# *Agrobacterium*-mediated transformation of banana *Musa acuminata* AA cv "Mas Lampung" with *hpt* gene using sterile corm as target tissue

**Amy Estiati[1], Ade Nena[1] and Witjaksono[2]**
[1]Research Center for Biotechnology, Indonesian Institute of Sciences (LIPI)
[2] Research Center for Biology, Indonesian Institute of Sciences (LIPI)
nugroho_amy@yahoo.com

## ABSTRACT

The protocol for *Agrobacterium*-mediated transformation of local banana plants cv "Mas Lampung" (AA) has been established. A selectable marker gene (*hpt*) has been used to study the transformation using *in vitro* corm slices as target tissues. Banana *in vitro* corm slices were co-cultivated with the EHA105 strain of *Agrobacterium tumefaciens* harbouring binary vector pCAMBIA 1301 containing hygromycin resistance gene (*hpt*) as a selectable marker and intron-containing β-Glucuronidase (*gus-intron*) gene as a reporter gene driven by CaMV 35S promoter. Polymerase Chain Reaction (PCR) were used to examine the existence of *hpt* gene in plants resulted from the transformation. Using primer pairs specific for *hpt* gene, our PCR analysis on leaves showed the presence of the *hpt* transgene in banana transgenic plants at first generation ($T_0$) of transformation. To prove the existence of *hpt* gene in the fruits of transgenic banana plants, PCR analysis were also carried out. The data showed that the *hpt* gene could be amplified from banana fruits of tested samples. These result demonstrates that the *Agrobacterium*-mediated transformation method used in this experiment has been successful to transfer gene into banana plants. Thus, the transformation method reported here could be used as a standard protocol to transfer another useful genes into local banana plants cv. "Mas Lampung". Furthermore, the presence of transgene in fruits of banana transgenic plants is important achievement especially for transgene that is expected to be expressed in the fruit including to introduce vaccine genes into banana fruits for edible vaccine.

**Key words:** *Agrobacterium*, *hpt* gene, transgenic banana Mas Lampung, *Musa acuminate* corm slices, PCR

## INTRODUCTION

Banana is an important fruit crop in Indonesia and worldwide, utilized for staple, dessert and processed food. Despite its important, bananas is plagued with pests and diseases. Conventional breeding for trouble shooting these problems have been difficult due to its reproductive biology of the plants: parthenocarpy, pollen sterility, and polyploidy. Therefore any advanced in biotechnology, i.e. genetic transformation would be beneficial to the improvement or other utilization of this plant.

Genetic transformation of banana (*Musa* spp.) has been experimented. Most of them using embryogenic cultures as the target tissues mediated by *Agrobacterium* (Ganapathi *et al*., 2001; Arinaitwe *et al*., 2004) or particle bombardment (Becker et al., 2000; Arinaitwe et al.,2004), or combination of both. This technique is difficult because the establishment of reliable embryogenic culture system have not been routine and obtained in low frequency and in an unpredictably long time. Alternatively, May *et al*. (1995) had transformed in vitro corm slices and apical meristems of *Musa acuminata* cv Grand

Naine (AAA) using integrated bombardment and *Agrobacterium* transformation system . Meanwhile, Sreeramanan *et al*. (2006, 2009) had transformed single buds and corm slices of banana cv Rasthali (AAB) using particle bombardment and *Agrobacterium*.

Relative success in the genetic engineering of bananas has been achieved in various cultivar of banana to transfer foreign genes into plant cells. Transformation of genes for specific purposes such as *HL* (Human Lysozyme ) gene for resistance to Panama wilt (Pei *et al*., 2005), rice chitinase gene for resistance to black sigatoka disease (Maziah *et al*., 2007) and *s* gene of hepatitis B surface antigen (HBsAg) for edible vaccine (Sunil-Kumar *et al*., 2005) had been carried out. Protocols have been developed for *Agrobacterium*-mediated transformation as well as microprojectile bombardment for some banana cultivars i.e. "Cavendish", "Grand Naine", "Rasthali", "Three Hand Planty", "Orishele", "Lady Finger", "Navolean" and "Bluggoe" . Unfortunatelly, transformation frequencies are reported to be cultivar-dependent. Thus there is a need to adapt and optimize transformation protocols for any particular type of banana (Arinaitwe *et al*., 2004).

