

SHORT COMMUNICATION

Enhancers of *Agrobacterium*-mediated Transformation of *Tibouchina semidecandra* Selected on the Basis of GFP Expression

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Abstrak: Kejuruteraan genetik adalah satu peralatan canggih untuk memperbaiki ciri tumbuhan. Walaupun dilaporkan berjaya dalam alam tumbuhan, teknologi ini hanya mencalar sedikit permukaan famili Melastomataceae. Kajian terhad telah dilakukan untuk memperolehi keputusan yang terbaik bagi parameter yang diketahui mempengaruhi kecekapan transformasi tumbuhan ini. Hasil utama kajian ini adalah penemuan keadaan yang terbaik dengan kehadiran perangsang yang tertentu [contohnya, monosakarida (D-glukosa, D-galaktosa dan D-fruktosa), tirosina, aluminium klorida (AlCl₃) dan asid askorbik] dalam meningkatkan kecekapan transformasi *Tibouchina semidecandra*. *Agrobacterium tumefaciens* strain LBA4404 dengan plasmid pCambia1304 telah digunakan dalam transformasi keratan pucuk and tunas ketiak *T. semidecandra*. Pelbagai kepekaan perangsang transformasi telah dikaji dengan menggunakan *green fluorescent protein* (GFP) sebagai penanda. Keputusan yang diperolehi adalah berdasarkan kepada peratusan ekspresi GFP yang diperhatikan pada hari ke-14 selepas transformasi dilakukan. Kombinasi 120 µM galaktosa dan 100 µM tirosina berserta dengan 600 µM AlCl₃ dan kehadiran 15 mg/l asid askorbik telah memberi peratus kejadian transformasi yang tertinggi bagi keratan pucuk *T. semidecandra*. Sedangkan 60 µM galaktosa dan 50 µM tirosina berserta dengan 200 µM AlCl₃ dan kehadiran 15 mg/l asid askorbik adalah terbaik bagi keratan tunas ketiak *T. semidecandra*. Kehadiran *hygromycin phosphotransferase II* (*hptII*) transgen di dalam DNA genomik bagi putatif *T. semidecandra* transforman telah dikaji dengan amplifikasi PCR menggunakan primer yang spesifik.

Kata kunci: Monosakarida, Tirosina, Aluminium Klorida, Asid Askorbik, Ekspresi Transien GFP

Abstract: Genetic engineering is a powerful tool for the improvement of plant traits. Despite reported successes in the plant kingdom, this technology has barely scratched the surface of the Melastomataceae family. Limited studies have led to some optimisation of

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parameters known to affect the transformation efficiency of these plants. The major finding of this study was to optimise the presence of selected enhancers [e.g., monosaccharides (D-glucose, D-galactose and D-fructose), tyrosine, aluminium chloride (AlCl₃) and ascorbic acid] to improve the transformation efficiency of *Tibouchina semidecandra*. *Agrobacterium tumefaciens* strain LBA4404 harbouring the disarmed plasmid pCAMBIA1304 was used to transform shoots and nodes of *T. semidecandra*. Different concentrations of the transformation enhancers were tested by using green fluorescent protein (GFP) as a reporter. The results obtained were based on the percentage of GFP expression, which was observed 14 days post-transformation. A combination of 120 µM galactose and 100 µM tyrosine supplemented with 600 µM AlCl₃ in the presence of 15 mg/l ascorbic acid gave the highest percentage of positive transformants for *T. semidecandra* shoots. Whereas 60 µM galactose and 50 µM tyrosine with 200 µM AlCl₃ in the presence of 15 mg/l ascorbic acid was optimum for *T. semidecandra* nodes. The presence of the hygromycin phosphotransferase II (hptII) transgene in the genomic DNA of putative *T. semidecandra* transformants was verified by PCR amplification with specific primers.

