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Agrobacterium tumefaciens-mediated Transformation of Embryogenic Callus and Somatic Embryos of the Banana cv "Ambon Lumut" (*Musa acuminata*)

Tifa R. Kusumastuti[1], Rizkita R. Esyanti[1], and Fenny M. Dwivany[2]

[1]Department of Plant Science and Biotechnology, School of life science and technology, Bandung Institute of Technology, Bandung 40132, West Java, Indonesia
[2]Department of Genetics and Molecular Biotechnology, School of life science and technology, Bandung Institute of Technology, Bandung 40132, West Java, Indonesia

Abstract

Banana is one of the major fruit crops, though its conventional breeding has limitations, such as sterility and high polyploidy levels. Biotechnological approach using genetic transformation crop for improvement offers an alternative solution. In this study a protocol was developed for establishing genetic transformation from embryogenic callus and somatic embryos of the banana cv Ambon Lumut. Embryogenic callus was obtained in ID4 medium (MS-based medium) supplemented with 1 mg L^{-1} IAA, 4 mg L^{-1} 2,4D, and 0.03 g L^{-1} active charcoal. Embryogenic callus was transferred into liquid mediu m to establish somatic embryos. Embryogenic callus and somatic embryos were used for *Agrobacterium tumefaciens*-mediated transformation. *A. tumefaciens* strain A GL1, containing pART-TEST7 p lasmid with gfp gene as a reporter and CaM V35S as a promoter, was used for transformations. The embryogenic callus and somatic embryos were transformed using heat-shock method followed by centrifugation (2000 rpm) and co-cult ivation in liquid medium containing acetosyringone (100 μM) for 3 days. Results of the GFP analysis showed transient expression from *gfp* gene reporter in transformed embryogenic callus and somatic embryos. Transformation efficiency in somatic embryos (85,9%) was higher than that in embryogenic callus (32.09%). PCR analysis using CaMV primer showed bands that compatible with CaMV35S promoter at 507 bp. This is a report showing establisment of embryogenic callus and somatic embryo culture transformation by using *A. tumefaciens*-mediated transformation protocol of the local banana cv Ambon Lumut. This study proved the huge potential for genetic transformation of banana cv Ambon Lumut for crop improvement, such as pest or disease resistance and abiotic factor stress tolerance.

Keywords: banana; embryogenic callus; somatic embryos.

Corresponding Author:
Rizkita R. Esyanti
rizkita@sith.itb.ac.id

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1. Introduction

Banana is one of the most important staple foods in the world. Since 2004, Indonesia supplied about 6% banana in international trades [1]. Data showed that banana production in Indonesia was increasing and reached 5.8 million tonnes in 2010. It was about 30% from national fruit production [2]. Unfortunately, Indonesia still cannot increase its export, due to low quality product for export standard. Conventional breeding to improve bananas quality has limitations, such as sterility of cultivated banana, high polyploidy levels and long generation time to grow new cultivar. In this study, a new method to improve local banana quality using biotechnological approach was developed. Genetic transformation using *Agrobacterium tumefaciens* offers the best solution for crop improvement in banana. Relative success in *A. tumefaciens* genetic transformation had been achieved in other studies using different cultivars such as Agbagba [3, 4], Rasthali, Robusta [5], Grand Naine [6], Cavendish and Lady finger [7] by inserting genes such as *Carica papaya* cystatin (CpCYS), rice chitinase (RCC) [8] β-1,3-glucanase, and Human lysozym (HL) [9].

The purpose of this study was to obtain a protocol for *A. tumefaciens*-mediated transformation in embryogenic callus and somatic embryos of banana cv. Ambon Lumut that had never been reported elsewhere. Embryogenic callus and somatic embryos were chosen as the subjects in this study. Embryogenic callus had been reported for its highly merismatic cells that could increase transformation efficiency [10]. Somatic embryos have been reported for their advantages in transformation, such as potential to develop more transformed plants via secondary somatic embryogenesis protocol (SE2). Secondary somatic embryogenesis is a process of induction of new somatic embryos from existing embryos, and since new embryos are continually formed from existing embryos, this method has the potential to produce many plants [11].Somatic embryos start from unicellular origin, which make them excellent candidate for genetic transformation since the potential for production of chimeric plants is low [12]. Somatic embryos have the potential to regenerate a large number of plants without passing through cell suspension that requires long culture times from culture initiation to plant regeneration [11, 13].

Plasmid pART-TEST7 used in this study contained *gfp* reporter gene, *nptII* selectable marker gene and CaMV35S promoter. CaMV35S was promoter that derived from cauliflower mosaic virus (CaMV), a specified promoter for plants. The CaMV35S promoter is widely used to drive transgene expression since it has been shown to be active in a wide range of tissue and in many crop species. The CaMV promoter is preferred because it is a more powerful promoter than others and is not greatly

influenced by environmental conditions or tissue types. CaMV has two promoters, 19S and 35S, of these two the 35S promoter is more frequently used in biotechnology because it is most powerful [14, 15].

The use of the green fluorescent protein (GFP) from *Aequorea victoria* as a vital marker has attracted interest and has been described in different organisms. GFP is a 27 kDa protein that fluorescent green when excited by ultraviolet or blue light. The GFP has several advantages over other visual reporter genes such as β-glucuronidase (GUS), β-galactosidase (LacZ) and luciferase (LUC). One of important advantages is that GFP does not require the addition of a substrate or cofactors for fluorescence to be detected. On the contrary, other reporter genes require either the addition of exogenous substrates or co-factors, and their applications are limited because the assays are generally destructive. This makes GFP the best choice for reporter gene if the explants or samples are limited [16, 17].

In this study, protocol was developed for transformation in liquid co-cultivation medium of banana local cultivar Ambon Lumut, detected by using transient expression of *gfp* gene reporter in both embryogenic callus and somatic embryos of banana local cultivar Ambon Lumut.

2. Materials and Method

2.1. Materials

2.1.1. pART-TEST7 plasmid

pART-TEST7 plasmid used in this study was acquired from Queensland University of Technology (QUT), Queensland, Australia. The plasmid has reporter gene *gfp,* selectable marker gene *nptII* (*neomycin phospotransferase*) and promoter CaMV35S (Fig 1.).

2.1.2. Banana inflorescens male flowers

Banana inflorescence male flowers were obtained from local farmers in Bandung, West Java Indonesia.

