OPTIMIZATION OF TRANSIENT *β-glucuronidase* (*gus*) GENE EXPRESSION IN TEAK (*Tectona grandis* L.f.) BY *Agrobacterium tumefaciens*-MEDIATED TRANSFORMATION SYSTEM

Yaowaphan Sontikun[1,2], Sontichai Chanprame[1,3], Peerasak Srinives[1,3] and Sermsiri Chanprame[1,4*]

[1] Center for Agricultural Biotechnology, Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom 73140, Thailand and Center of Excellence on Agricultural Biotechnology: (AG-BIO/PERDO-CHE), Bangkok 10900, Thailand
[2] Strategic Scholarships for Frontier Research Network for the Ph.D. Program Thai Doctoral degree from the Office of the Higher Education Commission, Thailand
[3] Department of Agronomy, [4]Department of Horticulture, Faculty of Agriculture at Kamphaeng Saen, Kasetsart University, Nakhon Pathom 73140, Thailand
Corresponding author: *agrsrc@ku.ac.th

(Received: July 20,2013; Accepted: November 2, 2013)

ABSTRACT

Teak is an important tree species for forest plantation in the tropics due to its high value timber. However, teak plantations encounter several problems including competition from weeds, as well as damage from pests and diseases. Genetic engineering is a possible approach to overcome these problems. However, as with many woody species, teak has appeared recalcitrant to genetic transformation. *Agrobacterium*-mediated gene transformation is effective in various forest tree species. Hence, the aim of this study is to determine the effects of explants type, wounded type, *A. tumefaciens* strains and concentrations, inoculation and co-cultivation period on transformation of teak. Two *A. tumefaciens* strains, EHA105 and AGL-1, possess the binary vector pCAMBIA1304 containing *gus* reporter gene and *hygromycin phosphotransferase* (*hpt*) selectable marker gene were used to transform leaf base and nodal segments of the *in vitro* teak at various concentrations of bacteria. The transformation efficiency was determined by transient expression of *gus* gene using GUS histochemical assay. The transient expression of *gus* gene revealed that nodal segment was more suitable to be used as explants than the leaf base tissue with the transformation efficiency of 58.32% and 44.45%, respectively. The extra wounding by sonication resulted in 90% transformation efficiency in nodal segment. The bacterial strain EHA105 at1.0 x 10^9 cfu/ml and the inoculation period of 5 hours followed by 3 days of co-cultivation were successfully used for teak transformation.

Key words: woody plant, transgenic plant, reporter gene

INTRODUCTION

Teak (*Tectona grandis* L.f.) is one of the world's premier hardwood timbers that attractive for its mellow color, fine grain and durability. Teak wood is used for making fine furniture, shipbuilding and interior and exterior luxury decorative objects. Currently, the demands for wood and wood products are increasing throughout the world, while the amount of natural teak forest is insufficient. Plantation production of teak is seen as an alternative to timber extraction from natural

forest. Teak plantations cover more than 3.3 million hectares in Asia, and it is the second most widely planted forest species in the world behind Eucalyptus (Behaghel, 1999). In Northern Thailand, there are more than 100,000 hectares of teak plantation (Kaosa-ard, 1981). However, the productivity of most plantations is low because of several problems including susceptible to various weeds, pests and diseases.