Keywords: Monosaccharide, Tyrosine, Aluminium Chloride, Ascorbic Acid, GFP Transient Expression

Tibouchina semidecandra (Schrank & Mart. ex DC.) Cogn. belongs to the Melastomataceae (Abdullah & Yong 2007) family, and Melastomataceae spp. has great potential as an ornamental plant. Locally known as 'senduduk biru', *T. semidecandra*, produces attractive purplish blue flowers at a very young age and blooms continuously, which is characteristic of tropical ornamental plants. Nurseries in Malaysia and other southeast Asian countries propagate and sell them as landscaping plants for borders and foundation planting. However, uncontrolled growth of the plants, limited colours and limited lifespan of each individual flower have reduced their commercial value (Yong *et al.* 2009). To improve the quality of the plants and/or develop new varieties, genetic transformation is recommended because it has opened new avenues to the modification of characteristics, such as flower colour (Fukusaki *et al.* 2004), fragrance (Zuker *et al.* 2002), longevity (Mitiouchkina & Dolgov 2000), shape and size (Giovannini *et al.* 1999). The most effective transformation technique is probably *Agrobacterium*-mediated transformation. *Agrobacterium*-mediated transformation was the method of interest in this study using *T. semidecandra* as the target plant species. To date, the only reports of genetic manipulation carried out on *T. semidecandra* are two studies by Yong *et al.* on system optimisation (2006) and on transformation of dihydroflavonol-4-reductase (DFR) genes (2009).

Plant transformation mediated by the soil-borne pathogen *Agrobacterium tumefaciens* was first reported in the early 1980s (Gelvin 2003). Since then, *Agrobacterium*-mediated transformation has been extensively used to genetically modify dicotyledonous as well as monocotyledonous plants such as tobacco (Slavov *et al.* 2005), torenia (Li *et al.* 2007), tomato (Sui *et al.* 2007), barley (Shrawat *et al.* 2007) and rice (Ozawa 2009). The efficacy of transformation is

highly dependent on many factors such as the bacterial strain, plant species and the type of tissue explant used. The key factors in the method are a 'super-binary' vector and the addition of acetosyringone to the co-cultivation medium as an enhancer. General parameters such as pre-culture time, co-cultivation time, immersion time, bacterial concentration, wound type and acetosyringone concentration were shown to influence the transformation efficiency by Yong *et al.* (2006), and the results obtained were based on the percentage of green fluorescent protein (GFP) gene expression, which was observed three days post-transformation.

In this study, instead of costly acetosyringone, different concentrations of D-monosaccharides, tyrosine, aluminium chloride (AlCl₃) and ascorbic acid were tested as enhancers to improve the transformation efficiency of *T. semidecandra*. These groups of compounds were expected to be able to replace the function of acetosyringone as transformation enhancers, as reported by previous researchers (Shimoda *et al.* 1990; Sarker *et al.* 2003; Okamoto *et al.* 2005; Abdullah & Yong 2007). Expression of the GFP gene, a useful non-invasive and non-destructive marker, was used to evaluate the transformation parameters. The optimised conditions have potential for use in the integration of desirable commercially important genes into this plant species to improve its quality and value as an ornamental plant.

In vitro-generated plantlets of *T. semidecandra* from Plant Biotechnology Laboratory, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, Selangor were used as sources of explants. The plantlets were multiplied and maintained on half-strength MS basal medium (Murashige & Skoog 1962) under a 16-h light/8-h dark photoperiod at 25 ± 2°C. Shoots (2 leaf stage) and nodes (all 3 nodes counting from the apex) from 6- to 8-week old plantlets showing high regeneration ability were used as explant material for transformation. All experiments were carried out with 3 replicates per treatment and 20 explants per replicate.

The binary vector pCAMBIA1304 (CSIRO, Australia) harbouring the reporter *mgfp5* driven by the *CaMV* 35S promoter (as illustrated in Fig. 1) was used. The plasmid also contains hygromycin phosphotransferase II (*hptII*) (the coding region of the *hptII* gene for hygromycin resistance in plant systems) as a selectable marker. The plasmid DNA was prepared from *Escherichia coli* using the alkaline lysis method (Sambrook *et al.* 1989) and mobilised into *A. tumefaciens* LBA4404 (Yong *et al.* 2006) prior to transformation of *T. semidecandra*. The plasmid yield was determined by a spectrophotometer (Pharmacia LBK-U1-trospec III, LKB Biochrom Ltd., England).