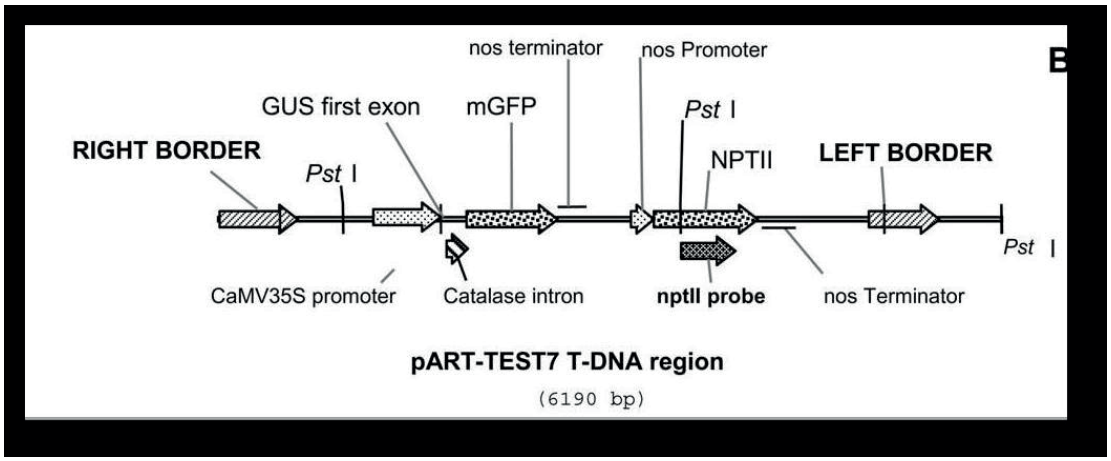

Figure 1: Schematic diagram of pART -T EST 7 plasmid T -DNA region.

2.1.3. *A. tumefaciens* strain AGL1

A. tumefaciens bacterium strain AGL1 were obtained from Genetics and Molecular Laboratory, School of life science and technology, Bandung Institute of Technology.

2.2. Methods

2.2.1. Plant materials and culture initiation

Male inflorescence bud was prepared by removing the bracts and the hands of the male flower. The buds disinfected with NaOCl, rinsed three times with sterile distilled water and further reduced in size to tips that were about, 5.0 cm long × 1.0 cm wide, in a laminar flow hood. The explants, the hands of immature male flowers (IMFs), were isolated aseptically from the buds by removing the bracts under a binocular stereomicroscope. The immature flower hands were isolated from positions 15 to 3 (1^{st} being the hand closest to the meristematic dome of the male bud) each bud were cultured in callus induction medium NID4 (MS Macronutrient 1x, MS Micronutrient 1x, MS Fe-EDTA 1x, Sucrose 30 gL^{-1}, Activated charcoal 0.03 g/ L, NAA 1 mg L^{-1}, IAA mg L^{-1}, 2,4-D 4 mg L^{-1}, Agar 8 g L^{-1}, pH 5.7-5.8). The cultures were kept in a growth room at 27 ± 2°C under total darkness and sub-cultured every four weeks. Non embryogenic callus emerged from explants after three times sub-cultured using NID4 medium. Non embryogenic callus were then transferred into ID4 medium (M S Macronutrient 1x, MS Micronutrient 1x, MS Fe -EDTA 1x, Sucrose 30 g L^{-1}, Activated charcoal 0.03 g/ L, IAA 1 mg L^{-1}, 2,4-D 4 mg L^{-1}, Agar 8 gL^{-1}, pH 5.7-5.8), kept in a growth room at 27 ± 2°C under total darkness and sub-cultured every four weeks to produce embryogenic callus.

Somatic embryos for transformation were obtained by transferring embryogenic callus into 100 mL erlenmayer flask containing 20 ml liquid medium ME (MS Macronutrient 1x, MS Micronutrient 1x, MS Fe-EDTA 1x, Sucrose 20 gL^{-1}, Activated charcoal 0.05 g L^{-1}, NAA 0.1 mg L^{-1}, BAP 1 mg L^{-1}, pH 5.7-5.8). The cultures were agitated on an orb ital shaker at 90 rpm in a growth room maintained at 27 ± 2°C in total darkness and sub cultured every two weeks until somatic embryos dominant in globular shape were obtained.

2.2.2. Transformation and confirmation of pART TEST7 plasmids in *A. tumefaciens* AGL1.

pART-TEST7 plasmid transformation was performed on competent A. tumefaciens strain AGL1. Competent A tumefaciens AGL1 cells were made using CaCl method and A. tumefaciens plasmid was carried out using heat-shock method [18]. The existence of pART-TEST7 plasmid in A. tumefaciens AGL1 was confirmed by crude PCR method using the pair of CaMV primer (primer forward 5'-CCTAACAGAACTCGCCGTAAAGA-3'and reverse 5'-CCCGTGTTCTCTCCAAATGAAATG-3'). Amplification of CaMV35S promoter was started with the 3 minutes initial denaturation at 95 °C temperature, then performed 25 cycles consisting of 30 seconds at a temperature of 95 °C for denaturation, 30 seconds at 60 °C for annealing, and two minutes at 72 °C for elongation. Final elongation process was carried out for 7 minutes at a temperature of 72 °C. DNA fragments from PCR products were separated using gel electrophoresis on 1% agarose concentration in TAE buffer solution.

2.2.3. *A. tumefaciens* bacteria culture preparation

A. tumefaciens A GL1 culture containing plasmid pART-TEST7 was grown in YEP solid medium (Yeast Extract 10 g/L, Pepton 10 g/ L, NaCl 5 g/ L, Agar bacto 1.5% (b/v), pH 7) containing antibiotics 100 mg L^{-1} ampicillin, 50 mg L^{-1} kanamycin, and 50 mg L^{-1} rifampicin. The bacteria were then incubated for 3 days at a temperature of 28°C in dark conditions. One colony of the solid culture was isolated using a loop and grown in 10 ml of liquid YEP medium (Yeast Extract 10 g/ L, Pepton 10 g/L, NaCl 5 g/L, p H 7) containing antibiotics (100 mg L^{-1} ampicillin, 50 mg L^{-1} kanamycin and 50 mg L^{-1} rifampicin). Cultures were grown at a temperature of 28°C, with agitation of 130 rp m and incubated in the dark for 48 hours. A total of 2 mL of the culture solution was transferred into a new liquid YEP containing antibiotics (100 mg L^{-1} ampicillin, 50 mg L^{-1} kanamycin, and 50 mg L^{-1} rifampicin) and made to volume of 45 ml (liquid YEP +

culture). Cultures were grown at 28°C, for ±10 hours, with agitation of 100 rpm to reach OD600nm = 1.0.