In order to establish a stable transformation system for local banana plants cv. "Mas Lampung", *Agrobacterium*-mediated transformation with a selectable marker gene (*hpt*) using *in vitro* corm slices as target tissues had been done. In this paper we report the successfully integration of a selectable marker gene (*hpt)* in local banana plants cv. "Mas Lampung" (AA), therefore, this transformation protocol reported here could be used as a standard protocol to introduce another useful genes into local banana plants cv. "Mas Lampung".

## MATERIAL AND METHODS

*Plant materials*

Suckers from banana (*Musa acuminata* AA cv Mas Lampung) clumps were collected from fields in Bogor. Shoot tips with conical shape with diameter aproximately 1 cm were isolated from sucker, surface sterilized with 20% Clorox (or Bayclin) (5.25% NaOCl) and rinse 3 times with sterile aquadest. The cones were then halves and inoculated on MS (Murashige and Skoog, 1962) medium containing 5 mg/L BA (Benzyl Adenine) and 250 mg/L cefotaxime. The medium was solidified with 2.5 g/L Gelzan. When new shoots with expanded leaves grew up from the explants, the new shoots were then isolated by discarding the old tissues from the initial explants and then transferred on MS medium containing 20 mg/L BA. The shoots then enlarge and form big corm that looks like a rootless plantlets. The rootless plantlets that has big corms were maintained in the same medium without antibiotic. Then, the developed corms were micro-dissected transverse to the shoot axes into corm slices (2-3 mm thick). This corm slices were used as target tissues for transformation experiments.

*Agrobacterium and plasmids construct for gene transfer*

*A. tumefaciens* EHA101 harboring pCambia 1301 was used for transformations. The plasmid pCambia 1301 contains hygromycin resistance gene (*hpt*) as a selectable marker and intron-containing β-Glucuronidase (*gus-intron*) gene as a reporter gene driven by CaMV 35S promoter (Jefferson *et al*., 1987) (Figure 1). The intron that resides in the *gus* A gene is used to optimize the expression of *gus* A gene and to ensure that the *gusA* gene would not expressed by the bacterium (Tanaka *et al*., 1990).

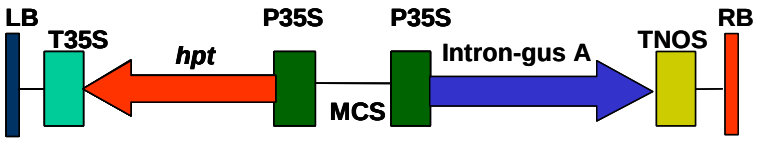


**Figure 1**. T-DNA region of binary plasmid pCAMBIA 1301. RB: right border; LB: Left border; P35S dan T35S : CaMV 35S promoter and terminator; TNOS: nopaline synthase terminator; *hpt*: hygromycin phosphotransferase; intron-*gusA*: ß-glucuronidase; MCS: multiple cloning sites

*A. tumefaciens EHA101* was plated on selective solid LB medium supplemented with 50 mg/L kanamycin and 20 mg/L rifampicin and incubated at 28°C for 3 days. The bacteria were collected and suspended in 25 ml liquid MS medium containing 20 mg/L BA, and 400 mM acetosyringone and grown in agitated (210 rpm) and 24°C to an $OD_{600}$ of 0.9-1.0 for approximately 1 h. After the $OD_{600}$ of 0.9-1.0 was reached, the *Agrobacterium* suspensions were ready to use for transformation experiment.

*Transformation and plant regeneration*

Banana *in vitro* corm slices were co-cultured with the *hpt*-harboring *A. tumefaciens* EHA 101 by immersing them in the bacterium suspension for 6 h. The corm slices were thereafter transferred to semi solid MS medium containing 20 mg/L BA, 400 mM acetosyringone, incubated for 6-7 days in the dark at 24°C. Thereafter, the corm slices were transferred onto solid MS medium containing 20 mg/L BA , 250 mg/L cefotaxime and incubated for 1 month with biweekly subculture in the dark condition at 24°C until shoots emerged. Then, the emerged shoots were transferred to the same medium but maintained under 16 h photoperiod at 24°C for another month. Under light condition, the color of shoots changed from whitish to greenish. After one month the green shoots were transferred to rooting medium i.e. solid MS medium containing 2 mg/L BA and 0.175 mg/L IAA (Indole Acetic Acid) until they grow roots. The plantlets with leaves and roots were maintained in solid MS medium without plant growth regulators. Planlets that reached 10 cm in height were then transferred into pots containing cocopeat, husk, manure and soil with ratio 1:1:1:3 and grown in the *biosafety containment*.

