

Agrobacterium-mediated stable transformation of medicinal plant *Andrographis paniculata* callus expressing β -glucuronidase (GUS) gene

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Abstract

This study was carried out to establish a stable genetic transformation in callus culture of *Andrographis paniculata* mediated by *Agrobacterium tumefaciens*. The leaf disks of *A. paniculata* were infected with *A. tumefaciens* LBA4404 carrying a binary vector pCAMBIA1304 that contain β -glucuronidase (*GUS*) and hygromycin phosphotransferase (*hpt*) genes. The infection was conducted by dipping method for one hour, followed by co-cultivation in the dark for three days. To examine transient *GUS* expression, the co-cultivated leaf disks were assayed for β -glucuronidase activity and to obtain stable transformed callus, the co-cultivated leaf disks were selected on the callus induction medium which contain 20 mg/l hygromycin for selection. The transformed callus was periodically subcultured every three weeks into the fresh selection medium over the 15 weeks period. To test a stable transformation, the callus was subjected to PCR analysis for *GUS* gene detection. The results indicated that the co-cultivated leaf disks expressed *GUS* activity and proliferated to produce callus on the selective medium. Analysis of PCR on the transformed callus indicated the presence 976 bp fragment that confirmed the presence of β -glucuronidase gene. These findings imply that the β -glucuronidase was stably integrated into *A. paniculata* callus culture.

Keywords: *Andrographis paniculata*, *Agrobacterium tumefaciens*, andrographolide, transformed callus, β -glucuronidase gene.

Introduction

Andrographis paniculata (Burm.f.) Wallich ex Ness has long been known as medicinal plant in southern Asia. Extract of this plant has shown pharmaceuticals properties such as anti-inflammatory, antipyretic, antibacterial, hepatoprotective, immunostimulant, antioxidants, anticancerous activity and anti-HIV (Niranjana *et al.*, 2010; Zhang and Tan, 2000; Calabrese *et al.*, 2000, Otake *et al.*, 1995). The primary medicinal constituents of *A. paniculata* that is responsible for the

pharmaceutical properties is andrographolide, a diterpene secondary metabolite.

In general, quantity of the plant secondary metabolites in nature is frequently inconsistent due to wide variation of environmental conditions at different location. This has prompted many scientists to consider the possibility of using *in vitro* plant tissue culture as an alternative sources for secondary metabolites production. Plant tissue culture such as cell culture and root culture have been developed as alternative sources to provide plant-specific valuable metabolites. This approach irrespective to environmental, ecological and climatic conditions. In addition, *in vitro* culture can proliferate the culture at higher growth rates than the whole plant in nature cultivation.

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However, our previous study indicated that production of andrographolide in cell and callus culture of *A. paniculata* demonstrated limited amount, only 0.49% in the callus culture and 0.23% in the cell culture. These amount of andrographolide were lower than that in original leaves plant which contain andrographolide as much as 2.3%. Therefore, it is important to develop an additional technique in plant tissue culture to enhance the secondary metabolite, andrographolide, production.

Several studies reported that many efforts have been tried to enhance production of secondary metabolites *in vitro* such as precursor feeding, elicitation, and cell immobilization. However, limited success was achieved due to the complex regulation in secondary metabolite biosynthesis. On the other hand, several reports indicated that genetically engineered plant cell culture showed a potency for enhancing secondary metabolites production. This approach can be done by modifying the genes related to secondary metabolites biosynthesis in cell cultures. One such modification can be done by introducing a gene of interest into plant cells to increase the activity of specific enzymes which regulate the synthesis of desired secondary metabolites. This may help to increase secondary metabolites production in cell culture (Mulabagal and Tsay, 2004).