Bacterial culture was transferred into 50 mL falcon tube, then it was centrifuged at a speed of 5000 rpm for 10 minutes. The remaining pellets were resuspended using 15 mL of media BRM (MS Macronutrient 1/10x, MS Micronutrient 1/10x, MS Vitamin 10 mL/ L, Sucrose 85.5 g/L, Thiamine 1125 µL/ L, Cysteine 500 mg L^{-1}, Glucose 45 g/L, pH 5.3) with the addition of 100 µM incubated acetocyringone for 2 hours at a temperature of 25 °C in dark conditions with 90 rpm agitation [19].

2.2.4. Embryogenic callus culture and somatic embryos banana Ambon Lumut preparation

Embryogenic callus was sub-cultured 3 weeks into the ID4 medium prior to transformation. Embryos for transformation were somatic embryos that had been sub-cultured one week earlier into liquid MS medium (MS Macronutrient 1x, M S Micronutrient 1x, MS Fe -EDTA 1x, Sucrose 20 g/ L, Activated charcoal 0.05 g/L, p H 5.7-5.8). Embryogenic callus and somatic embryos were transferred into 50 mL falcon tube aseptically, liquid MS medium was added until cultures were submerged. Embryogenic callus and somatic embryos were then given a heat-shock treatment for 5 minutes at a temperature of 45-48 °C [7, 19].

2.2.5. Transformation and co-cultivation

Embryogenic callus and somatic embryos were inoculated with A. tumefaciens culture (OD600nm = 1.0). A total of 0.02% F68 pluoronic acid was added as a surfactant. Embryogenic callus and somatic embryos were inoculated with A. tumefaciens followed by centrifugation at 2000 rp m for 10 minutes, homogenized using a vortex for 20 seconds, and then rested for 30 minutes [7]. Embryogenic callus and somatic embryos were then transferred into the liquid medium for co-cultivation (MS Macronutrien 1x, MS Micronutrien 1x, MS Fe-EDTA 1x, Sucrose 20 g/ L), and incubated for 3 days at a temperature of 23 °C in dark conditions.

2.2.6. Decontamination and recovery

Culture was removed aseptically into the falcon tubes containing sterile distilled water, so that all parts of the culture were submerged. Falcon tube was agitated while occasionally homogenized by vortex for one minute, distilled water was then discarded.

The culture was added with washing liquid MS medium, which had been added with 200 mg L^{-1} timentin. Falcon tube was agitated at a speed of 90 rpm for 15 minutes while occasionally homogenized by vortex. The entire wash step was repeated 3 times.

2.2.7. Observations of gfp reporter gene expression

Observations of gfp reporter gene expression was performed using a microscope fluorescents Nikon Eclipse E-800. Callus and embryos transformant as well as non-transformant were placed on the glass object and observed using fluorescence microscopy with ultraviolet light (UV) at a wavelength of 450-490 nm. Parts that emitted green luminescence during observation indicated the presence of gfp reporter gene that was expressed in transformed culture.

2.2.8. Estimation percentage of GFP expression and transformation efficiency in transformed embryogenic callus and somatic embryos

Estimation percentage of GFP expression and transformation efficiency in transformed embryogenic callus and somatic embryos was carried using Adobe Photoshop CS2 software. Transformed callus and somatic embryos pictures were viewed using Adobe Photoshop CS2, part that emitted green luminescence and non emitted green luminescence were counted using grid layer. Results were calculated using formula below:

$$\text{Estimation of percentage of GFP expression and transformation efficiency} = \frac{\text{Total grid in transformed culture emitted green luminescence}}{\text{Total all grid in transformed culture}} \times 100\%$$

2.2.9. CaMV 35S promoter molecular PCR testing

Isolation of transformed DNA was performed using Tiangen Plant Genomic DNA Kit. DNA of embryogenic callus and somatic embryos were confirmed by PCR using the pair of primer (forward 5'-CCTAACAGAACTCGCCGTAAAGA-3' and reverse 5'-CCCGTGTTCTCTCCAAATGAAATG-3'). Amplification of CaMV35S promoter was started with the 3 minutes initial denaturation at 95 °C temperature, then performed 25 cycles consisting of 30 seconds at a temperature of 95 °C for denaturation, 30 seconds at 60 °C for annealing, and two minutes at 72 °C for elongation. Final elongation process was carried out for 7 minutes at a temperature of 72 °C. DNA fragment s of PCR products were separated using gel electrophoresis on 1% agarose concentration in TAE buffer solution.

3. Result and Discussion

3.1. Embryogenic callus and somatic embryos banana ambon lumut initiation

The calluses were initiated from explants of immature male flowers of banana cv. Ambon Lumut. Male flowers had been widely used as explants in somatic embryogenesis in bananas to obtain a culture of embryogenic callus, somatic embryos [13] and embryogenic cell suspension culture [6, 7, 21]. The immature male flowers of banana was selected because in theory the cells were still meristematic, easily proliferated and had a high ability to divide. The closer the position of the male flowers to the meristem, the higher the ability to divide and so embryogenic callus would be easily obtained [20].

Callus obtained in laboratory, had a blackish color, hard and non embryogenic (Figure 2.A) Callus were then sub-cultured into ID4 medium every 4 weeks to obtain embryogenic callus which was white, round with a smooth surface (Fig 2.B) as common characteristic of embryogenic callus [21]. The use of auxin in the medium was to increase cell proliferation and induction of embryogenic callus. Auxin particularly 2,4-D and IAA played a role in the initiation of somatic embryogenesis in bananas [6, 21, 23], carrots [24], alfalfa [25] and chrysanthemum [26].

Embriogenic callus was transferred into liquid medium ME. Use of liquid medium for somatic embryo initiation has been used in several study, such as the banana cultivar 'Rasthali' [27], 'Dwarf Brazilian' [13], and the cultivar 'Mas' [21]. In the liquid medium, proembryos cells that form nodules on the callus were separated into single proembryos which had a yellowish white color (Fig 2C). Single proembryos was transferred into a liquid medium MS. In the liquid medium not all proembryos formed globular embryos at the same time, which might be due to several conditions, including media composition, source of explants, and genetic factors [13]. Somatic embryo culture were homogenized by stages, this protocol had been done by Fujimura and Komamine [28]on carrot embryogenic cell cultures to produce a uniform development of the embryo in a culture.