*PCR analysis*

Fresh leaf material from untransformed banana plant and plants regenerated from the transformation experiments were collected from the bio-safety containment. Genomic DNA was extracted from leaves as described by Zheng *et al*. (2000). Successful PCR was performed using specific primers for *hpt* gene (forward: 5'- GATGCCTCCGCTC GAAGTAGCG -3' and reverse 5'- GCA TCTCCCGCCGTGCAC-3'). The reactions were carried out in a Biometra Temperature Gradient®. Plant genomic DNA in the amount of 100 ng was amplified in a PCR reaction mixture containing 0.5x buffer GoTaq Hot Start Polymerase [Promega], 2.5 ng/µl of each primer of *hpt* gene and dH2O. PCR analysis was carried out under standard condition with 1 min denaturation at 95 $^{o}$C, 1 min annealing at 60 $^{o}$C , 1 min extension at 72 $^{o}$C for 35 cycles. After PCR, the DNA was loaded on a 1.5% agarose gel with 0.5X TBE at 100 volt for running time about 1 hour.

To prove the presence of *hpt* gene in fruits of banana transgenic plants, PCR analysis was carried out. Fruits were harvested from transgenic banana plants number 69 and 70, respectively (Figure 2). DNA was isolated from fruits of banana transgenic plants that had been taken randomly. Genomic DNA was extracted from fruits as described by Zheng *et al*. (2000). Successful PCR was performed

using specific primers for *hpt* gene (forward: 5'- GATGCCTCCGCTCGAAGTAGCG-3' and reverse 5'- GCATCTCCCGCCGTGC AC -3'). The PCR reactions solutions, PCR condition and detection of *hpt* fragment on agarose gel was the same as the ones for leaf.

## RESULT AND DISCUSSION

*Transformation and regeneration of transformant*

We have developed a fast transformation system of banana using *in vitro* corm as a target tissue by taking advantage of its organogenic ability. The sequence of the process are described in Figure 2. The use of halves conical shoot tips (Figure 2 a) dissected out of suckers collected from field has advantage of its speed in recovering banana in vitro shoots. It took only about 1 month to get in vitro shoots (Figure 2b) from that explants. Aproximately 95% of the explants developed in vitro shoots with expanding leaves upon transfer to the same medium. The use of cefotaxime in this medium may have played an important role in recovering shoots that were free of bacterial contamination. New shoots were then isolated by discarding the old tissues from the initial explants and then transferred on MS medium containing 20 mg/L BA. In this medium, the shoots form big corm that looks like a rootless plantlets (Figure 2c). Then, the developed corms were micro-dissected transverse to the shoot axes into corm slices (2-3 mm thick) (Figure 2d).

The use of corm dish ( Figure 2 d) as the target tissues and co-cultured in the bacteria suspension (Figure 2 e, f) has resulted in regeneration of shoots about one month in the dark (Figure 2 g). The use of banana tissue culture media for growing bacteria suspension and co-culture with the corm may be beneficial to the banana tissue in regard to reduction of stress. The number of shoots that developed range from 2-4 per corm. The shoots then turned green and developed further under light (Figure 2 h). It took approximately one month for the shoots to enlarge to about 1-2 cm height and have 1-2 leaves (Figure 2 i). Upon transfer to rooting medium for about a month, the shoots developed roots and ready for acclimatization (Figure 2 j) . The planlets with leaves and roots had been successfully acclimatized and grew in the biosafety containment to maturity( Figure 2 k). The mature plants have fruited with normal bunch and fruit morphology (Figure 2 l,m).

In co-cultivation media, the color of corms slices turned from white into brown color. Acetosyringone is known to activate the virulence genes of the Ti plasmid and to initiate the transfer of the T-DNA. Cefotaxime were required in solid medium in order to inhibit growth of *Agrobacterium tumefaciens* which could affect explant growth and cause contamination, reducing transformation efficiency. Cefotaxime itself did not show negative effect in apple tissues (Maximova et al., 1998), adventitious buds and shoots induction in *Pinus radiate* (Holland *et al*., 1997) and chrysanthemum (Teixeira da Silva and Fukai, 2002). In this media, little shoots with white colour emerged. Under light condition, the colour of shoots will change from whitish to greenish.