Genetic improvement of teak by conventional breeding is still an obstacle due to the long reproductive cycle, difficulties in performing controlled pollination, low germination percentage and also teak germplasm has insufficient genetic variability (Gyves and Rugini, 2009). These limitations make genetic engineering a possible approach to overcome these problems. Genetic transformation is an attractive tool because it allows relatively rapid, specific changes in proven cultivars without disrupting their fundamental genetic background (Schuerman and Dandekar, 1993). However, as with many woody species, teak has appeared recalcitrant to genetic transformation. Mostly, successful genetic transformation in trees have been reported by using *A. tumefaciens* as mediator, for example, walnut (McGranahan *et al*., 1988), sandalwood (Lakhsmi-Sita *et al.,* 1998), *Populus* spp. (Dai *et al*., 2003; Thakur *et al*., 2005) and pine (Cerda *et al*., 2002; Charity *et al*., 2002; Tang *et al*., 2004). Teak transformation has been reported by using biolistic and *A. tumefaciens* - mediated methods as tools to transform the *gus* gene into explants. The results showed low percentage of transient *gus* expression (Norwati *et al*., 2007; Widiyanto *et al*., 2009). However, to date, there have no reports of successful stable genetic transformation in teak. The successful of teak transformation will lead to the transformation of high value traits such as insect resistance trait achieve from *Bt* genes.

Recently, some other factors have been found important in influencing the efficiency of *Agrobacterium* -mediated genetic transformation such as tissue specific factors, wounded type, *A. tumefaciens* strain and density, plasmid vectors, medium composition, and inoculation and co-cultivation conditions (Opabode, 2006). Hence, this study is aimed at optimizing those parameters affecting *A. tumefaciens* -mediated gene transformation in teak.

MATERIALS AND METHODS

Plant materials

Plant materials used in this study were provided by the forest industry organization, Thailand. *In vitro* teak was subcultured at monthly intervals by transferring nodal segment onto Murashige and Skoog medium (Murashige and Skoog, 1962) and cultured at 25±2ºC under 16 hour photoperiod with 55 µmol/ m^2/ s light intensity. Two month-old *in vitro* teak, consisting of 4 - 6 young leaves were used for the *A. tumefaciens* -mediated transformation experiment.

A. tumefaciens strains and plasmid DNA

A. tumefaciens strains, EHA 105 and AGL-1 were maintained in 70% glycerol and stored in -80ºC for long term storage. Both bacterial strains possess the binary vector pCAMBIA 1304 containing β-*glucuronidase* (*gus*) gene as a reporter gene and *hygromycin phosphotransferase* (*hpt*) gene that provides resistance to hygromycin as a selectable marker gene. The gene are driven by the cauliflower mosaic virus (CaMV) 35S promoter and NOS terminator sequences (Fig 1) (Jefferson *et al.,* 1987).

A. tumefaciens was cultured in Luria-Bertani (LB) medium containing 50 mg l^{-1} kanamycin and incubated at 28ºC and 120 rpm overnight. Cultures were streaked on solid LB medium containing 50 mg l^{-1} kanamycin and incubated at 28ºC for two days. Single colony was then grown in LB medium containing 50 mg l^{-1} kanamycin and incubated at 28ºC and 120 rpm for 16 hours to reach an optimal density of 2.0 units at 600nm (OD_{600}). This bacterial suspension (200 µl) was transferred to

50 ml LB medium containing 50 mg l^{-1} kanamycin and 100 µM acetosyringone and incubated at 28ºC and 120 rpm for 16 hours. Bacteria cells were pelleted by centrifugation at 12,100 g at 4 ºC for 10 min then resuspended in MS liquid medium before inoculation.