Embrogenic callus and somatic embryos were treated with heat-shock for 5 minutes at 45-48°C. Heat-shock techniques performed on callus culture and embryo were expected to disturb the balance of the cell membrane, metabolism and other physiological processes in the cell [29, 30]. This treatment could induce cells response to stress in the form of HspL namely small crystalline alpha-type heat-shock protein (Hsp-α). In the process of transformation, HspL was required for VirB protein accumulation,

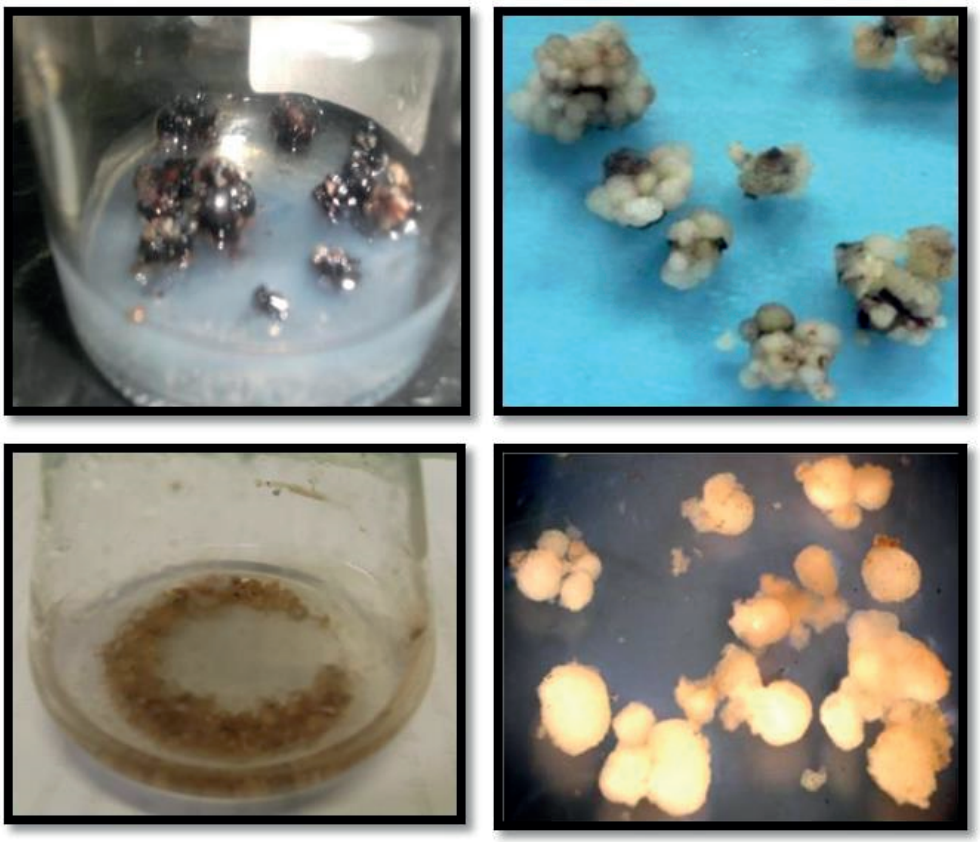

Figure 2: Embryogenic callus and somatic embryos of Ambon Lumut. (A) non embryogenic callus; (B) embryogenic callus on petri dish; (C) somatic embryos culture in liquid medium; (D) somatic embryos observed under stereo microscope.

which allegedly played an important role in the transfer of T-DNA by VirB / D4 [31]. Treatment of heat-shock had previously been successful in increasing the efficiency of transformation in embryogenic cell cultures of banana cultivar 'Cavendish' and 'Lady Finger' [7], somatic embryos of bananas 'Cavendish' [19] and the culture of ryegrass and rice embryogenic callus [31].

3.2. Confirmation of plasmid part-test7 in *A. tumefaciens* strain AGL1

Agrobacterium tumefaciens strain AGL1 were grown in YEP medium that contained antibiotics (100 mgL^{-1} ampicillin, 50 mgL^{-1} kanamycin, and 50 mgL^{-1} rifampicin) (Fig 3).

Confirmation of the presence of plasmid pART-TEST7 was conducted through crude PCR method using a pair of CaMV primers. Visualization of electrophoresis results was presented in Fig 4 showed colonies of *A. tumefaciens* strain containing plasmid positive AGL1 pART-TEST7. The result of *A. tumefaciens* strain AGL1 amplification using primers

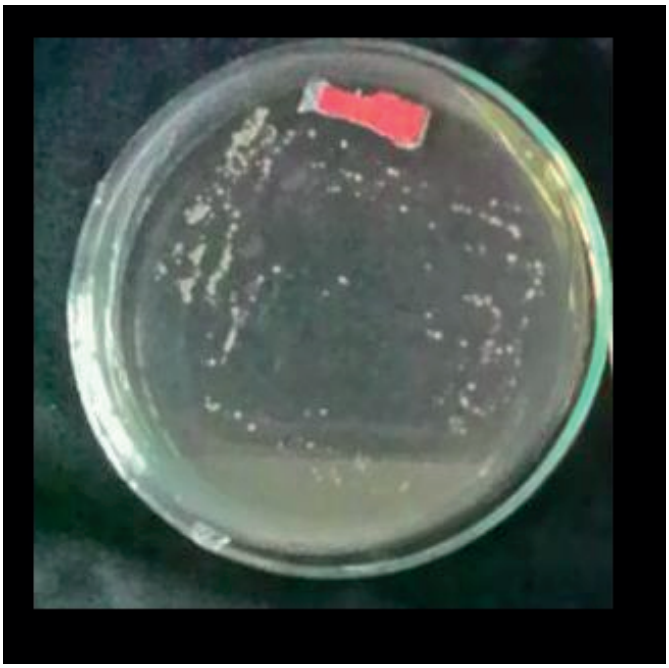

Figure 3: *A. tumefaciens* strain AGL1 colonies harbouring plasmid pART-TEST7 cultured in solid YEP medium contained antibiotics.