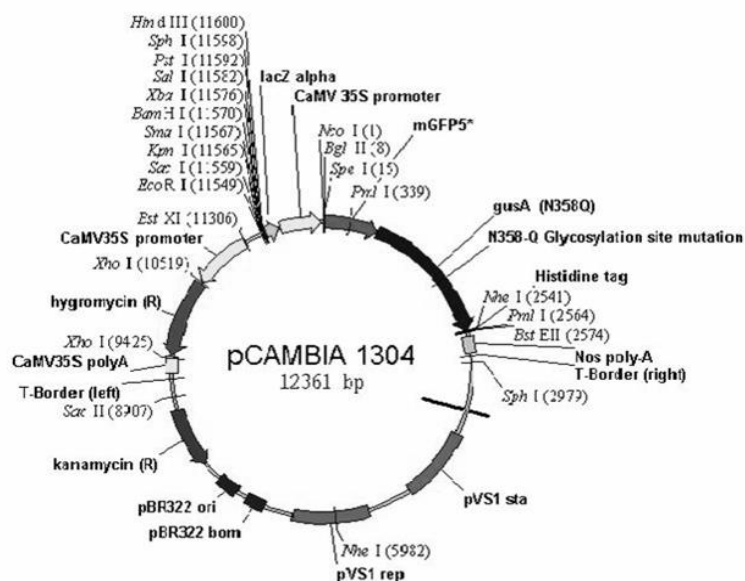


Figure 1: Plasmid map of pCambia1304.

Explants of *T. semidecandra* as described above were prepared and pre-cultured for three days on half-strength MS basal medium prior to being transferred into *Agrobacterium* suspension (OD_{600} 0.8) with the addition of monosaccharide and tyrosine. The bacterial suspension and explants were mixed and gently shaken at 150 rpm to ensure that all the explants were fully submerged (Yong *et al.* 2006). After an hour of immersion, the explants were blotted dry on sterile filter paper and subsequently transferred to the co-cultivation medium supplemented with $AlCl_3$ and ascorbic acid for three days. The effects of the selected transformation enhancers were tested in combination, where addition of 30 μ M glucose and 50 μ M tyrosine into *Agrobacterium* suspension together with 200 μ M $AlCl_3$ and 85 μ M (15 mg/l) ascorbic acid in the co-cultivation medium were treated as the control. In this study, different concentrations of monosaccharides (0, 30, 60, 90, 120 and 150 μ M), tyrosine (0, 50, 100, 150 and 200 μ M) and $AlCl_3$ (0, 200, 400, 600 and 800 μ M) were assessed, as was the presence of ascorbic acid. The concentration ranges for these compounds were based on previous studies carried out by Shimoda *et al.* (1990), Sarker *et al.* (2003), Okamoto *et al.* (2005) and Abdullah and Yong (2007). The cultures were incubated at $25 \pm 2^\circ C$ under a 16-h light/8-h dark photoperiod. After co-cultivation for 3 days, the explants were

washed by gentle shaking for 30 min in Petri dishes containing liquid culture medium 5 times to remove the bacteria (Puchooa 2004) before being transferred to bacterial elimination medium (half strength MS containing 100 mg/l cefotaxime). The bacterial elimination treatment was repeated every three days to remove the bacteria completely.

All parameters were optimised by screening for transient green fluorescent protein (GFP) expression using a fluorescence stereomicroscope (Leica MZFL III, Leica Microsystems Inc., Bannockburn, IL, USA) equipped with a GFP2 filter set. GFP observations were carried out daily and targeted to the newly regenerated shoots. The results obtained were based on the highest GFP expression at 14 days post-transformation, when the *Agrobacterium* was completely eliminated. The data were analysed using one-way ANOVA, and the differences were assessed using Tukey's multiple comparison test. The statistical analyses were performed using SPSS 17.0 (SPSS Inc., USA) at the 5% level.

Newly regenerated shoots from randomly selected GFP-positive transformants at 14 days post-transformation were subjected to genomic DNA extraction and amplification of the *hptII* transgene by PCR with specific primers (*hpt1*: 5'-TAG GAGGGCGTGGATATGTC-3' and *hpt2*: 5'-TAC ACAGCCATCGGTCCAGA-3') as suggested by Zou *et al.* (2004) using standard protocols (Sambrook & Russell 2001), which provided an amplification product of ~800 bp. PCR was performed using a Mastercycler Gradient (Eppendorf, Germany) machine. The amplifications were carried out in 25 µl reaction volumes containing 100 ng genomic DNA, PCR buffer (10 mM Tris-Cl, 50 mM KCl and 0.8% Nonidet P40), dNTP mix (200 µM each), 1.5 mM MgCl₂, 15 pmole of each primer and 5 U *taq* DNA polymerase. The PCR was carried out with an initial denaturation step of 5 min at 94°C, followed by 35 cycles of denaturation (94°C, 30 s), annealing (55°C, 30 s) and elongation (72°C, 30 s) and a final extension step of 10 min at 72°C.