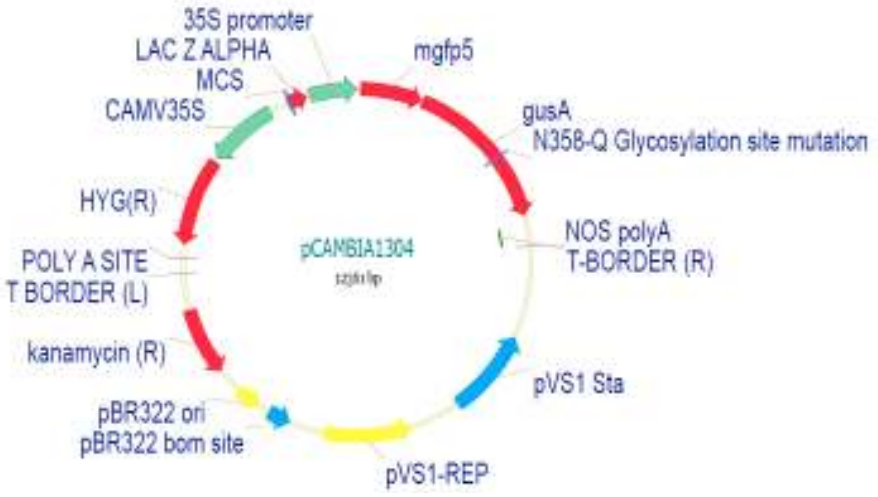

Fig. 1. Schematic diagram of the plasmid pCAMBIA 1304. The binary vector pCAMBIA 1304 (CSIRO, Australia) harbouring the reporter *gus* gene driven by the CaMV 35S promoter. (http://www.cambia.org/daisy/cambia/585.html)

Transformation method

The leaf base and nodal explants of *in vitro* teak were wounded by cutting 5 times for each explant or punching 5 times with a needle or sonication in MS liquid medium for 5 sec. Wounded explants were immersed in the *A. tumefaciens* suspension of 0.5 x 10^9 and 1.0 x 10^9 cfu ml^{-1} for 1, 3, and 5 hours to begin the bacteria inoculation process. They were then co-cultivated on solid MS medium containing 100 µM acetosyringone in the dark for 3 and 5 days. After co-cultivation, the explants were washed in liquid MS medium containing 500 mg l^{-1} cefotaxime 3 times and transferred to solid MS medium containing 300 mg l^{-1} cefotaxime for 3 days. Explants were then assessed for transient GUS expression to determine the optimum conditions for transformation. The data was laid out in Completely Randomized Design (CRD) with four replications, each replication has 30 explants.

Histochemical GUS assay

Transient expression levels of *gus* gene in teak transformants were assessed three days after co-cultivation. The transformed explants were immersed in X-Gluc staining solution containing 0.1 M $NaPO_4$ buffer pH 7.0, 10 mM EDTA, 0.5 mM Kferricyanide, 0.5 mM Kferrocyanide, 1.0 mM X-Glucuronide and 0.1% Triton X-100 as a substrate for the enzyme (Jefferson *et al*.,1987). After incubation at 37ºC overnight they were de-stained in 70% ethyl alcohol. Staining with dichloro-dibromo-indigo allowed stereo-microscope identification of GUS activity. Explants with positive GUS activity were evaluated to determine the transient transformation efficiency. The percentages of explants with *gus* positive expression were defined as the numbers of *gus* positive per total numbers of evaluated explants x 100. The blue area of each explant was scored as the percentages of transformation frequency. On all staining occasions, non-transformed leaves were also included as a control assay against background staining.

RESULTS AND DISCUSSION

In order to optimize the conditions for teak transformation, the effects of several parameters known to influence *A. tumefaciens* - mediated methods were compared.

Effect of explants on percentage of transient *gus* expression

The explant material itself is one factor that plays an important role in the transformation process. This study used two types of explant: leaf base and nodal. Transformation efficiency was assessed as the area of the blue inclusion in the transformed explants (Fig. 2). Nodal tissue resulted in *gus* positive expression (91.71%) and transformation frequency (58.32%) that was higher when compared with material originating from the leaf base (75.19% and 44.45% , respectively) (Fig. 3A). However, the influence of organ and tissue origin in compatibility of *Agrobacterium* with plant-host tissues varies depending on the plant species. In perilla (*Perilla frutescens*), hypocotyl, cotyledon and leaf explants were examined in *A. tumefaciens* -mediated transformation experiments and hypocotyl explant, resulted in the highest transformation efficiency. (Lee *et al*., 2005). Therefore, we may also need to consider both explant tissue and genotype when developing procedures for *A. tumefaciens*-mediated transformation. While the current study utilized a single genotype, fine-tuning of the protocol will require examining multiple diverse genotypes.