Introducing a gene into plant cell can be carried out directly by using particle bombardment or indirectly by using *Agrobacterium tumefaciens* as a vector. Among the available gene transfer systems, *Agrobacterium*-mediated gene transfer is considered as more efficient for stable integration of genes into plant genome (Vasudevan *et al.*, 2007). However, there is little information concerning a genetic modification in *A. paniculata*. Thus, the purpose of this study was to develop genetic transformation method on *A. paniculata* which are mediated by *Agrobacterium tumefaciens* to obtain stable transformed callus. So that, the method can facilitate genetic transformation

of *A. paniculata* generating stable transformant of callus or even whole plant.

Materials and Methods

Plant materials

Andrographis paniculata (Burm.f.) Wallich ex Nees as the source of plant material was obtained from Balai Penelitian Tanaman Obat dan Rempah (Balitro) Bogor, West Java. The leaf explants (second to fourth leaf adjacent to the apical shoot bud) were used as explants for transformation. Intact leaves were surface-sterilized with 1.7% sodium hypochlorite containing 2 drops of Tween 20 for 10-15 min, rinsed four times with sterile distilled water.

Agrobacterium tumefaciens

Agrobacterium tumefaciens strain LBA4404 harboring the binary vector pCAMBIA 1304 was used for transformation experiments. This vector carries the reporter gene for β -glucuronidase (*GUS*) and the selectable marker gene for hygromycin phosphotransferase (*hpt*). For plant transformation, a single colony of the bacteria was inoculated into Luria Bertanie (LB) medium supplemented with kanamycin (25 mg/l) and rifampicillin (50 mg/l) and cultured overnight at 28°C. The *Agrobacterium* cells, approximately 10^6 cell /ml medium ($OD_{600nm} = 0.8$), were collected by centrifugation at 4,000 rpm for 20 min and resuspended in liquid half-strength MS medium containing 100 μ M acetosyringone.

Preculture, transformation and co-cultivation

Leaves from 2-month-old plants were cut into 1x1 cm (as leaf explants) and precultured on MS medium solidified with 0.8% (w/v) agar contained 0.5 μ M 2,4-D and 0.1 μ M BAP for three days at 28°C. Afterwards, the leaf explants were dipped for 60 min in the *A. tumefaciens* suspension which was supplemented with 100 μ M acetosyringone and agitated at 100 rpm. Subsequently, the leaf explants were blotted

on sterile filter paper and co-cultivated for three days in dark at 28°C on agar-solidified (0.8% w/v) MS medium supplemented with 100 µM acetosyringone, 0.5 µM 2,4-D and 0.1 µM BAP.

Selection of transformant and subculture of transformed callus

After 3 days of co-cultivation, explants were washed twice (5 min, per wash) with sterile water containing 400 mg/l cefotaxime, blotted dry on sterile filter paper, and were then transferred on a selection medium (MS supplemented with 0.5 µM 2,4-D, 0.1 µM BAP, 20 mg/l hygromycin and 400 mg/l cefotaxime). After three weeks in the dark at 28°C, they were subcultured on the fresh selection medium without the addition of cefotaxime. The induced transformed callus was maintained on full-strength MS medium with the addition 5.0 µM 2,4-D, 0.1 µM BAP, and 20 mg/l hygromycin for cell proliferation. In order to continue the growth of callus and to obtain a stable transformed callus, the hygromycin resistant-callus was subsequently subcultured on the fresh selection medium at 3-weeks interval for five repeated subculture. During these treatments, only a small proportion of target cell typically received the inserted DNA and only a small proportion of these cells survived and grew to form a cell clones with stably integrated DNA fragments. In the process of subculture, the death tissues (necrosis) were removed, so that only the survived callus on the selection medium was transferred to the fresh medium. The growth of normal and transformed callus was observed by measuring their fresh weight. The collected data were plotted on a graph to obtain a growth curves of normal and transformed callus.