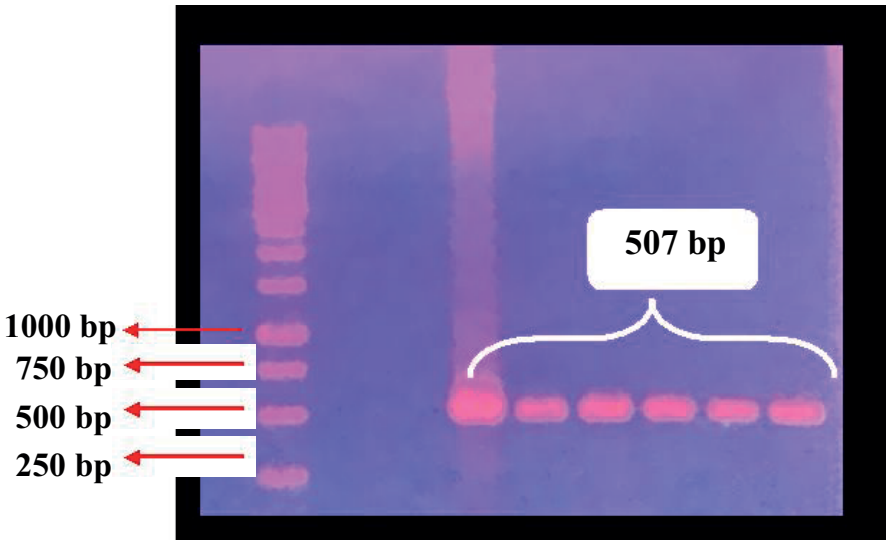

Figure 4: PCR confirmation of pART-TEST7 plasmid in *A. tumefaciens* AGL1. L: DNA Ladder 1 kb (FERMENTAS); (co+) positive control plasmid pART-TEST7; (1-5) *A. tumefaciens* strain AGL1 colonies contained plasmid pART-TEST7.

CaMV against CaMV35S promoter in the plasmid pART-TEST7 produced bands of 507 bp which is corresponded to the positive control (plasmid pART-TEST7).

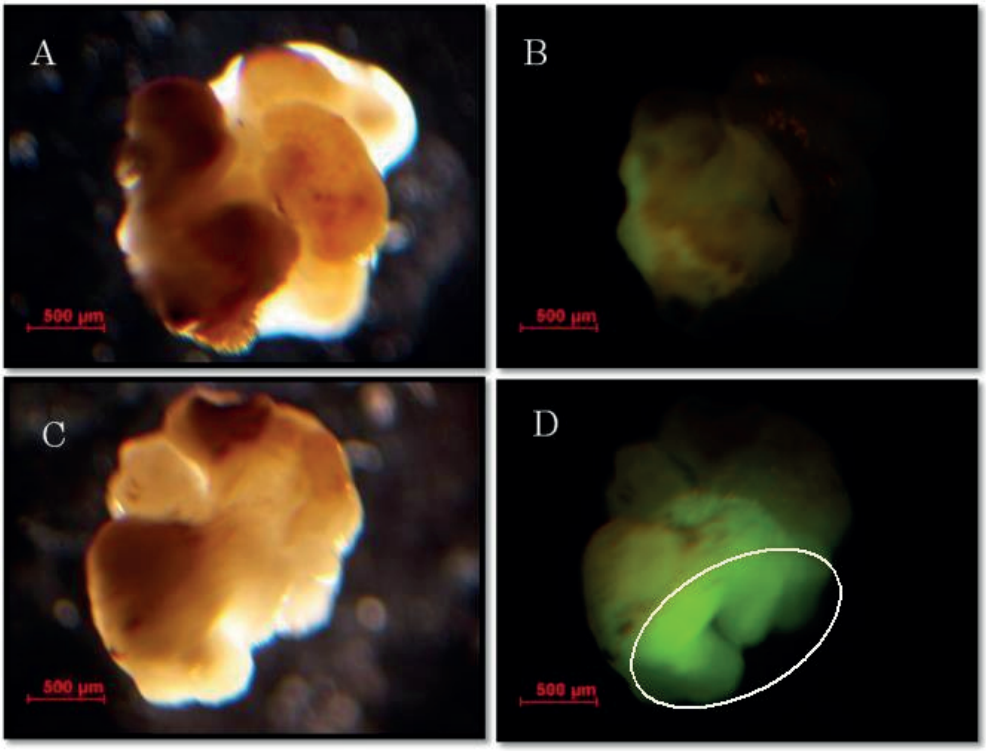

Figure 5: Observation of GFP on embryogenic callus of banana cultivar Ambon Lumut;(A-B) non-transformed embryogenic callus (C-D) embryogenic callus transformed by *A. tumefaciens* strain AGL1/pART-TEST7.

3.3. Observations of gfp reporter gene expression in embryogenic callus and somatic embryos banana Ambon Lumut

Observations conducted using the fluorescence microscope under UV light with a wavelength of 450-490 nm indicated the success of the transformation in embryogenic callus culture (Fig 5.D) and somatic embryos (Fig 6.D). The positive result of the transformation was shown by green luminescens from GFP protein, which was the result from transient expression of the *gfp* reporter gene expression in transformed embryogenic callus and somatic embryos, *gfp* gene is a gene that encodes a protein green fluorescence, when the gene is expressed it will produce a glowing green in parts of transformed plants or culture. GFP expression had been successfully used to test the success of transformation, including the embryogenic callus of sweet orange (*Citrus sinensis* (L.) Osbeck) [32] and somatic embryos of grapes (*Vitis vinifera* L. cv.Thompson Seedless) [33].