Wounding explants before bacteria inoculation can promote transformation efficiency. Because plant cells have a hard and thick cell wall, wounding produces a channel across the tissue that allows *A. tumefaciens* access to deeper target tissue. As a result, wounding by sonication improved transformation frequency in explants (89.90%) (Fig. 3B). The strength of this method is that it results in thousands of microwounds on and below the surface of the plant tissue (Trick and Finer, 1998). .

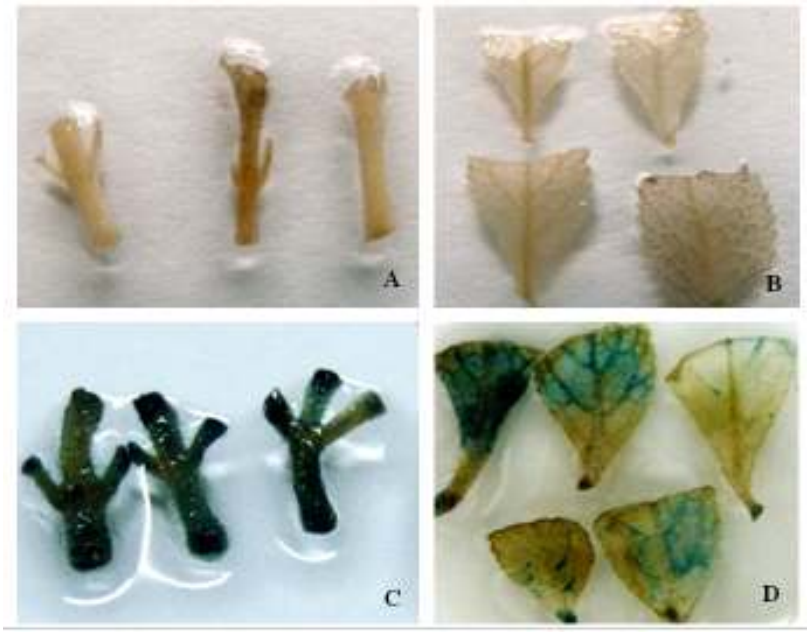

Fig. 2. Nodal segment and leaf base tissue of teak after subjected to GUS histochemical assay after *A. tumefaciens* -mediated transformation for 3 days, A, B: control tissue, C, D: blue stained tissue resulted from gus^{+} expression

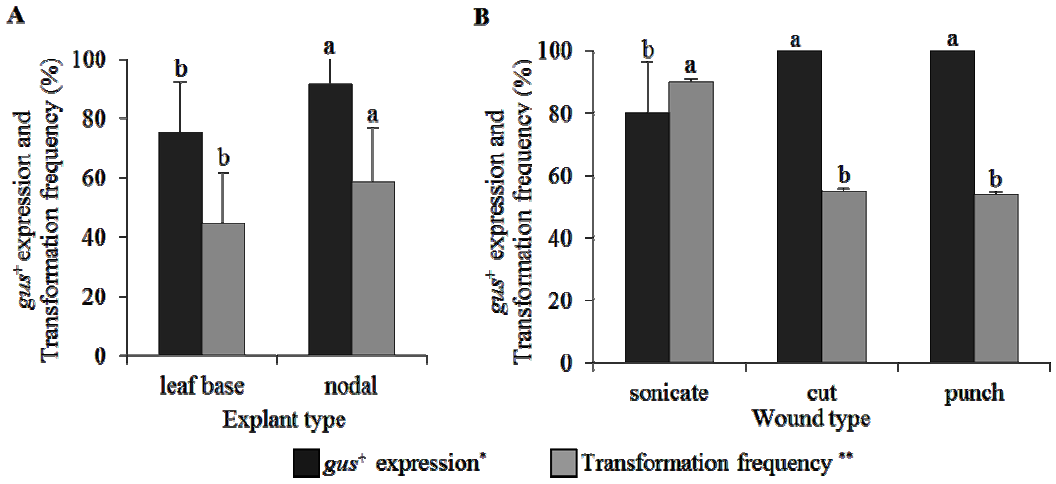

Fig. 3. Influence of various parameter of A: explant type and B: wound type on the percentage of transient *gus* expression. Data are the means ± SD, different letters on the bars are significantly different at 1% probability level by Duncan's Multiple Range Test