GUS histochemical assay

β-glucuronidase activity was detected by staining the co-cultivated leaf explants and the transformed callus with 5-bromo-4-chloro-3-indol-β-d-glucuronic acid (X-Gluc)

as substrate as described by Jefferson (1987). The staining was performed by reacting the co-cultivated leaf explants and the transformed callus with a mixture of 1 mM X-Gluc, 0.1 mM K₃Fe(CN)₆, 0.1 mM K₄Fe(CN)₆, 50 mM fosfat buffer (NaH₂PO₄/NaHPO₄, pH 7). The reaction was kept over night at 37°C in the dark condition. Following the reaction, the tested samples were soaked with 95% methanol to remove any chlorophyll and others plant pigments. The *GUS* activity was observed visually by formation of blue color spots in the tissue that carries *GUS* gene. In this experiment, the T-DNA of pCAMBIA 1304 contained *GUS* gene with a size 976 bp and CamV35S promoter with a size 1866 bp CamV35S promoter. The *GUS* gene has an intron in the N-terminal region of the coding sequence. This *intron-gus* reporter gene expresses *GUS* activity only in plant cell but not in the cell of *A. tumefaciens* (Ohta *et al.*, 1990 in Hiei *et al.*, 1994). Thus, blue spots staining only appeared on transformed plant tissue but not in cell of *A. tumefaciens* when those samples reacted with 5-bromo-4-chloro-3-indol-β-d-glucuronic acid (X-Gluc) as substrat.

Molecular analysis of transformed callus by PCR

Stable transformation of the callus was confirmed by detection of the *GUS* gene using PCR (Polymerase chain reaction) analysis on callus derived from transformed leaf disks. Putative transformed callus survived on medium containing hygromycin were randomly picked up to analyze whether the *GUS* gene has been inserted into the callus of *A. paniculata*. Total DNA was extracted individually from the callus based on the CTAB method with modification of Lodhi *et al.* (1994). For PCR analysis, the extracted genomic DNA was amplified using specific primers for the *GUS* gene (*forward*: 5' - CAA CGA ACT GAA CTG CAG - 3'; *reverse* : 5' - GGC ACA GCA CAT CAA AGA GA - 3'). The reaction mixture for PCR was incubated in a DNA thermal cycler under the following

conditions: initial denaturation at 94°C for 1 min, followed by 25 cycles of denaturation at 94°C for 30 seconds, annealing at 62°C for 30 seconds, and extensions at 72°C for 3 min, and final extensions at 72°C for 7 minutes. The PCR products were analyzed on 1.5% agarose gel electrophoresis with ethidium bromide and Tris-acetate-EDTA buffer followed by photographed under UV lamp at λ 380 nm.

Result and discussion

Transformation and selection of putative transformed callus

The method of co-cultivation of three days in this experiments showed that the leaf disks could be successfully transformed. This was indicated that when the infected leaf discs cultured on medium containing selection agent (20 mg/l hygromycin) could survive and dedifferentiated to produce callus from the cut edge. The transformed of leaf disks seem became resistance to hygromycin due to the vector of pCAMBIA 1304 that carried by *A. tumefaciens* has transferred the *hpt* gene (for resistance to hygromycin) into the genom of *A. paniculata*. On the other hand, uninfected leaf disks could not survive on the selection medium and eventually died (Figure 1). This results was in agreement with previous study that 1-3 days co-cultivation was the optimal time for higher plant transformation using *A. tumefaciens* (Boyko *et al.*, 2009).

Of the total infected leaf disks (176), as much as 114 (64.4% of frequencies) of the leaf explants produced hygromycin-resistant

callus. This results indicated that the method of infection applied by dipping method and the addition of acetosyringon at the inoculation and co-cultivation steps was highly efficient in the process of T-DNA insertion to the plant cell of *A. paniculata*. Similar findings have been reported earlier by Hong *et al.* (2007), Li *et al.* (2007), and Sharma *et al.* (2009) for the efficiency of dipping method which followed by co-cultivation and the addition of acetosyringone in doing genetic transformation mediated by *Agrobacterium*. The addition of acetosyringone was reported to improve transformation efficiency as acetosyringone could activate the *vir* genes of *Agrobacterium* that were required to initiate the transformation process (Gelvin, 2000).