The optimisation of *T. semidecandra* explant transformation efficiency with monosaccharide (D-glucose, D-galactose and D-fructose) treatment is presented in Figure 2. Among the different concentrations of the 3 types of monosaccharide, 120 µM and 60 µM galactose gave the highest percentages of GFP expression for both shoot (67.78%) and node (61.11%) explants. Based on this observation, the trends of GFP expression for glucose and galactose treatments increased simultaneously with increases in their respective monosaccharide concentrations, up to 120 µM for shoots and 150 µM for nodes, although the trend observed for the nodes fluctuated. The results indicated that the GFP expression profile for fructose treatment was different from that of glucose and galactose, where fructose above the concentration of 30 µM decreased GFP expression in both shoot and node explants. This phenomenon may be due to the structural differences between the different functional carbonyl groups of these monosaccharides; glucose and galactose contain an aldehyde group, whereas fructose contains a keto group. A previous study reported that the aldehyde monosaccharide form in aldoses can markedly enhance the expression of *vir* genes in *Agrobacterium* for T-DNA transfer into plant cells

(Cangelosi *et al.* 1990; Shimoda *et al.* 1990). The key molecule that allows *Agrobacterium* to sense environmental conditions favourable for T-DNA transfer is the VirA protein (Lee *et al.* 1996). For optimal *vir* gene induction, the periplasmic domain of VirA is a sensor of a variety of monosaccharides on the cell walls of plants, including arabinose, galactose, galacturonic acid, glucose, glucuronic acid, mannose, fucose, cellobiose and xylose (Ankenbauer & Nester 1990). In this study, the addition of monosaccharides significantly enhanced the transformation efficiency of *T. semidecandra*, and galactose was found to be the most favourable monosaccharide utilised by *Agrobacterium* in this process.

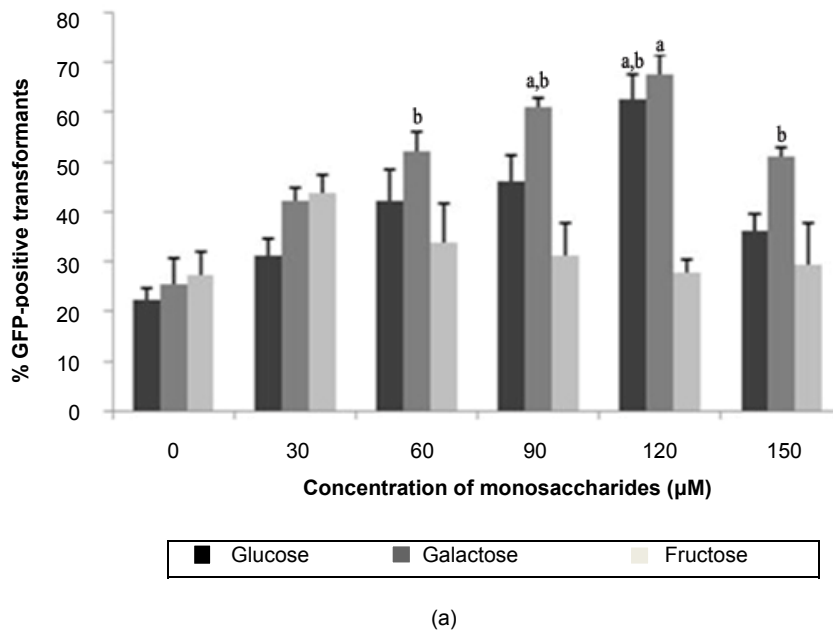
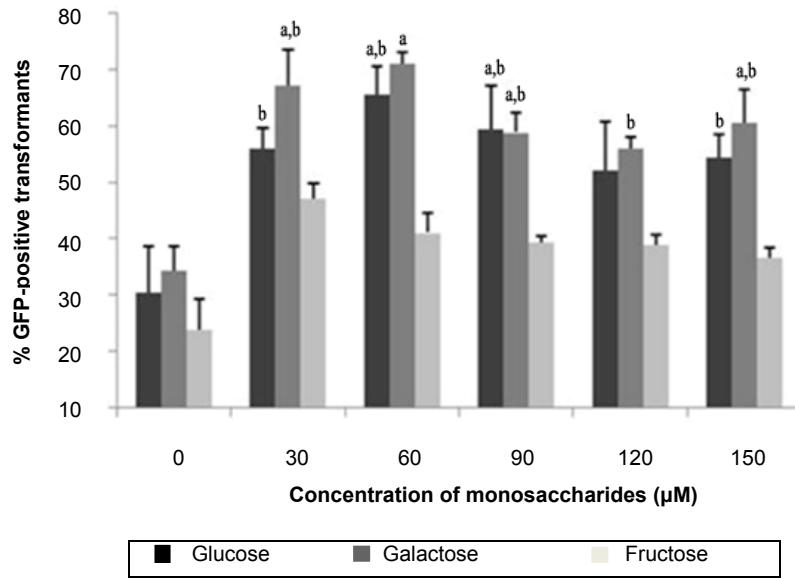