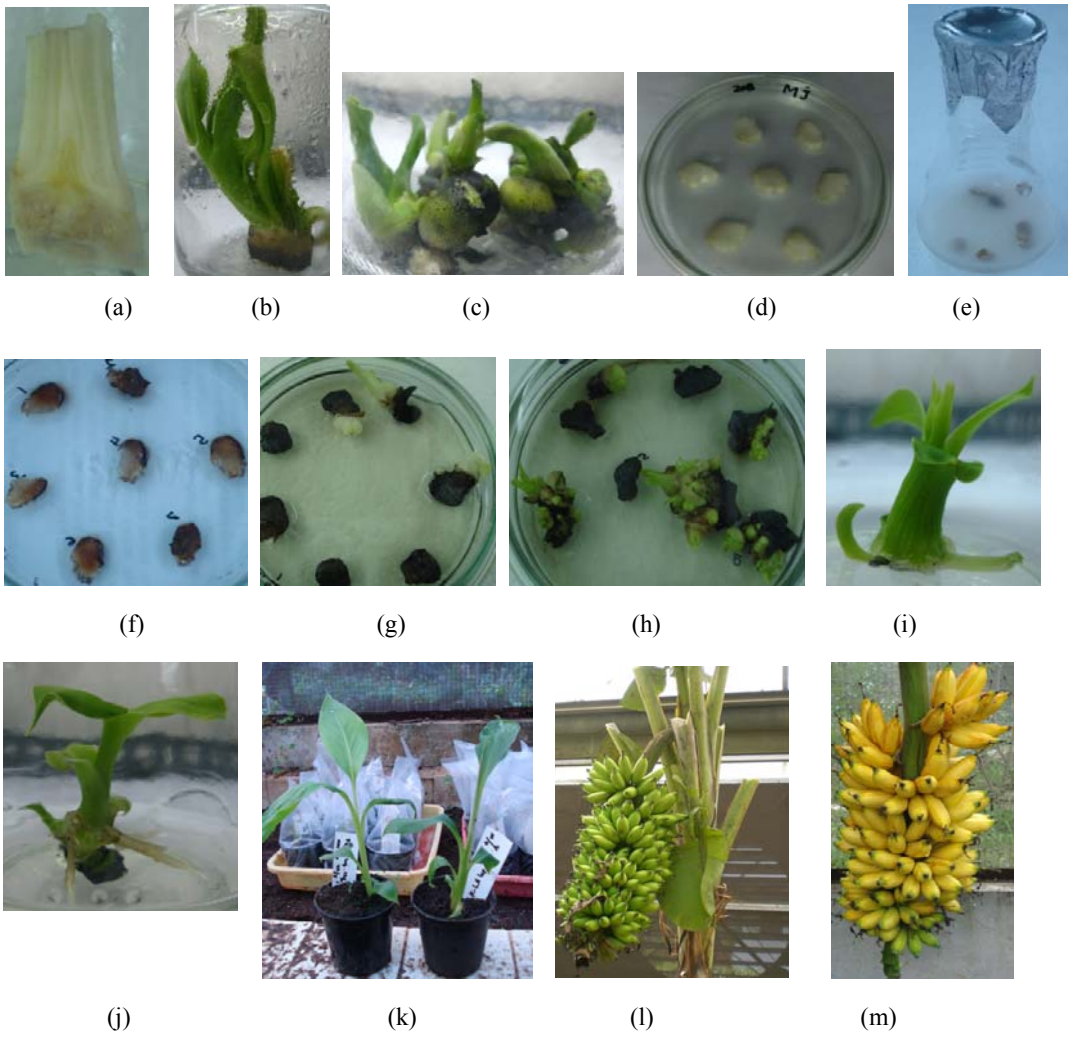


**Figure 2.** Procedure transformation, shoot initiation, regeneration and acclimatization. (a) shoot tip explant; (b) in vitro shoot that grew out of the explant; (c) rootless plantlet with big corm; (d) corm slices micro-dissected from rootless plantlet in vitro; (e) co-cultivation of the corm slices with *Agrobacterium*; (f) corm slices growth on co-cultivation medium under dark condition; (g) shoot initiation in the dark condition; (h) shoot initiation under light condition; (i) shoot regeneration; (j) root regeneration; (k) acclimatization; (l) banana transgenic plants with fruit bunch; (m) ripening transgenic fruits