* *gus*$^+$ expression percentage was defined as the total number of shoots showing GUS activity/total number of shoots ×100

**Transformation frequency was calculated as the area of *gus*$^+$ expression per shoots/the total area of explants ×100

Effect of *A. tumefaciens* strain on percentage of transient *gus* expression

In this study, two strains (EHA 105 and AGL1) of *A. tumefaciens* harboring the same binary plasmid (pCAMBIA 1304) were tested for their capability to infect nodal and leaf base. Transformation efficiency was evaluated by the percentage of *gus* positive expression and the transformation frequency in regardless of explant types. The results indicate that *A. tumefaciens* strain, EHA 105 (83.29%) was more effective on transformation, as measured by *gus* expression, than AGL1 (72.9%) (Fig. 4A). *A. tumefaciens* strains differ in their ability infect plants and transfer T-DNA (Suzuki *et al*., 2001; Khanna *et al*., 2004). In cauliflower (*Brassica oleracea* var. botrytis), a high level of *gus* expression was observed in explants infected with *A. tumefaciens* strain GV2260, while co-cultivation with LBA4404 strain resulted in very low levels of expression (Chakrabarty *et al*., 2002). Generally, the use of a highly virulent strain of *A. tumefaciens* enhances the frequency of transformation in recalcitrant plants (De la Riva *et al*., 1998).

A. tumefaciens density was also determining the transformation efficiency. Explants were inoculated with *A. tumefaciens* (strain EHA 105) culture of varying bacteria suspension concentration as 0.5 x 10^9 and 1.0 x 10^9 cfu ml^{-1}. It was observed that the highest percentage of *gus* positive expression was observed when bacteria concentration was 1.0 x 10^9 cfu ml^{-1}. (85.04%) (Fig. 4B). *A. tumefaciens* density higher than 1.0 x 10^{10} cfu ml^{-1} usually damaged the plant cells. Nevertheless, when a higher density of *A. tumefaciens* is necessary for recalcitrant explants or species, transformation frequency can be improved by a short inoculation time and gently rinsing the explants after inoculation (Zhao *et al*., 2001; Zhang *et al*., 2003).