Figure 2: Percentage of GFP expression observed 14 days post-transformation with different concentrations of monosaccharides: (a) shoots, (b) nodes (*continued on next page*).

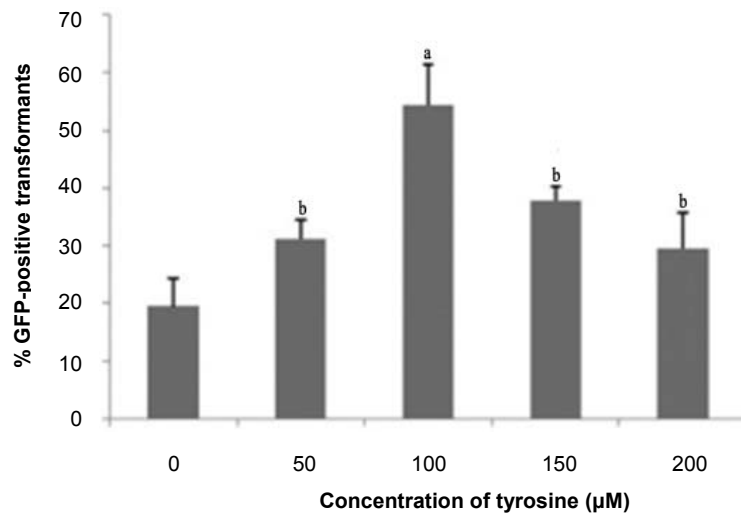
Notes: Error bars indicate standard deviations (n = 3). Different letters indicate value that are significantly different ($p \leq 0.05$).



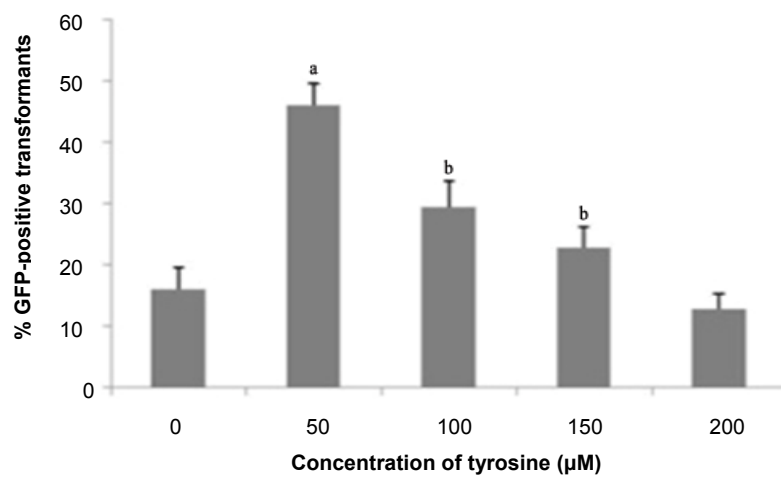
(b)

Figure 2: (continued)