*PCR analysis on leaves and fruits of putative transformants*

Total DNA isolated from the putative transformants and untransformed plant were tested for the presence of the transgenes. The efficient and simplicity of the PCR analysis, enabled screening for transformed plants in a shorter period. Using primer pairs that specific for amplified the *hpt* gene, PCR amplification using DNA from leaves had proved the presence of the *hpt* transgene in banana transgenic plants at first generation ($T_0$) with showing PCR product of 492 kb in length. Meanwhile, no bands could be detected from DNA extracted from the untransformed plant. The PCR results of some transgenic banana plantlets are shown in Figure 3.

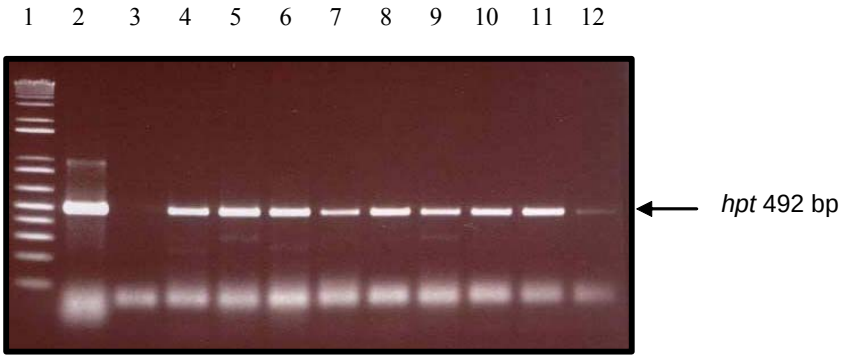


**Figure 3.** PCR analysis of the transformants for the presence of the transgene *hpt*. DNA was amplified with *hpt* primers. Lane 1: 100 bp ladder marker; lane 2: pCAMBIA 1303 as a positive control; lane 3: untransformed plant as a negative control; lane 4-12: individual transgenic plants (no.5, 69, 65, 70, 64, 71, 2, 61, 68) (Source: Estiati and Witjaksono, 2008)

Meanwhile, to confirm the presence of the *hpt* gene in the fruits of transgenic banana plants, PCR analysis was also carried out. Using primer pairs that specific for amplified the *hpt* gene, PCR amplification had proved the integration of *hpt* gene in fruits of banana plants number 69 and 70 with showing PCR product of 492 kb in length . Meanwhile, no bands could be detected from DNA extracted from fruits of untransformed plant. The PCR results of some transgenic banana fruits are shown in Figures 4 and 5, respectively. This result demonstrated that the transformation method used in this experiments has been successfully. This method could be used as a standard protocol to transfer genes of interest into banana plants cv. "Mas Lampung".

The presence of transgene in fruits of banana transgenic plants also proved that the transgene was carried through the plant development including in the fruit. This is important achievement especially for transgene that is expected to be expressed in the fruit for example for introducing vaccine genes in the banana fruits for edible vaccine.

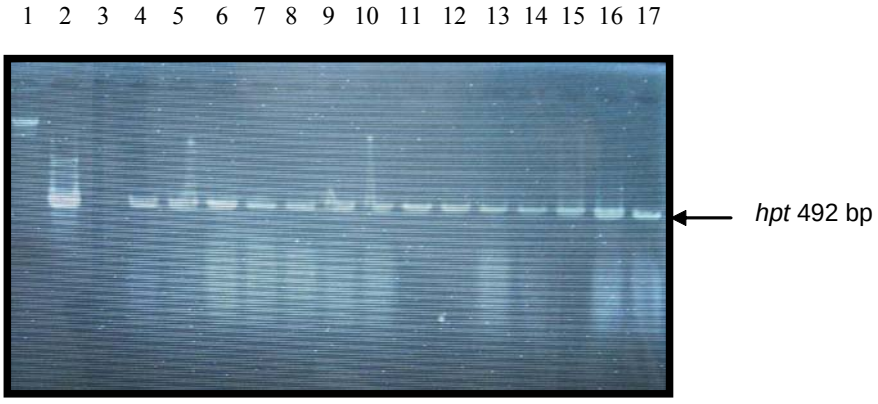


**Figure 4.** PCR analysis of the transformants for the presence of the transgene *hpt*. DNA was isolated from fruits of banana transgenic plant no. 69 DNA was amplified with *hpt* primers. Lane 1: λ DNA digested with *Hin*dIII; lane 2: pCAMBIA 1301 as a positive control; lane 3: fruits from untransformed plant as a negative control; lane 4-17: fruits taken randomly from transgenic banana plants no.69