Growth of the putative transformed callus

The results showed that the growth of transformed callus was inhibited on the medium containing hygromycin but remain growing. This was indicated by slower growth of transformed callus compared to that of untransformed callus on medium without hygromycin (Figure 2). This was suspected that hygromycin caused stress to the growth of transformed callus and may induced secretion a substances such as phenolics that may inhibited the regeneration or proliferation of transformed cell. Consequently, the growth of the transformed callus decreased when compared to non-transformed callus. This results are consistent with the growth of transgenic Potato callus (Turhan, 2004). In

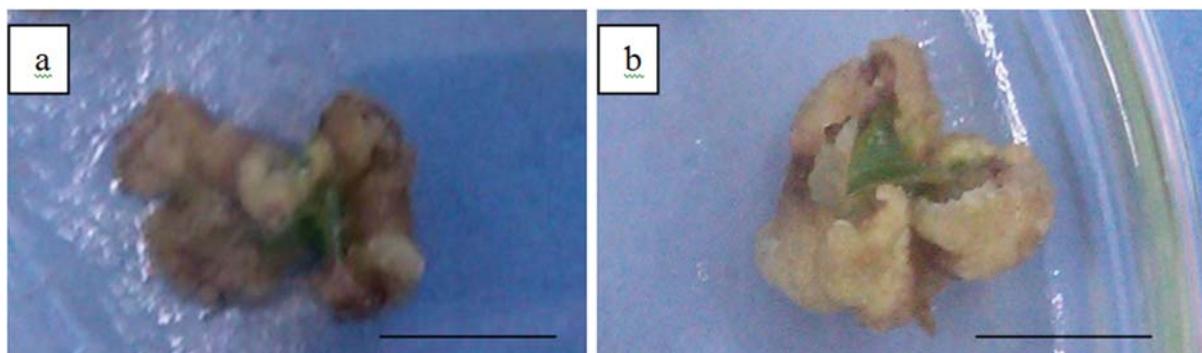


Figure 1. Non-transformed leaf disk (a) and transformed leaf disk which de-differentiate to produce callus (b) on the selection medium containing 2 mg/l hygromycin. Barr =10mm

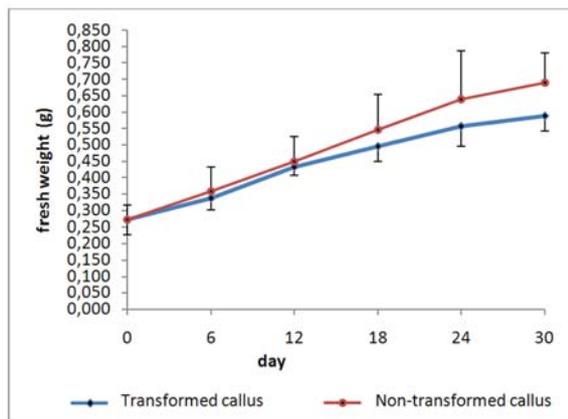


Figure 2. Growth curve of transformed callus on medium containing hygromycin and untransformed callus on medium without hygromycin

contrast, when untransformed callus placed on selection medium containing hygromycin, the untransformed callus could not survive and eventually died. The hygromycin

resistant-callus was subsequently transferred on the selection medium for five repeated subculture. Eventhough the growth of transformed callus decreased but the callus remain growing on subculture-1, 2, 3, 4, and 5 (Figure 3). These results suggest that the *hpt* gene has been inserted into callus, so that the callus has resistance to hygromycin that was contained in the selection medium.