Successful transformant part in expressing GFP protein p roved the potential of CaMV35S promoter to be used as a promoter in transformation banana cultivars Ambon Lumut. Transient expression from reporter gene could be used to estimate the promoter strength. Strong activity from promoter can lead to strong expression of reporter

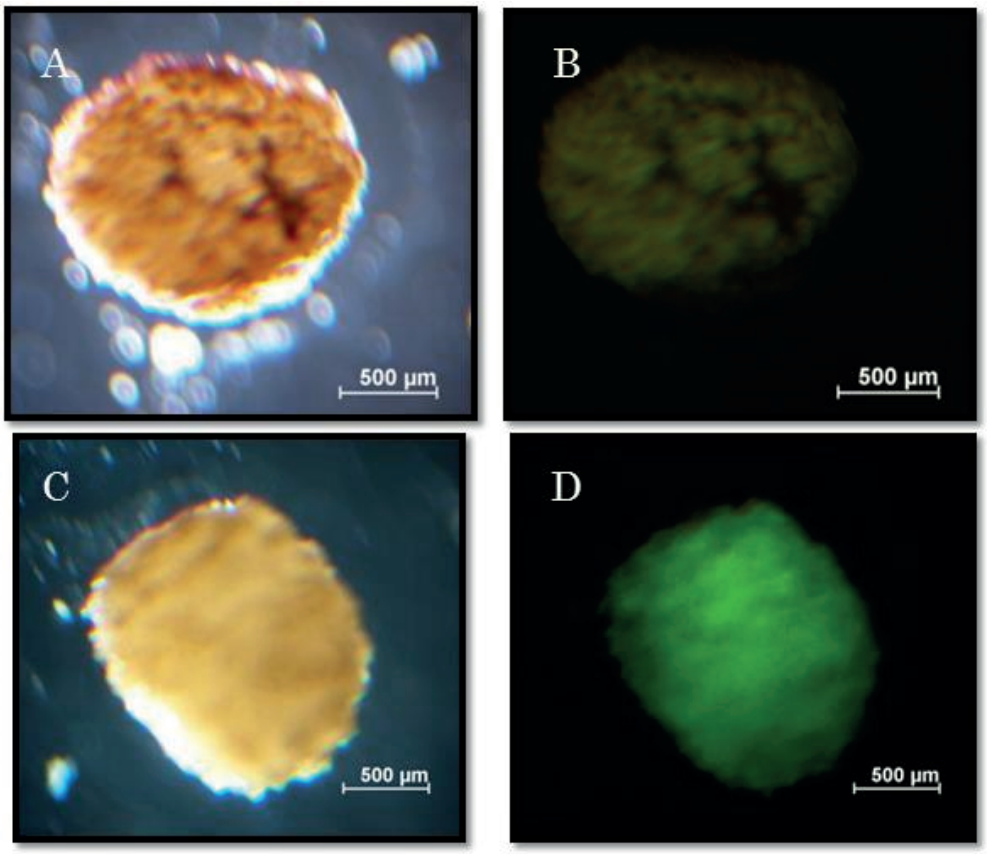

Figure 6: Observation of GFP on somatic embryos of banana cultivar Ambon Lumut; (A-B) non-transformed somatic embryo (C-D) somatic embryo transformed by *A. tumefaciens* strain AGL1/pART-TEST 7.

gene. Promoter activity was one of the key factors that included in gene expression regulation. Other research of transformation in grape somatic embryos using the promoter double cauliflower mosaic virus 35S (CaMV35S) and double cassava vein mosaic virus (CsVMV) produced transgenic plant expressing a transgene which last up to 5 years [33]. GFP expression was stable until the plant survived and regenerated and it expressed in various tissues and organs of the plant (buds, leaves, roots, petal, stamen), even in flowers, fruits and seeds [16].

3.4. Estimation of percentage areas of GFP expression and transformation efficiency in embryogenic callus and somatic embryos of Ambon Lumut banana

Estimation of percentage area that expressed GFP in transformed somatic embryos was higher than embryogenic callus. Estimate number area that emitted green luminescence in somatic embryos tissue was about 85,9% per embryos, while transformed embryogenic callus was about 32,09% per callus (Table 1). These result showed that

Table 1: Percentage of area expressed GFP in transformed somatic embryos and embryogenic callus.

Name	Total transformed culture culture tested	Average total area per culture	Average area emitted green luminescens per culture	Percentage of GFP expression and transformation efficiency
Somatic Embryos	120	1136	976	85,9%
Embryogenic Callus	120	1296	416	32,09%

Figure 7: Result of DNA isolation from embryogenic callus and somatic embryos of banana cultivar Ambon Lumut. (L) DNA Ladder 1 kb FERMENT AS, (E+) transformed somatic embryos; (K+) transformed embryogenic callus; (E-) non transformed somatic embryos; (K-) non transformed embryogenic callus.

transformation efficiency and GFP expression in somatic embryos was higher than embryogenic callus.

3.5. Molecular PCR testing on embryogenic callus and somatic embryos banana Ambon Lumut

Samples of embryogenic callus and somatic embryos (transformed and non- transformed) were isolated using Tiangen Plant Genomic DNA Kit, specifically for the isolation of DNA geno mic of plants. This kit uses silica membrane technology to eliminate complicate stages in the process of DNA isolation. Especially, if the sample contains polyphenols, resins, or other metabolites [34, 35]. Isolated DNA (Fig 7) showed the DNA bands that were faint, but clean of impurities. This result was used for further testing to confirm existence of pART-TEST7 plasmid using PCR method.

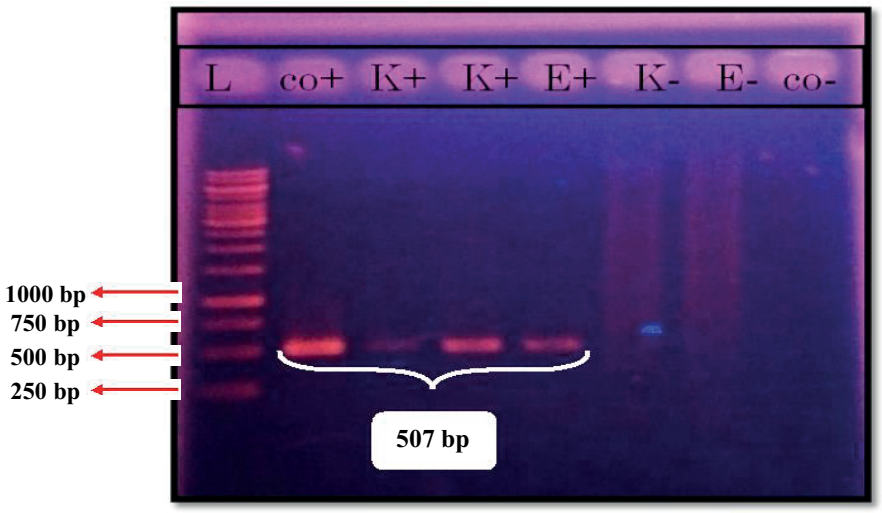

Figure 8: PCR plasmid pART-TEST7 confirmation result in embryogenic callus and somatic embryos of Ambon Lumut bananas. (L) DNA Ladder 1 kb FERMENTAS, (co+) positive control primer CaMV/ pART - TEST7; (K+) transformed embryogenic callus; (E+) transformed somatic embryos; (K-) non transformed embryogenic callus; (E-) non transformed somatic embryos; (co-) negative control H_2O.