The highest GFP expression was observed with 100 μM tyrosine treatment for shoot explants (54.45%) and 50 μM tyrosine treatment for the nodes (46.11%), as presented in Figure 3. Tyrosine can be used as an alternative transformation enhancer due to its structure, which is similar to acetosyringone (Baker *et al.* 2005). In previous research carried out by Sarker *et al.* (2003), transformed explants were co-cultivated on tyrosine to enhance transformation efficiency and the plant regeneration process. Both acetosyringone and tyrosine contain an aromatic benzene structure similar to phenolic compounds present in plants that are responsible for *Agrobacterium vir* gene induction (Joubert *et al.* 2002). However, using tyrosine for transformation is more economical than using acetosyringone. While supplementation with phenolic compounds did enhance transformation efficiency, the degree of influence was dependent upon its concentration. Guivarc'h *et al.* (1993) reported that more than 75 μM acetosyringone rarely promoted gene transfer, although levels up to 200 μM are not considered to be significantly toxic to *Agrobacterium* cells. In addition, the decreased transformation efficiency observed above the optimum level of tyrosine may be associated with the accumulation of avirulent mutants, as indicated by Fortin *et al.* (1992).



(a)

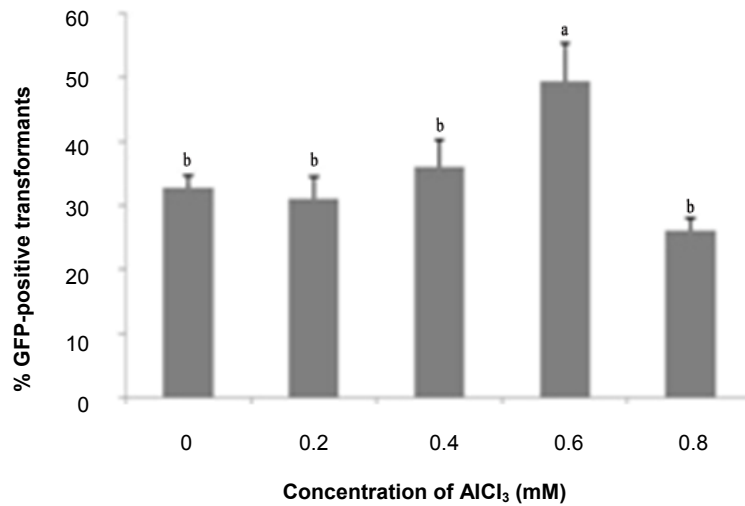


(b)

Figure 3: Percentage of GFP expression observed 14 days post-transformation with different concentrations of tyrosine: (a) shoots, (b) nodes.

Notes: Error bars indicate standard deviations (n = 3). Different letters indicate values that are significantly different ($p \leq 0.05$).

The effect of AlCl_3 on the transformation efficiency of *T. semidecandra* explants is demonstrated in Figure 4. Supplementation with 600 μM AlCl_3 gave the highest GFP expression for shoot explants (49.44%), while 200 μM AlCl_3 was the optimum level for nodes (46.11%). Addition of AlCl_3 to the co-cultivation medium notably facilitates the processes of replication, transcription and translation of the transgenic protein in plant cells (Davey *et al.* 2005). Aluminium toxicity is often a primary factor affecting crop productivity, but the growth of Melastomataceae spp. was reported to improve when the growth medium was supplemented with aluminium at 500 μM (Watanabe & Osaki 2002; Watanabe *et al.* 2005). Jansen *et al.* (2002) surveyed members of this family and found at least 127 species (including *Tibouchina* sp.) with the capacity to accumulate aluminium. However, above tolerable levels, excess aluminium may cause toxicity to plants via inhibition of cell elongation and cell division, leading to tissue stunting accompanied by reduced water and nutrient uptake (Samac & Tesfaye 2003). This is evidenced by decreased explant survival rates above the optimum levels of aluminium. The mechanisms of aluminium toxicity and tolerance in plants were specifically discussed by Delhaize and Ryan (1995).



(a)

Figure 4: Percentage of GFP expression observed 14 days post-transformation with different concentrations of AlCl_3 : (a) shoots, (b) nodes (*continued on next page*).

Notes: Error bars indicate standard deviations ($n = 3$). Different letters indicate values that are significantly different ($p \leq 0.05$).