Expression of β -glucuronidase (GUS) in transformed tissue

Detection of β -glucuronidase (*GUS*) expression can be used to indicate both transient and stable transformation (Serres *et al.*, 1997). In present study, *GUS* activity was detected in leaf explants after three days of co-cultivation and in putative transformed callus on the subculture 1,2,3,4, and 5. The co-cultivated leaf explants exhibited *GUS* expression as indicated

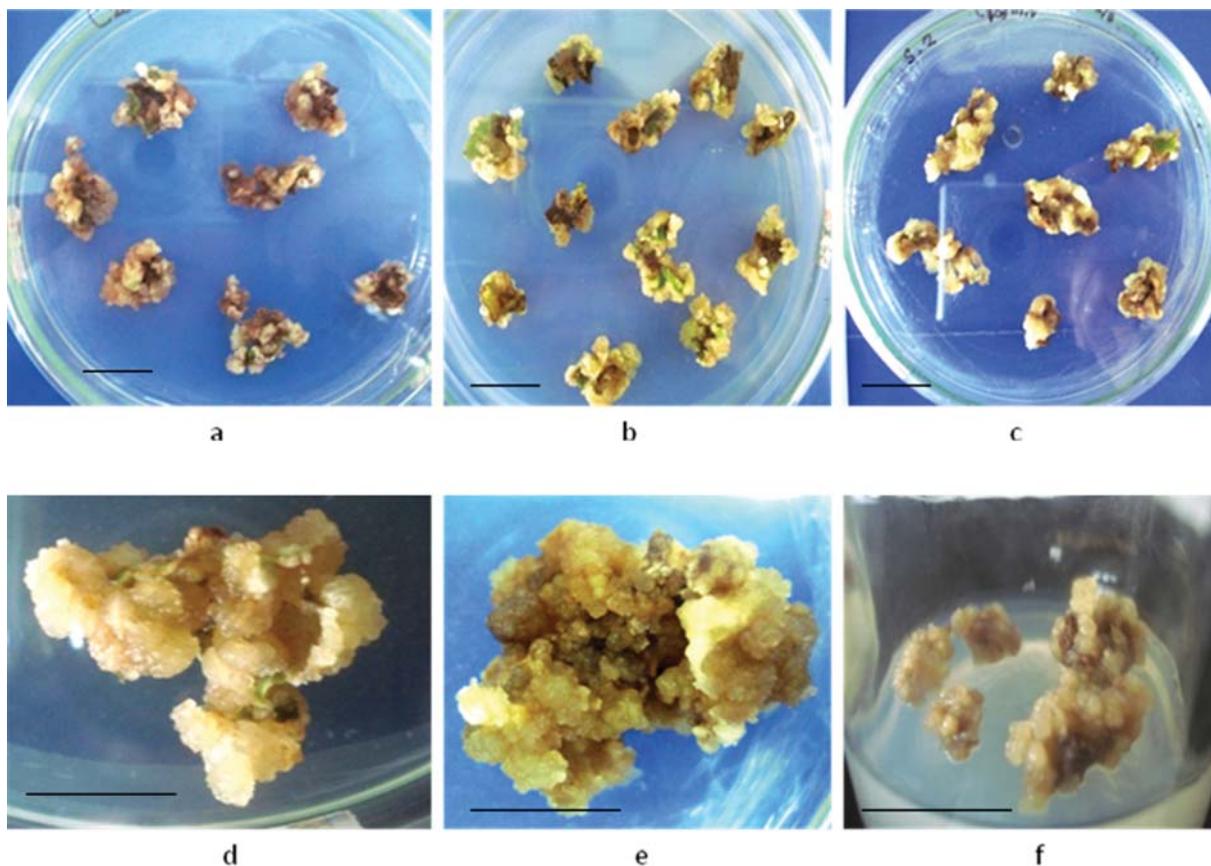


Figure 3. Transformed callus on subcultured-1 (a), subcultured-2 (b), subcultured-3 (c), subcultured-4 (d), subcultured-5 (e), and untransformed callus (f), Bar = 10 mm



Figure 4. Results of *GUS* assay of non-transformed leaf disk (a) and transformed leaf disk (b), Bar = 10 mm