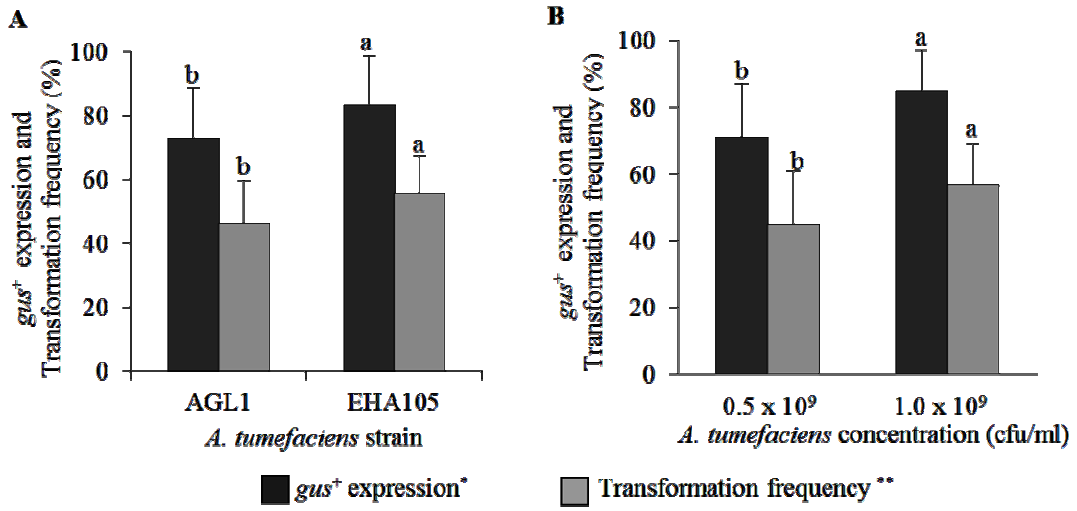

Fig. 4. Influence of various parameters A: *A. tumefaciens* strain and B: *A. tumefaciens* strain EHA 105 concentration on the percentage of transient *gus* expression and the transformation frequency. Data are the means ± SD, different letters on the bars are significantly different at 5 % probability level by Duncan's Multiple Range Test.

* gus^+ expression percentage was defined as the total number of shoots showing GUS activity/total number of shoots ×100

**Transformation frequency was calculated as the area of gus^+ expression per shoots/the total area of explants ×100

Effect of inoculation and co-cultivation period on percentage of transient *gus* expression

The importance of the length of the inoculation period to *gus* expression in the explants was also tested. We inoculated explants with *A. tumefaciens* strain EHA 105 suspension at the concentration of 1.0 x 10^9 cfu ml^{-1} for 1, 3, and 5 hours. Inoculation for 3 and 5 hours developed the high percentage of *gus* positive expression (82 % and 84.9 %, respectively), while inoculation for 5 hours showed transformation frequency higher than 3 hours (Fig. 5A). However, the inoculation period is also associated with loss of viability of the explants and problems with the elimination of the bacteria (Yong *et al*., 2006).

The periods of co-cultivation differed according to plant species. Using the optimal conditions, the length of the co-cultivation period was investigated. After inoculation period, explants were transferred to MS medium containing 100 µM acetosyringone for 3 and 5 days. The results showed, the co-cultivation period for 3 and 5 days promote high the percentage of *gus* positive expression (82%, 84.9%, respectively) and transformation frequency (51.26%, 51.82%, respectively) (Fig. 5B). Co-cultivation 2-7 days is generally considered to be suitable for *A. tumefaciens* -mediated transformation as reports in many plant species. However, co-cultivation for 5 days showed over growth of bacteria and difficult to eliminate. Thus, the inoculation period of 5 hours followed by 3 days of co-cultivation was successfully used for teak transformation.