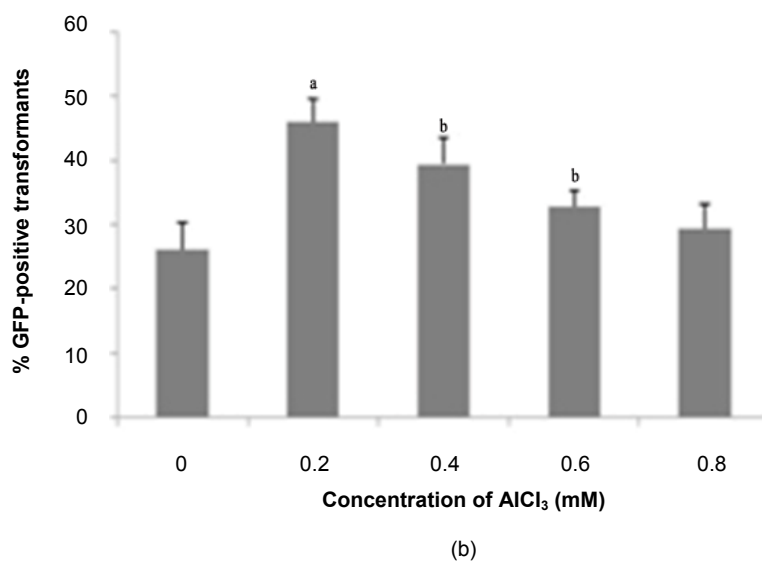
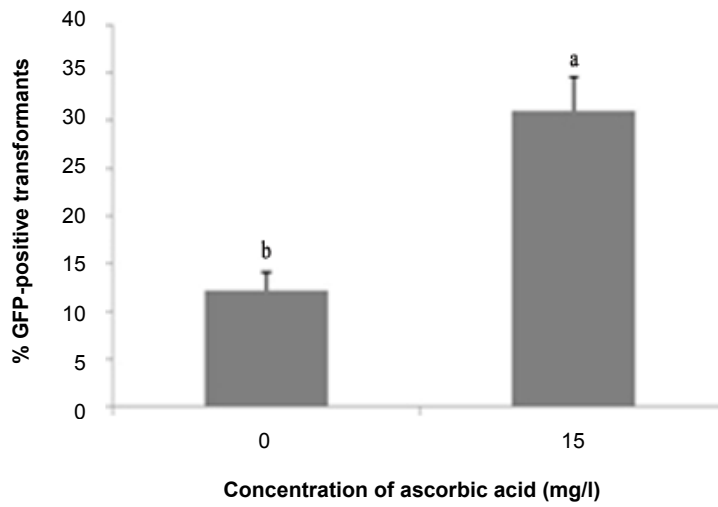
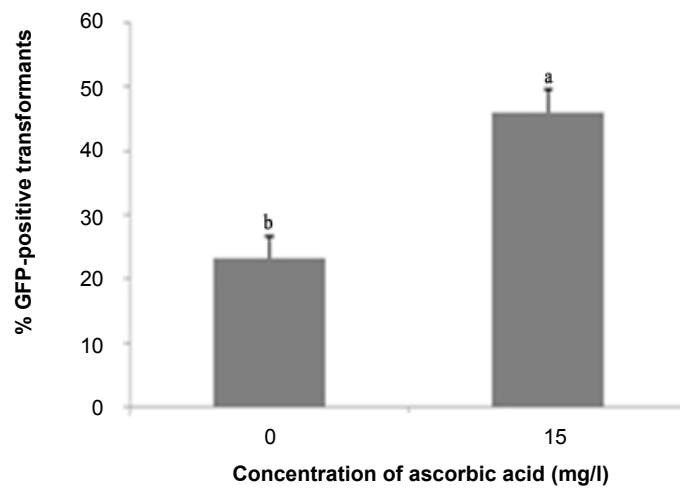


Figure 4: (continued)