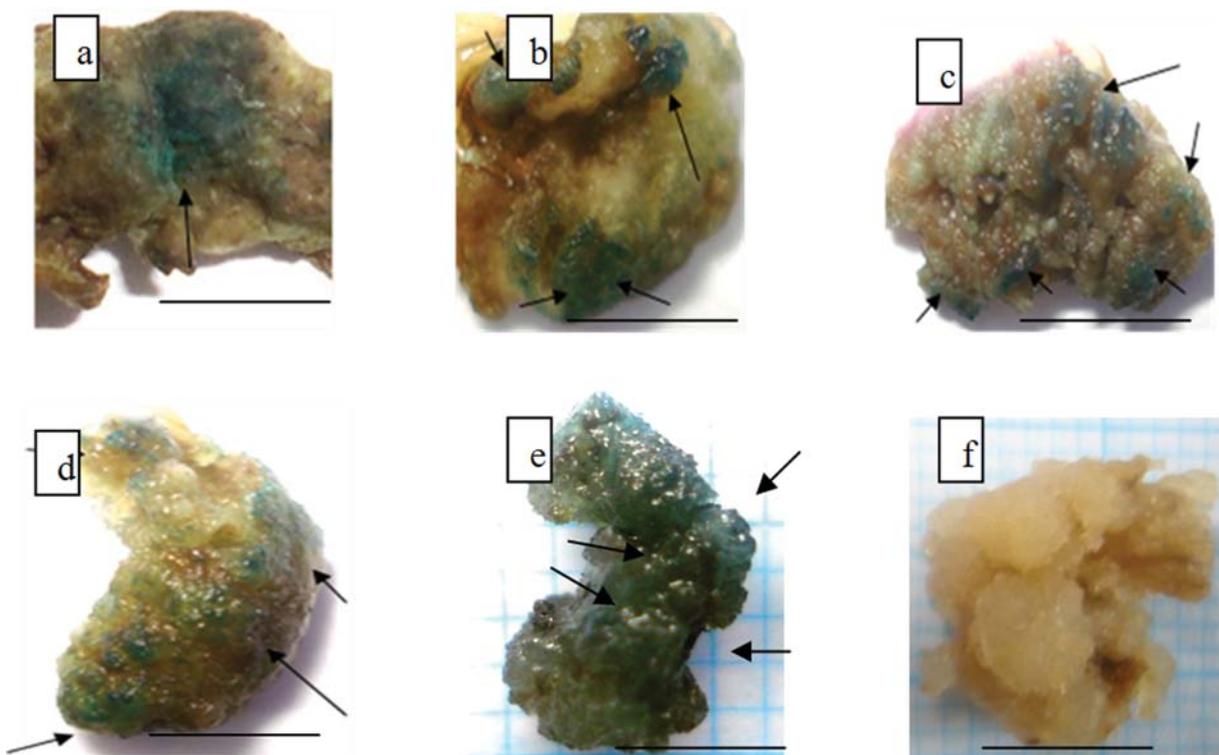


Figure 5. Results of *GUS* assay in transformed callus on subculture 1-5 (a-e) and untransformed callus (f), the arrow indicates *GUS* positive expression, Bar= 10 mm

by blue staining spots on almost entire of surface area of the explants, however *GUS* expression was not detected in non-infected leaf explants (Figure 4). This positive transient expression of *GUS* indicated the efficiency of T-DNA delivery from *A. tumefaciens* into leaf explants of *A. paniculata*.

The results of *GUS* histochemical assay in putative transformed callus on subculture 1,2,3,4, and 5 showed blue staining spots

in many callus, however some of the callus showed unstained areas which suggested that some of the putative transformed callus were chimeric. On the other hand, *GUS* assay for uninfected callus that had been cultured on callus induction medium without hygromycin resulted no blue staining spots (Figure 5). Tripathi *et al.* (2005) reported the same results in the histochemical assay for *GUS* for stable expression in transgenic

leaves of Banana that showed chimeric phenomenon. They thought the cells involved in the bud developments only partially were transformed. This phenomenon was also reported by Hiei *et al.* (1994) that the blue staining spots was not uniform at the hygromycin-resistant cell colonies because of the uneven process of the transformation that occurs.