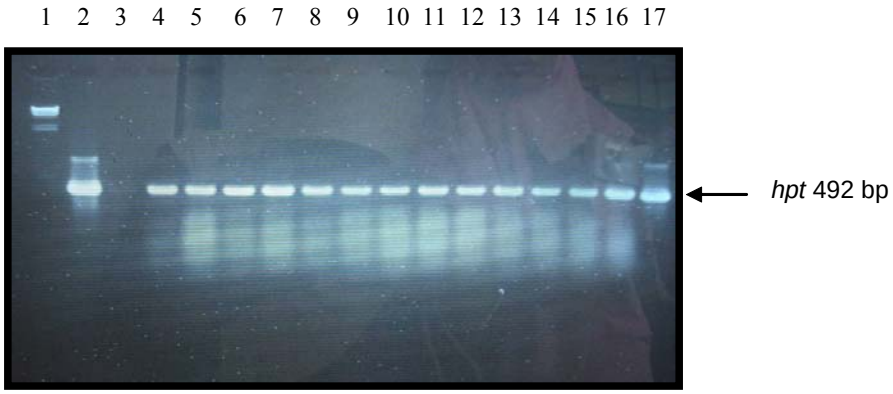


**Figure 5.** PCR analysis of the transformants for the presence of the transgene *hpt*. DNA was isolated from fruits of banana transgenic plant no. 70 DNA was amplified with *hpt* primers. Lane 1: λ DNA digested with *Hin*dIII; lane 2: pCAMBIA 1301 as a positive control; lane 3: fruits from untransformed plant as a negative control; lane 4-17: fruits taken randomly from transgenic banana plants no.70 (Source: Estiati and Witjaksono, 2008)

However, the presence of the gene of interest should remain stable throughout generation. One of the advantageous characters of banana plant is its vegetative propagation by which the character inserted in the parent will be retained in the progeny. To prove this, PCR analysis to confirm the existence of *hpt* gene need to be conducted in the second and the third generation of transformans.

Though *Agrobacterium* mediated transformation is the most common method for the generation of transgenic plants with single integration of a precisely delimited DNA sequences (Smith and Hood, 1995; Lawrence *et al*., 2001), the structure of the inserted T-DNA varies widely from single or multiple copies, individual or tandem repeats, at a unique or several loci in the plant genome (Iglesias et al., 1997). Since multiple copies of transgene could affect the expression of transgene in plants, Southern hybridization should be carried out.

**Acknowledgements**

This work was supported by Competitive Research Grant from the Indonesian Institute of Sciences to Witjaksono (2004-2006). Rita Yuliasari's technical assistance in transformation experiments were appreciated.

**REFERENCES**

Arinaitwe G., S. Remy, H. Strosse, R. Swennen, L. Sagi. 2004. *Agrobacterium*- and particle bombardment-mediated transformation of a wide range of banana cultivars. Arinaitwe, G., Remy, S., Strosse, H., Swennen, R., Sagi, L. (2004). In: Mohan Jain S., Swennen R. (Eds.), *Banana Improvement:Cellular, Molecular Biology, and Induced Mutations* (pp. 351-357). Enfield, NH, USA: Science Publishers Inc.

Becker, D.K., B. Dugdale, M.K. Smith, R.M. Harding, J.L. Dale. 2000. Genetic transformation of Cavendish banana (*Musa* spp. AAA group) cv 'Grand Nain' via microprojectile bombardment. Plant Cell Reports 19: 229-234

Estiati and Witjaksono. 2008. Pasca Genomic dan Molecular Farming: Biologi Molekuler untuk Produksi Obat-obatan dan Mendukung Ketahanan Pangan. Lembaga Ilmu

Pengetahuan Indonesia. Pusat Penelitian Bioteknologi. Cibinong, Bogor. 2008. pp: 19-45

