCR of

Table 1: Specific GUS activity of two transformed seedlings (A_1 and B_1) showing blue colour deposits in the vascular bundle. GUS activity was calculated as mmol/min/μg protein

Sample	Absorbance values	Concentration of MU, μM (from equation formulated)	Specific GUS activity (mmol/min/μg)
A_1	0.756	541.43	9.92×10^{-10}
B_1	0.497	356.43	6.44×10^{-10}

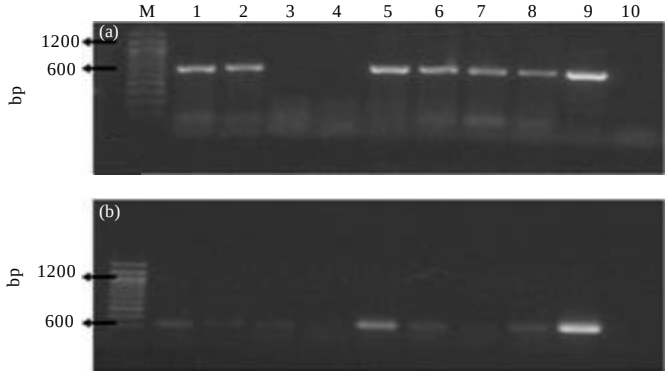

Fig. 1(a-b): Agarose gel electrophoresis (1.5% w/v) of PCR products from putatively transformed seedlings containing binary vector pBI121. Lane M is 100 bps DNA ladder (Seegene), (a) Lane 1-8 are PCR products using GUS primer (~600 bps) and (b) lane 1-8 are PCR products using NptII primers (~200 bps). Lane 9 in both row is the positive control using DNA from pBI121 and lane 10 is the negative control (wild type *M. citrifolia* DNA)

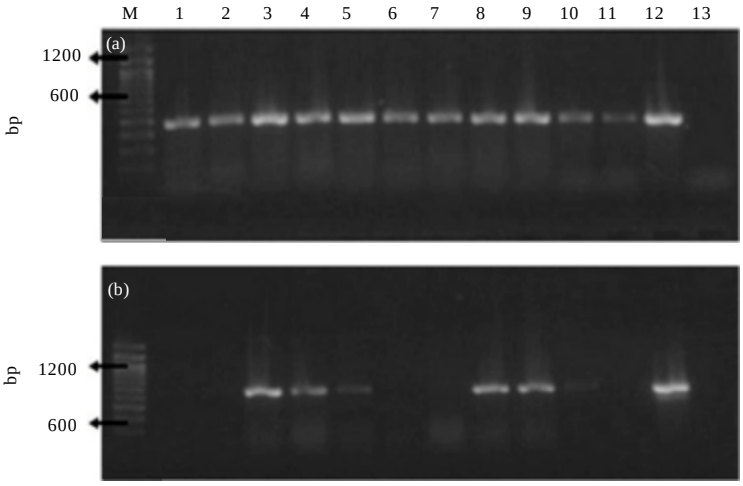

Fig. 2(a-b): Agarose gel electrophoresis (1.5% w/v) of PCR products from putatively transformed seedlings harbouring binary vector pGSA1131. Lane M is the 100 bps DNA ladder (Seegene) (a) Lane 1-11 are PCR products using GUSe primers (~400 bps) and (b) lane 1-11 are PCR products using Bar-3 primer (~600 bps). Lane 12 in both rows is the positive control using DNA from pGSA1131 and lane 13 is the negative control (wild type *M. citrifolia* DNA)

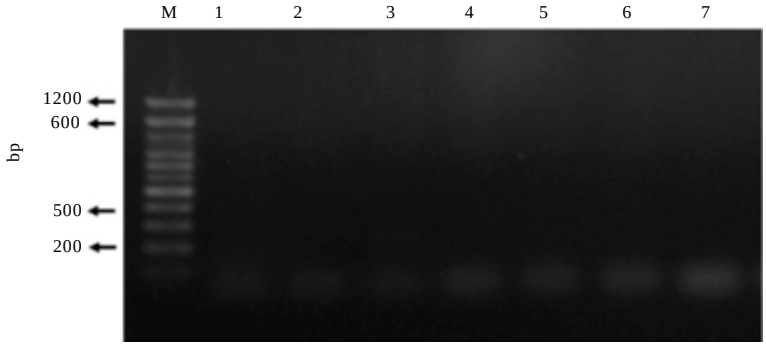

Fig. 3: Agarose gel electrophoresis (1.5% w/v) of virulence gene PCR of some of the putatively transformed seedlings. Lane 1-7 are DNA sample of putative transgenic seedlings containing GUS and selectable marker. No amplicons were detected using virulence gene primers except for lane 3 which was determined later to be artefact (data not shown)