In this experiment, *GUS* expression in callus on subculture-5 showed the blue staining spots almost uniformly throughout the callus (Figure 5e). This indicates that the cells of transformed callus have been selected by repeated subculture. The non-transformed cells were eliminated but the transformed callus was kept to give rise uniform cells that contain *GUS* gene. Therefore, it is assumed that the growing callus was transgenic callus. To confirm this assumption, molecular analysis of transgene (*GUS* gene) that has been subjected into leaf disks together with *hpt* gene were performed by PCR analysis.

PCR analysis of putative transformed tissue

In this experiment, transformed callus was obtained after being selected on selection medium (MS medium containing hygromycin). The calli on the first to fifth subculture were survive and continuously divided. These suggested that T-DNA from pCAMBIA1304 plasmid has been integrated into the calli. To confirm the presence of T-DNA in the callus, a molecular analysis through PCR amplification has been carried out by detection the *GUS* gene. The results of PCR amplification demonstrated the presence of 976 bp fragment of the *GUS* gene in all putative transformed calli from subculture 1 to 5. On the other hand, corresponding fragment was not detected in non-transformed callus (Figure 5). These results indicated that the T-DNA of pCAMBIA1304 plasmid in *A. tumefaciens* has been integrated into calli of *A. paniculata* on subculture 1,2,3,4, and 5 (approximately

within 15 weeks of culture). Those findings led to the thought that callus of *A. paniculata* has been stably transformed.

This phenomenon seems consistent with the earlier report by Yusibov *et al.* (1994) that explained that the T-DNA can be found in cytoplasm within 30 minutes after co-cultivation, but after two hours of co-cultivation the T-DNA declined because the T-DNA was either degraded or transported into the nucleus and integrated into the genome of plant cell. In this experiment, T-DNA carrying *GUS* gene appeared in transformed callus on subculture 1,2,3,4, and 5 or within 15 weeks of culture. Thus, this work confirmed that callus of *A. paniculata* has successfully been transformed. Similarly, Mc. Cormick *et al.* (1986) reported that through a long selection of putative transformed tissue on selection medium, a stable transformation of the tissue can be obtained. In this investigation, the putative transformed callus demonstrated the presence of *GUS* gene on subculture one to five or within 15 weeks of culture in selection medium. Thus, this results indicated that stable transformation in callus of *A. paniculata* has been obtained through *A. tumefaciens* mediated transformation that carries *GUS* gene in pCAMBIA1304 plasmid.

Conclusion

In conclusion, efficient protocol for genetic transformation of *A. paniculata* mediated by *A. tumefaciens* strain LBA4404 was established to give rise stable transgenic callus. This was indicated by expression of β -glucuronidase (*GUS*) and hygromycin phosphotransferase (*hpt*) genes in transformed callus from first subcultured to fifth subcultured within 15 weeks period. Moreover, molecular analysis through PCR amplification demonstrated the presence *GUS* gene (976 bp fragment) in the transformed callus from first subcultured to fifth subcultured which confirm the stable transformation of callus *A. paniculata*. Therefore, this reliable transformation