Confirmation presence of the plasmid pART-TEST7 in embryogenic callus and somatic embryos was done using the PCR method with CaMV primer.Amplification result using CaMV primer against CaMV35S promotercontained in plasmid pART-TEST7 showed bands at 507 bp corresponded to the positive control (plasmid pART-TEST7). These bands were not found in non transformed embryogenic callus and somatic embryos (K-and E-) as well as negative control using water (H20) (Fig 8).

The use of surfactants and the addition of acetocyringone compounds can improve the efficiency of transformation in plants. Surfactants such as Pluronic acid F68 used in this study were added at transformation. The use of surfactant serves to facilitate the attachment of bacteria *A. tumefaciens* cultures by reducing the surface tension of the solution. The addition of surfactant can also eliminate other compounds that inhibit the attachment of bacteria to the plant cell [10, 36].

Addition of exogenous compounds such acetosyringone, especially for monocotyledonous plants were proven to improve the efficiency of transformation. Acetosyringone can activate VirA protein located on the cell surface of membrane, and trigger activation of other *vir g*enes [36]. Acetosyringone compounds were commonly p roduced in dicotyledonous plants, but only a small portion of monocotyledonous plants produce these compounds. Monocotyledonous plants, such as bananas require administration of exogenous acetosyringone [37]. Acetosyringone should be added in optimum concentration, since acetosyringone compounds could effectively activate the *vir* genes at the minimum concentration, but resulted in bacteriostatic when used at high concentrations [37].

The transformation protocol had been used by Khanna et al., [8] and Apriyani [19], but it was slightly modified in this study. A liquid medium to improve the efficiency of transformation was used in co-cultivation step. This protocol was also used in other transformation using different types of media on the leaves of tobacco plants. The efficiency of transformation in tobacco leaf using a liquid medium during co–cultivation was higher when compared with tobacco leaves that were co-cultivated on solid media [39]. This protocol can be developed further as the protocol of transformation for the local banana cultivars Ambon Lumut to produce stable banana plant transformation, because liquid media was used in the process of transformation and co-cultivation. This step could enhance culture contact with the bacteria in the transformation process and minimizing stress to the culture due to the media changes. The use of liquid media could also reduce browning and culture cell death caused by overgrowth of *A. tumefaciens* that co-cultivated in solid or semi-solid medium [40].

4. Conclusion

In this present study, protocol for transformation was obtained for embryogenic callus and somatic embryos of banana local cultivar Ambon Lumut that never been reported elsewhere. Protocol for banana cultivar Ambon Lumut plant regeneration via indirect somatic embryogenesis (data not shown) was also obtained. This report showed potential of CaMV35S promoter and *gfp* gene reporter for transformation in local cultivar banana Ambon Lumut. This report also proved potential of local cultivar banana Ambon Lumut for crop improvement using *A. tumefaciens*-mediated genetic transformation and a step towards stable transformation in transgenic banana local cultivars.

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References

[1] Smith, S., 2010. Fairtrade Bananas: A Global Assesment of Impact. Institute of Development Studies. University of Sussex. United Kingdom

[2] Direktorat Budidaya dan Pascapanen Buah., 2012. Pedoman Penanganan Pascapanen Pisang. Direktorat Budidaya dan Pascapanen Buah. Kementerian Pertanian. Jakarta.

[3] Tripathi, J.N., Muwonge, A., and Tripathi, L., 2012. Efficient regeneration and transformation of plantain cv."Gonja manjaya" (Musa spp. AAB) using embryogenic cell suspensions. In Vitro Cell.Dev.Biol. 48 (2), 216-224.

[4] Tripathi, L., Tripathi, J.N., and Hughes, J.d'A., 2012. Agrobacterium transformation of plantain (Musa spp.) cultivar Agbagba. African Journal of Biotechnology. 4 (12), 1378-1383

[5] Ghosh, A., Ganapathi, T. R., Nath, P., and Bapat, V. A., 2009. Establishment of embryogenic cell suspension cultures and Agrobacterium-mediated transformation in an important Cavendish banana cv. Robusta (AAA). Plant Cell T iss Organ Culture 97, 131–139.

[6] Sutherland, R., 2006. Genetic modification of Cavendish bananas (Musa spp) in South Africa. Thesis. Faculty of Nature and Agricultural Science. University of Pretoria. Pretoria. South Africa.

[7] Khanna, H., Becker, D., Kleidon, J., and Dale, J., 2004. Centrifugation Assisted Agrobacterium tumefaciens mediated Transformation (CAAT) of embryogenic cell suspensions of banana (Musa spp. Cavendish AAA and Lady Finger AAB). Molecular Breeding. 14, 239–252.

[8] Sreeramanan, S., Maziah, M., and Xavier, R., 2009. A protocol for Agrobacterium-mediated transformation of banana with a rice chitinase gene. Emirates Journal Food Agriculture. 21 (2), 18-33.

[9] Pei, X. W., Chen, S. K., Wen, R. M., Ye, S., Huang, J. Q., and Zhang, Y. Q., 2005. Creation of transgenic bananas expressing human

[10] Deo, P. C., Tyagi, A. P., T aylor, M., Harding, R., and Becker, D., 2010. Factors affecting somatic embryogenesis and transformation in modern plant breeding. The South Pacific Journal of Natural and Applied Sciences. 28, 27-40.

[11] Sidha, M., Suprasanna, P., Bapat, V.A., Kulkarni, U.G., and Shinde, B. N., 2006. Developing somatic embryogenic culture system and plant regeration in banana. BARC Newsletter. Founder's day issue special Issue. 285, 153-161.

[12] Escalant, J.V., Teisson, C., and Côte, F., 1994. Amplified somatic embryogenesis from male flower of triploid banana and plantain cultivars (Musa sp.). In Vitro Cell Dev Biol 30:181–186

[13] Khalil, S. M., Cheah, K. T., Perez, E. A., and Gaskill, D. A., 2002. Regeration of banana (Musa spp. AAB cv.Dwarf Brazilian) via secondary somatic embryogenesis. Plant Cell Report. 20, 1128-1134.

[14] Steinbrecher, R.A., 2002. The CaMV 35S Promoter Government and Corporate Scientific Incompetence: Failure to assess the safety of GM crops. http://www.econexus.info Accsessed 22 July 2015.