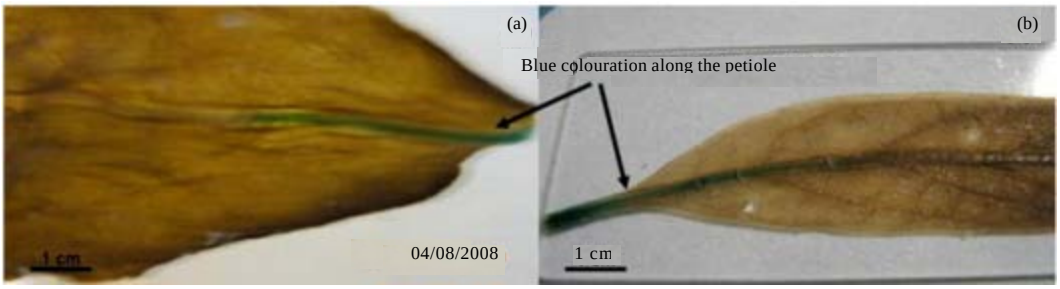

Fig. 4(a-b): (a) GUS histochemical assay. Staining conducted on leaf sample from putatively transformed seedlings containing pBI121 and (b) pGSA1131, Arrow indicates the blue colour deposits along the vascular bundle near the leaf petiole

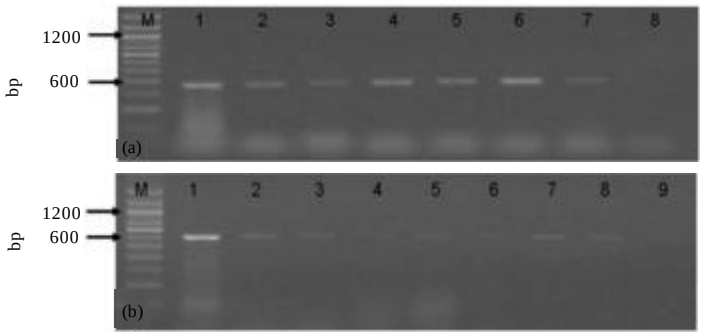

Fig. 5(a-b): Agarose gel electrophoresis (1.5% w/v) of RT-PCR assay using GUS-specific primers from putatively transformed seedlings. Lane M is 100 bps DNA ladder (Seegene) (a) Lane 1-7: cDNA samples of seedlings transformed with pGSA1131, Lane 8: Negative control and (b) Lane 1-8: cDNA samples of seedlings transformed with pBI121, Lane 9: Negative control

Table 2: Morphological description of selected putatively transformed and wild type seedlings that were verified via., PCR

Putative seedlings	Primers used for PCR	Morphology description of transformants	Wild type characters
I30_ii_1	GUSe, Bar-3	Small leaflet grew from an internodal nodes resulting in shorter length of the internode	Rachis between nodes of leaf
I77_6	GUSe, Bar-3	Three stems branched out from the same node (trifoliate leaf) with higher growth rate	Two leaf stems branch out from same node
I58_1	GUS, NptII	Shrivelled leaf on alternate node	Shiny leaf pair on each node
B66_1	GUS, NptII	The emergence of third leaf from the stem apex with existing two leaves at the same bulb.	Two leaves from stem apex
B21_4	GUS, NptII	Leaf curling from the stem apex.	Ovate leaf shape

GUSe and Bar-3 primers-primers specific for pGSA1131, GUS and NptII primers-primers specific for pBI121

reporter and selection genes, including the samples that did not produce blue coloration in the histochemical assay. Seven putative transformants were selected from seeds transformed with pGSA1131 and eight putative transformants transformed using pBI121. Controls were included in the RT-PCR using DNA derived from wild type and non-transformed plants. The RT-PCR assay of the putative transformant samples showed variable intensity of banding patterns for GUS gene amplification that indicated differential expression of the GUS gene in each transformant (Fig. 5).

Morphological observations of putative transformants: Morphological differences were also observed in several putative transformants that could be the result of random integration of the T-DNA region into plant genome. Table 2 lists the descriptions of morphological changes observed in transformed plants as compared with the wild type. The wild type leaf has the following characters; large, simple, dark green, shiny, deeply veined, opposite leaf arrangement.

DISCUSSION

The scarified seeds were slightly desiccated via., air drying before co-cultivation with *Agrobacterium* (Urushibara *et al*., 2001). This desiccation process helped in increasing T-DNA delivery and subsequent cell recovery (Cheng *et al*., 2003). A total of 2180 seedlings were germinated, in which three parameters were analysed to optimize the *A. tumefaciens*-mediated seed transformation; bacterial density, co-cultivation temperature and incubation period. It was reported that the bacterial density has significant impact on transformation efficacy, unrelated to the *Agrobacterium* strains used for the transformation (Gao *et al*., 2009). However, our research study found that there is no significance difference in transformation efficiency of *M. citrifolia* with varying bacterial density parameter. The result showed that the optimum optical density for *M. citrifolia* transformation was 1.0 for both binary vectors used.