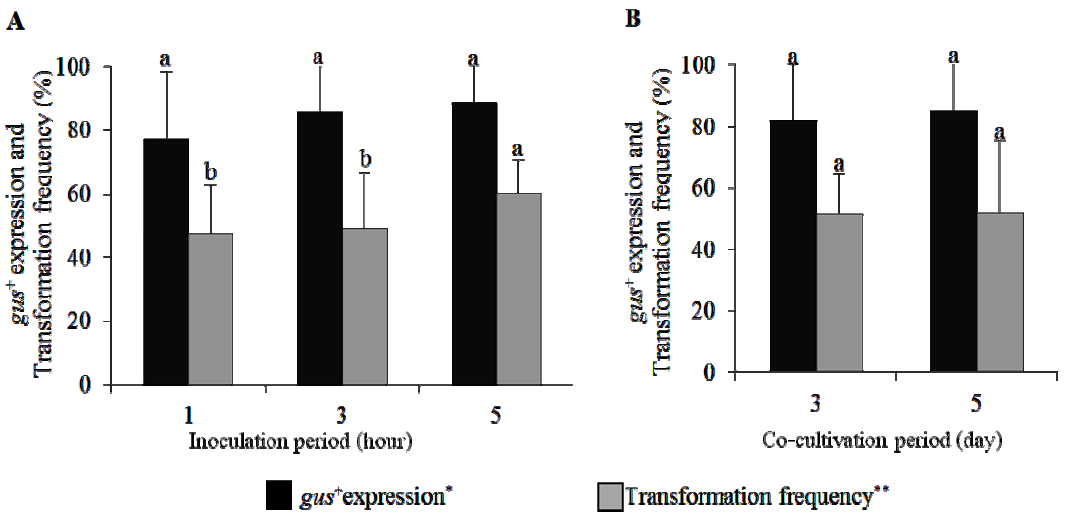

Fig. 5. Influence of various parameters A: inoculation period and B: co-cultivation period on the percentage of transient *gus* expression. Data are the means ± SD, different letters on the bars are significantly different at 5% probability level by Duncan's Multiple Range Test.

* *gus*$^+$ expression percentage was defined as the total number of shoots showing GUS activity/total number of shoots ×100

**Transformation frequency was calculated as the area of *gus*$^+$ expression per shoots/the total area of explants ×100

CONCLUSION

The results obtained in this study revealed the successful transformation of *Agrobacterium tumefaciens*–mediated system in teak. The major key factors: explant type, wounded type, *A. tumefaciens* strains, *A. tumefaciens* concentration, inoculation and co-cultivation period, the optimization of these factors is a critical step as it breaches the limitation of *A. tumefaciens* T-DNA delivery into recalcitrant species as teak. In conclusion, we demonstrated that the teak nodal segment that extra wounding by sonication was more suitable to be used as explants to transform gene into teak. The bacterial strain EHA105 at 1.0 x 10^9 cfu $ml^{-1}$and the inoculation period of 5 hours followed by 3 days of co-cultivation were successfully used for teak transformation.

ACKNOWLEDGEMENT

This research was supported by the grant from the program Strategic Scholarships for Frontier Research Network for the Ph.D. Program Thai Doctoral degree from the Office of the Higher Education Commission, and the Center of Excellence on Agricultural Biotechnology, Science and Technology Postgraduate Education and Research Development Office, Office of Higher Education Commission (AG-BIO/PERDO-CHE), Ministry of Education, Thailand. We thank Prof. D. Hole from Utah State University for the kindness on critical review of the manuscript.

REFERENCES

Behaghel, I. 1999. The state ofteak (*Tectona grandis* L.F.) plantation in the world. Bois Forest Tropical 269: 5-18.

Cerda, F., F. Aquea, M. Gebauer, C. Medina and P. Arce-Johnson. 2002. Stable transformation of *Pinus radiata* embryogenic tissue by *Agrobacterium tumefaciens*. Plant Cell, Tissue and Organ Culture 70: 251-257.

Chakrabarty, R., N. Viswakarma, S.R. Bhat, P.B. Kirti, B.D. Singh and V.L.Chopra. 2002. *Agrobacterium* mediated transformation of cauliflower: optimization of protocol and development of Bt-transgenic cauliflower. Journal of Bioscience 27: 495-502.

Charity, J.A., L. Holland, S.S. Donaldson, L. Graceand C. Walter. 2002. *Agrobacterium*-mediated transformation of *Pinus radiate* organogenic tissue using vacuum-infiltration. Plant Cell, Tissue and Organ Culture 70: 51-60.

Dai, W., Z. M. Cheng and W. Sargent. 2003. Plant regeneration and *Agrobacterium*-mediated transformation of two elite aspen hybrid clones from *in vitro* leaf tissues. *In Vitro* Cellular & Developmental Biology - Plant 39: 6-11.

De la Riva, G. A., J. González-Cabrera, R. Vázquez-Padrn and C. Ayra-Pardo. 1998. *Agrobacterium tumefaciens*: a natural tool for plant transformation. Electronic Journal of Biotechnology 1: 118-133.

Gyves, E. M.-d. and E. Rugini. 2009. Teak, pp. 321-340. *In* C. Kole and T. C. Hall Jr., *eds.* Compendium of Transgenic Crop Plants: Transgenic Forest Tree Species. Blackwell Publishing Ltd.