[15] Zannati, A., 2009. "Sang penyelenggara" itu disebut promotor. BioTrends. 4 (1), 31-34.

[16] Pérez-Clemente, R. M., Pérez-Sanjuán, A., García-Férriz, L., Beltrán, J. P., and Cañas, L. A., 2004. Transgenic peach plants (Prunus persica L.) produced by genetic transformation of embryo sections using the green fluorescent protein (GFP) as an in vivo marker. Molecular Breeding. 14, 419–427.

[17] El-Shemy, H.A., Khalafalla, M.M., and Ishimoto, M., 2008. The role of Green Fluorescent Protein (GFP) in transgenic plants to reduce gene silencing phenomena. Curr.Issues Mol. Biol. 11 (Suppl. 1), 21–28.

[18] Hofgen, R., and Millmitzer, L., 1988. Storage of competent cells for Agrobacterium transformation. Nucleic Acids Research. 16 (20), 9877.

[19] Apriyani, R. K., 2012. Efisiensi transformasi pada kultur embrio pisang Cavendish (Musa acuminata colla (AAA Group)) melalui Agrobacterium tumefaciens strain AGL1 dan GV3101 yang membawa plasmid pBI121. Thesis. Department of Biotechnology.

[20] Chang, S., and Shu, H., 2013. A method to supress the browning in banana (Musa, AAA) embryogenic callus induced. Research Journal of Biotechnology. 8(4), 63-69.

[21] Wong, W.C., Jalil, M., Ong-Abdullah, M., Othman, R.Y., and Khalid, N., 2006. Enhancement of banana plant regeneration by incorporating

[22] Ikeuchi, M., Sugimoto, K., and Iwase, A., 2013. Plant callus: Mechanisms of induction and repression. The Plant Cell. 25, 3159–3173.

[23] Khalil, S. M., and Elbanna, A. A. M., 2003. Highly efficient somatic embryogenesis and plant regeneration via suspension cultures of banana (Musa spp.). Arab Journal Biotechnology. 7(1), 99-110.

[24] Zimmerman, J.L., 1993. Somatic embryogenesis: a model for early development in higher plants. The Plant Cell. 5, 1411-1423.

[25] Pasternak, T. P., Prinsen, E., Ayaydin, F., Miskolczi, P., Potters, G., Asard, H., Onckelen, H. A. V., Dudits, D. and Fehér, A., 2002. The role of auxin, pH and stress in the activationof embryogenic cell division in leaf protoplast derived cells of Alfalfa. Plant Physiology 129, 807-1809.

[26] Shinoyama, H., Nomura, Y., Tsuchiya, T. and Kozuma, T., 2004. A simple and effecient method for somatic embryogenesis and plant regeneration from leaves of chrysanthemum [Dendranthema x grandiflorum (Ramat.) Kitamura]. Plant Biotechnology. 21, 25-33.

[27] Ganapathi, T.R., Higgs, N.S., Balint-Kurti, P.J., Arntzen, C.J., May, G.D., and Van Eck, J.M., 2001. Agrobacterium-mediated transformation of embryogenic cell suspensions of T he banana cultivar Rasthali (AAB). Plant Cell Reports. 20, 157–162.

[28] Fujimura, T., and Komamine, A., 1979. Synchronization of somatic embryogenesis in a carrot cell suspension culture. Plant Physiology. 64, 162-164.

[29] Wahid, A., Gelani, S., Ashraf, M., and Foolad, M., 2007. Heat tolerance in plants: An overview. Environ. Exp. Bot. 6, 199-223.

[30] Usman, M. G., Rafii, M. Y., Ismail, M. R., Malek, M. A., Latif, M. A., and Oladosu, Y., 2014. Heat shock proteins: functions and response against heat stress in plants. International Journal Of Scientific and T echnology Research. 3(11), 204-218.

[31] Patel, M., Dewey, R. E., and Qu, R., 2013. Enhancing Agrobacterium tumefaciens-mediated transformation efficiency of perennial ryegrass and rice using heat and high maltose treatments during bacterial infection. Plant Cell Tissue Organ Culture. 114, 19–29

[32] Li, D. D., Shi, W., and Deng, X. X., 2003. Factors influencing Agrobacterium-mediated embryogenic callus transformation of Valencia sweet orange (Citrus sinensis) containing the pTA29-barnase gene. Tree Physiology. 23, 209–1215

[33] Li, Z. T., Dhekney, S., Dutt, M., Van Aman, M., T attersall, J., Kelley K. T., and Gray D. J., 2006. Optimizing Agrobacterium-mediated transformation of grapevine. In Vitro Cell. Dev. Biol.—Plant. 42: 220–227.

[34] Berensmeier,S., 2006. Magnetic particles for the separationand purification of nucleic acids. Appl. Microbiol.Biotechnol. 73, 495-504

[35] Esser, K.H., Marx, W.H., and Lisowsky, T., 2006. MaxXbond: first regeneration system for DNA binding silica matrices.

[36] Opabode, J. T., 2006. Agrobacterium -mediated transformation of plants: emerging factors that influence efficiency. Biotechnology and Molecular Biology Review. 1: 12-20.

[37] Suzuki, S., Supaibulwatana, K., Mii, M,. and Nakano, M., 2001. Production of transgenic plants of the Liliaceous ornamental plant Agapanthus praecox ssp. Orientalis (Leighton) Leighton via Agrobacterium-mediated transformation of embryogenic calli. Plant Science. 161, 89-97.

[38] Amoah, B. K., Wu, H., Sparks, C. A., and Jones, H. D., 2001. Factors influencing Agrobacterium-mediated transient expression of uidA in wheat inflorescence tissue. Journal of Experimental Botany, 52, 1135-1142.

[39] Uranbey, S., Sevimay, C.S., Kaya, M.D., Ipek, A., Sancak, C., Basalma, D., Er, C., and Ozcan, S., 2005. Influence of different co-cultivation temperature, periods and media on Agrobacterium tumefaciens mediated gene transfer. Biologia Plantarum. 49 (1), 53-57.

[40] Wenck, A. R., Quinn, M., Whetten, R.W., Pullman, G., and Sederoff, R., 1999. High efficiency Agrobacterium-mediated transformation of Norway spruce (Picea abies) and loblolly pine (Pinus taeda). Plant Mol Biol. 39, 407–416.