Incubation temperature during co-cultivation was also analysed in which previous studies have shown that T-DNA delivery was thermo-sensitive due to the temperature dependent regulation of the virulence regulon (Dillen *et al*., 1997). We tested two temperatures, room temperature (RT, approximately 23°C) and 28°C. The study result indicated that varying temperatures during co-cultivation step did not affect the transformation efficiency and that transformation can be conducted at temperatures above 25°C as mentioned by Gonzalez *et al*. (2008) and Jakubowski *et al*. (2003).

The co-cultivation period had been shown by Jabeen *et al*. (2009) to affect the transformation efficiency. Four co-cultivation periods were selected; 0.5, 1, 2 and 24 h. Although, no significant impact on the transformation efficiency was detected, the work indicated that the optimal co-cultivation period was between 1 and 2 h. The co-incubation of the *Agrobacterium* with *M. citrifolia* seeds was conducted with gentle agitation that facilitates *Agrobacterium* growth and allow homogenous distribution of *Agrobacterium* in the inoculation medium (Gonzalez *et al*., 2008). Incubation at 24 h showed decreased transformation with prolonged co-cultivation period that could cause *Agrobacterium* overgrowth and eventually has detrimental effects on plant cell recovery (Sreeramanan *et al*., 2008).

Previous seed transformation work conducted by several research groups on plant species such as in *Z. mays* (Wang *et al*., 2007) and *Hibiscus sabdariffa* (Gassama-Dia *et al*., 2004) produced transformation efficiencies of 29 and 60%. Whereas Katavic *et al*. (1994) had performed that *in planta* transformation of *A. thaliana* using germinated seedlings with removed inflorescence shoots reported approximately 4.31 and 15.97% transformation frequency. Meanwhile, another type of seed transformation, the fruit inoculation, conducted by Ahmad and Mirza (2005) in which the incubation periods were varied, produced 25 and 23% transformation rate with 72 and 96 h incubation periods, respectively. A comparison of the reported transformation rate of other plant species seemed to indicate that the seed transformation for *M. citrifolia* is an efficient transformation method.

Several limitations are noted for the plant transformed namely in the non-use of antibiotics. Although, the seeds were not treated with antibiotics to eliminate the *Agrobacterium* after co-cultivation process, nevertheless plants transformed with the binary vectors were analysed via PCR using primers specifically to detect for virulence gene from *Agrobacterium* (Nain *et al*., 2005). Positive PCR amplification result indicated that the T-DNA region from pGSA1131 and pB1121, had integrated into the plant genome. PCR-positive putative transformants on transgene integration were then selected for further histochemical, fluorometric and expression studies.

The deposition of GUS assay indicated that the integration of the T-DNA in the transformed plant was in the region that is specific to the vascular bundle along the petiole. However, GUS histochemical assay that were conducted in other putative transformants did not yield satisfactory results (data not shown). This might be because the T-DNA was integrated into different cell types where the GUS activity was not detectable or expressed at an extremely low level (Jefferson, 1987).

The seedlings that did not produce blue colouration in the GUS histochemical assay were also tested for transgene expression via., reverse transcription PCR (RT-PCR) to detect the expression of GUS at low levels, since the PCR technique can detect very low levels of gene expression (Cha and Thilly, 1995). The differential expression patterns of GUS in RT-PCR further supports the histochemical assay finding that putatively transformed seedlings indeed harbour the T-DNA but expressions in the seedlings were very low to be detected via histochemical assay.

Previous T-DNA insertional mutations studies in *Solanum lycopersicum* L. (Yasmeen *et al*., 2009), *Malus micromalus* (Zhang *et al*., 2006) and cotton (*Gossypium hirsutum* L.) had shown that transformants caused morphological changes such as wrinkled or curled leaves, shorter height and other phenotypic changes that were similar to the changes observed on putative transgenic seedlings of *M. citrifolia*. Nevertheless, further analyses are still required for this study.

CONCLUSION

Direct seed transformation using *Agrobacterium* mediated method is a reliable method for transformation of *M. citrifolia* without much difference in the type of vector used. Histochemical assay produced blue staining along the vascular bundle. Nevertheless, the method requires further modification to analyse for other transformants. GUS transcript analysis showed that the reporter gene is expressed in the transformants with different levels of expression. Co-cultivation of seeds with *Agrobacterium* at optimal conditions has shown to produce high transformation efficiency based on the optimised conditions.

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