Jefferson R.A., T.A. Kavanagh and M.W. Bevan. 1987. Gus fusions: *β-glucuronidase* as a sensitive and versatile gene marker in higher plants. EMBO Journal 6: 3901-3907.

Kaosa-ard, A. 1981. Teak It's natural distribution and related factors. Natural History Bulletin of the Siam Society 29: 55-74.

Khanna, H., D. Becker, J. Kleidon and J. Dale. 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.

Lakhsmi-Sita G, G. L. Sreenivas and S. Bhattacharya. 1998. *Agrobacterium*-mediated transformation of sandalwood (*Santalum album* L.) a tropical forest tree. Plant Tissue Culture and Biotechnology. 4: 189-195.

Lee, B.K., S.H. Yu, Y.H. Kim, B.O. Ahn, H.S. Hur, S.C. Lee, Z. Zhang and J.Y. Lee. 2005. *Agrobacterium*-mediated transformation of perilla (*Perilla frutescens*). Plant Cell, Tissue and Organ Culture 83: 51-58.

McGranahan, G.H., C.A. Leslie, S.L. Uratsu, L.A. Martin and A.M. Dandekar. 1988. *Agrobacterium*-mediated transformation of walnut somatic embryos and regeneration of transgenic plants. Nature Biotechnology 6: 800-804.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum 15: 473-497.

Norwati, A., A. Ruslan, M. Norwati and B. Norlia. 2007. A biolistic approach for the transfer and expression of *uidA* gene in nodal segments of teak (*Tectona grandis*). Asia-Pacific Journal of Molecular Biology and Biotechnology 15: 115-120.

Opabode, J.T., 2006. *Agrobacterium*-mediated transformation of plants: Emerging factors that influence efficiency. Biotechnology Molecular Biology Review 1: 12-20.

Schuerman, P.L. and A.M. Dandekar. 1993. Transformation of temperate woody crops: progress and potentials. Scientia Horticulturae 55: 101-124.

Suzuki, S., K.Supaibulwatana, M. Mii and M. Nakano. 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.

Tang, W., H. Luo and R.J. Newton. 2004. Effect antibiotics on the elimination of *Agrobacterium tumefaciens* from loblolly pine (*Pinus taeda*) zygotic embryo explants and on transgenic plant regeneration. Plant Cell, Tissue and Organ Culture 79: 71-81.

Thakur, A. K., S. Sharma and D. K. Srivastava. 2005. Plant regeneration and genetic transformation studies in petiole tissue of Himalayan poplar (*Populus ciliata* Wall.). Current Science 89: 664-668.

Trick, H.N.and J.J. Finer. 1998. Sonication assisted *Agrobacterium* mediated transformation of soybean [*Glycine max*(L.) Merrill] embryogenic suspension culture tissue. Plant Cell Reports 17: 482-488.

Widiyanto, S. N., A. Sukmawan, N. Haro and H. Rahmania. 2009. Transient expression of β-glucuronidase reporter gene in *Agrobacterium*-inoculated shoots of various teak clones. African Journal of Biotechnology 8: 2143-2150.

Yong, W. T. L., J. O. Abdullah and M. Mahmood. 2006. Optimization of *Agrobacterium*-mediated transformation parameters for *Melastomataceae* spp. using *green fluorescent protein* (*GFP*) as a reporter. Scientia Horticulturae 109: 75-85.

Zhang ,W., S. Subbarao, P. Addae, A. Shen, C. Armstrong, V. Peschke and L. Gilbertson. 2003. *Cre/lox* mediated gene excision in transgenic maize (*Zea mays* L.) plants. Theoretical and Applied Genetics 107: 1157-1168.

Zhao, Z-Y, W. Gu, T. Cai, L. Tagliani, D. Hondred, D. Bond, S. Schroeder, M. Rudert and D. Pierce. 2001. High throughput genetic transformation mediated by *Agrobacterium tumefaciens* in maize. Molecular Breeding 8: 323- 333.