Ganapathi T.R., N.S. Higgs, P.J. Balint-Kurti, C.J. Arntzen, G.D. May, J.M. Van Eck. 2001. *Agrobacterium*-mediated transformation of embryonic cell suspensions of the banana cultivar Rasthali (AAB). Plant Cell Reports 20: 157- 168

Holland L., J. E. Gemmell, J. A. Charity, C. Walter. 1997. Foreign gene transfer into *Pinus radiate* cotyledons by *Agrobacterium tumefaciens*. NZ J. For Sci. 27:289-304

Iglesias V. A., A. Gisel, R. Bilang, N. Leduc, I. Potrykus, C. Sautter. 1997. Transient expression of visible marker genes in meristem cells of wheat embryos after ballistics micro-targeting. Planta.192:84-91

Jefferson R.A., T.A. Kavanagh, M.W. Bevan MW. 1987. GUS fusions: *β-glucuronidase* as a sensitive and versatile gene fusion maker in higher plants. EMBO J 6: 3901-3907

Lawrence P. K. , K. R. Koundal. 2001. *Agrobacterium tumefaciens*mediated transformation of pigeon pea [*Cajanus cajan* (L.) *Mill sp*.] and molecular analysis of regenerated plants. Curr Sci. 80:1428-1432

May G., R. Afza, H.S. Mason, A. Wiecko, F.J. Novak, C.J.Arntzen. 1995. Generation of Transgenic Banana ( *Musa acuminata* ) Plants via *Agrobacterium*- Mediated Transformation, Biotechnology 13: 486-492

Maximova S. N., A. M. Dandekar, M. J. Guiltinan. 1998. Investigation of *Agrobacterium* mediated transformation of apple using green fluorescent protein: high transient expression expression and low stable transformation suggest that factors other than T-DNA transfer are rate-limiting. Plant. Mol. Biol. 37:549-559

Maziah M., S. Sreeramanan, A. Puad, M. Sariah, 2007. Production of transgenic banana cultivar, rastali (AAB) via agrobacterium-mediated transformation with a rice chitinase gene. J. Plant Sci., 2: 504-517

Murashige T., F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant .15**:**473-497

Pei X.W., S.K. Chen, R.M. Wen, S. Ye, J.Q. Huang, Y.Q. Zhang, B.S. Wang, Z.X. Wang, S.R. Jia. 2005. Creation of Transgenic Bananas Expressing Human Lysozyme Gene for Panama Wilt Resistance. Journal of Integrative Plant Biology, 47: 971–977

Smith R. H., E. E. Hood. 1995. *Agrobacterium tumefaciens* transformation of monocotyledons. Crop Sci. 35:301-309

Sreeramanan S., M. Maziah, M. P. Abdullah, M. Sariah, R. Xavier. M.F. Nor Aini. 2005. Physical and biological parameters affecting transient GUS and GFP expression in banana via particle bombardment. Asia Pasific Journal of Molecular Biology and Biotechnology 13 (1): 35-57

Sreeramanan S., M. Maziah, R. Xavier. 2009. A protocol for *Agrobacterium*-mediated transformation of banana with a rice chitinase gene. Emir. J. Food Agric. 21(2): 18-33

Sunil Kumar G.B., T.R. Ganapathi, C. J. Revathi, L. Srinivas, V.A. Bapat. 2005. Expression of hepatitis B surface antigen in transgenic banana plants. Planta 222: 484-493

Tanaka A., S. Mita, S. Ohta, J. Kyozuka, K. Shimamoto, K. Nakamura.1990. Enhancement of foreign gene expression by a dicot intron in rice but not in tobacco is correlated with an increased level of mRNA and an

efficient splicing of the intron. Nucleic Acids Res 18: 6767-6770

Teixeira da Silva J. A., S. Fukai. 2002. Increasing transient and subsequent stable transgene expression in chrysanthemum (*Dendranthema* x *grandflora* (Ramat.) *Kitamura*) following optimization of particle bombardment and *Agro*infection parameters. Plant Biotechnol. 19:229- 240

Zheng S.J., L.I. Khrustaleva, B. Henken, E. Sofiari, E. Jacobsen, C. Kik, F.A. Krens. 2000. *Agrobacterium tumefaciens*- mediated transformation of *Allium cepa* L.: the production of transgenic onions and shallots. In Towards onions and shallots (*Allium cepa* L.) resistant to beet armyworm (*Spodoptera exigua* Hübner) by transgenesis and conventional breeding. Thesis. pp. 73-96