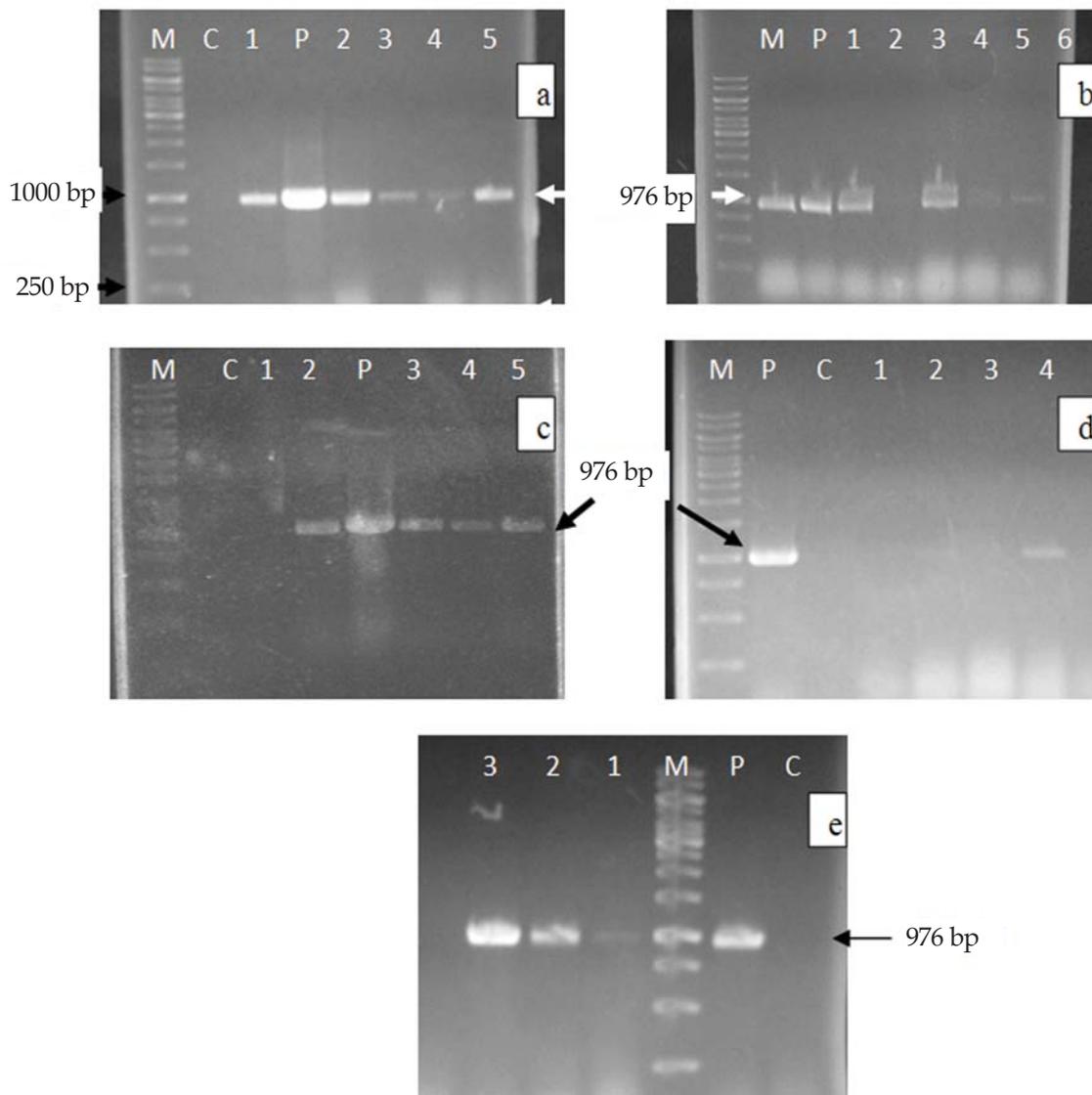


Figure 6. PCR analysis of the genomic DNA from transformed callus using primer amplifying 976 bp fragment of the *GUS* gene that presence in transformed callus (lane 1-6) on subculture 1 to 5 (a-e). Lane M (*molecular weight marker*), C (nontransformed callus) , P (pCAMBIA1304).

protocol using *A. tumefaciens* strain LBA4404 can be applied for transferring any genes of interest into *A. paniculata* tissue to produce transgenic cell, callus and whole plants with a particular characteristics.

Acknowledgement

This work was funded by the Priority Research Program of The Research Center in Biotechnology , Institut Teknologi Bandung, Indonesia.

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