With the exclusion of ascorbic acid, the percentage of GFP expression in transformed shoots decreased by about 60%, with only 12.22% positive expression compared to 31.11% with ascorbic acid treatment (Fig. 5). For transformed nodes, the percentage of GFP expression increased about 50% (from 23.33% to 46.11%) with the addition of ascorbic acid to the co-cultivation medium. Tissue browning is apparently a part of the plant defence machinery, as this makes a dead cell barrier around wounded sites that protects plants from further spreading of the injury (Okamoto *et al.* 2005). Thus, plants with high browning activity are recalcitrant to *Agrobacterium*-mediated transformation because their defence responses are often induced by *Agrobacterium* infection (Pu & Goodman 1992; Hansen 2000). Application of reducing compounds, such as ascorbic acid, cysteine and dithiothreitol, has contributed to the establishment of *Agrobacterium*-mediated transformation of some crop plants (Perl *et al.* 1996; Enriquez-Obregon *et al.* 1999; Frame *et al.* 2002), probably due to suppression of tissue browning. Inclusion of ascorbic acid in the post-transformation culture medium decreases the cell death rate in explants by inhibiting hypersensitive responses to damage generated during tissue manipulation. A previous study by Enriquez-Obregon *et al.* (1997) reported that reducing compounds were able to decrease the hypersensitivity reaction in cut zones of sugarcane meristematic explants, and they subsequently improved the competence of the plant tissue for *Agrobacterium*-mediated gene transfer.



(a)



(b)

Figure 5: Percentage of GFP expression observed 14 days post-transformation for ascorbic acid treatment: (a) shoots, (b) nodes.

Notes: Error bars correspond to standard deviation ($n = 3$). Different letters indicate values are significantly different ($p \leq 0.05$).

The result obtained from the PCR analysis revealed that ~800 bp of the *hptII* transgene was successfully amplified from putative transformants randomly selected from newly regenerated shoots (Fig. 6). Analyses of regenerated shoots from putative transformants are recommended to reduce the chimerism rate in the future recovery of transgenic plants (Ahn *et al.* 2007). Untransformed plants were used as controls, and no bands were amplified from them in the PCR analyses. The *hptII* gene was co-integrated with the GFP gene in the selected putative transformants. Co-transformation of the *mgfp5* and *hptII* genes indicated that there was no breakage of T-DNA during the transformation process. The *Agrobacterium*-mediated delivery of foreign DNA into target tissue was found to be superior to microprojectile bombardment, which tended to promote DNA breakage and subsequently reduce the transformation efficiency (Yong *et al.* 2009).

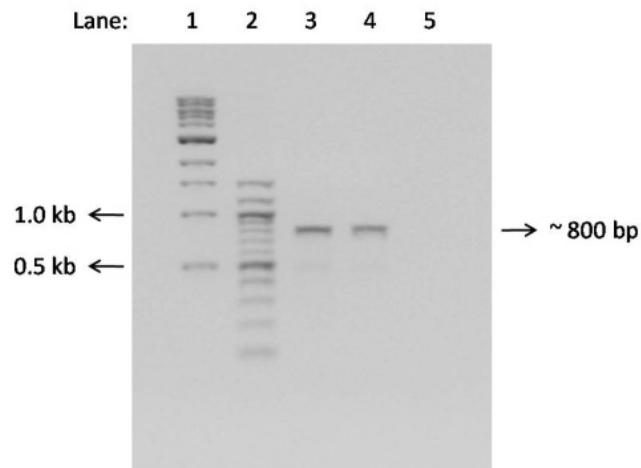


Figure 6: PCR analysis of the *hptII* transgene in GFP-positive transformants; lane 1, 2 = molecular weight markers (1: 1-kb DNA ladder; 2: 100-bp DNA ladder), lane 3, 4 = genomic DNA samples of GFP-positive transformants, lane 5 = untransformed plants (control).

The efficiency of plant transformation is dependent on multiple factors, including transformation enhancers that stimulate the activity of the *vir* gene in *Agrobacterium* to enable the transfer of T-DNA into plant cells. In this study, selected enhancers with significant effects on transformation efficiency as reported by previous researchers were optimised for *T. semidecandra*

transformation. The concentrations of monosaccharides, tyrosine, AlCl_3 were optimised for the transformation of *T. semidecandra* explants, as was the presence of ascorbic acid. However, further molecular studies including Southern and Northern blot analyses are highly recommended to demonstrate the integration and functionality of the transgene in the new host. With the optimised parameters, transformation of economically important genes that improve the quality of *T. semidecandra* is feasible. Furthermore, the plant must be in a healthy condition in order for its cells to undergo gene expression. It is important to have a good understanding of basic transformation, and more parameters should be optimised in order to obtain the best protocol for plant transformation.

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