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The Establishment of *Agrobacterium*-Mediated Gene Transformation in Rubber Tree Through Organized Explants

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Abstract Rubber tree, belongs to the genus *Hevea*, is an economically important crop of Thailand. To improve it agronomical trait for glyphosate-resistant *in vitro* gene transformation through *Agrobacterium* was conducted. The bacteria carrying plasmid pCAMBIA 1304, harboring *gus* as screenable marker genes and *EPEPS* gene was used. Shoots and nodal segments were inoculated with *A. tumefaciens* for 15, 30, 45 minutes and 2 hours and kept in darkness on rotary shaker at 100 rpm and 28 ^{o}C for 6 hours. The results revealed that inoculation for 30 minutes gave the higher survival rate from shoot segments after being cultured on glyphosate containing MS medium for one month. Assessment of transformed shoots revealed positive results in GUS histochemical assay. The integration of genes in plant genome was positively detected by PCR technique and dot blot hybridization.

Keywords*:* Gene transformation, Agrobacterium tumefaciens, shoot segments, rubber tree

Introduction

Rubber tree (*Hevea brasiliensis* Muell. Arg.), belonging to the family Euphorbiaceae, is an economically important perennial tree grown in Thailand and Southeast Asia as the commercial source of natural rubber. Although rubber is formed in over 2,000 species of plants distributed among 300 genera of seven families (Priya *et al.,* 2006), in the present day only one species of plant, *Hevea brasiliensis*, is used widely for the industrial raw material of natural rubber. Natural rubber (cis-1,4-polyisoprene) is an important commercial production. It has molecular structure and high molecular weight. This rubber has high performance properties that cannot be easily replaced by synthetically produced polymers. The *Hevea* latex is a rubber containing cytoplasm eliminated from laticiferous cells, upon managed bark wounding or tapping. The laticifers are periodically excreted from the cambium, forming

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latex cell mantels in the soft bark of the rubber tree. It's contained polysomes and numerous rubber particles accounting for 35–45% of the total latex volume (Venkatachalam *et al.,* 2009). The financial records for 42% of the rubber consumed worldwide (Lardet *et al*., 2011).

Rubber tree is a heterozygous perennial plant with a long reproductive maturity. Rubber tree is propagated mostly by grafting high producing latex buds on heterogeneity of the rootstocks produced from seeds (Hua *et al.*, 2002). Breeding program takes up to 25 years to select and recommend clones. Accordingly, improvement of this tree via conventional breeding is very difficult and takes so long time (Lardet *et al*., 2011). In addition, latex yield in rubber plantations has been significantly increased by releasing high latex yielding clones for cultivation over the past decades, an estimated annual rubber production loss due to tapping panel dryness (TPD) was 15–20% (Chen *et al.,* 2003). The immediate effect of TPD is the appearance of partial dry zones (no latex flow) and in the advanced stage, the tapping panel may even become completely dry and other symptoms such as browning, thickening, or even flaking of bark can occur (Sookmark *et al.*, 2002).

An alternative strategy that may potentially shorten breeding time is genetic engineering. It can be used to produce desirable agronomic traits quickly and efficiently (Arokiaraj *et al.,* 2002). An efficient *Agrobacterium tumefaciens*-mediated genetic transformation procedure has been developed from friable integument callus line for clone PB260 with a good frequency of integration and with a majority of transgenic calli (Montoro *et al.,* 2003). An efficient genetic transformation procedure using a recombinant green fluorescent protein (*gfp*) has been developed in *Hevea brasiliensis* clone PB260 (Leclercq *et al.,* 2010). Glyphosate selection has a number of advantages over other commonly used selectable markers. For example, in maize, glyphosate selection can yield a very low frequency of non-transgenic escapes compared to kanamycin, where selection is quite inefficient. Glyphosate is not detoxified, and consequently, there is no cross-protection afforded to adjacent cells. In addition, glyphosate is a highly mobile selection agent and translocates throughout the plant and so is less dependent on direct contact of the target tissue than some other selection agents (Howe *et al.,* 2002). A genetic transformation protocol was developed using the transfer of a synthetic *CP4 EPSPS* transgene, as a conditional positive selectable marker, into commercially relevant zonal pelargoniums using an *Agrobacterium* tumefaciens strain in combination with a novel step-down glyphosate selection system. Moreover, the presence of *Agrobacterium* in transformed tissues, even after the use of bacteriostatic antibiotics, can give false-positive PCR results (Boase *et al.,* 2012). So, the aim of present study was to improve gene

transformation procedure in *Hevea brasiliensis* using *Agrobacterium* for transferring some important genes in the future.

Materials and methods

Plant Material

Seeds from a native clone of rubber tree, naturally grown at Prince of Songkla University, Hatyai campus, Songkhla province, Thailand, were collected and used as explants for zygotic embryo culture. After 2 weeks of culture, seedings were obtained and they were excised into two parts, shoot tip and hypocotyl node. The two explants were cut into 0.5 cm in length and cultured on shoot induction medium (SIM) supplemented with 5 mg/l 6-benzyladenine (BA), 3% sucrose and 0.05% activated charcoal as described by Te-chato and Muangkaewngam (1992). The medium's pH was adjusted to 5.7 with 0.1 N HCl or KOH before adding 0.75% agar and autoclaved at 1.05 kg/cm^2, at 121 ℃ for 15 minutes. The cultures were maintained at 28±0.5^o C under fluorescent lamps at light intensity of 12.5 µmol/m^2/s, 14 hour photoperiod for 1 month.

Bacterial plasmid

Agrobacterium tumefaciens strain EHA105 containing the plasmid pCAMBIA1304-EPEPs which harbored ß-glucuronidase (*gus*) and 5-enolpyruvylshikimate-3-phosphate synthase (CP4 EPSPS) genes (Figure 1) was used in this study. A single colony of this bacteria was pick out and suspended in 25 ml liquid LB medium (10 g/L tryptone, 5 g/L Bacto yeast extract, 5 g/L NaCl, pH 7.0) containing 50 mg/L kanamycin and incubated on a rotary shaker at 100-150 rpm in the dark at 28 ℃. After proliferation in LB medium overnight the cells were collected and adjusted density by spectrophotometer at optical density (OD) of 600 nm at 0.6.

Inoculation and selection of transgenic calli

The shoot tips and nodal segments were immersed in 25 ml the *A. tumefaciens* suspension at optical density (OD) of 600 nm at 0.6 and kept in darkness on rotary shaker at 100 rpm at 28^{o}C for 15, 30, 45 and 102 min. The two explants were placed on sterile tissue papers before transfer to co-cultivation medium (SIM containing 200 µM acetosyringone) and kept in the dark at 28^{o}C for 3 days. After co-cultivation, The explants were washed with liquid SIM containing 400 mg.L^{-1}cefotaxime for 10 min to remove excess

bacteria. Then explants were transferred to SIM supplemented with 200 mg.L^{-1} cefotaxime to eliminate bacteria for 1 month and subcultured every 2 weeks. The inoculated explants were then transferred to selective medium (SIM containing 0.5 mM glyphosate) for early screening of transformed tissues. The cultures were maintained under 12.5 µmol/m^2/s illumination, 14 hours photoperiod at 26±2 ℃.

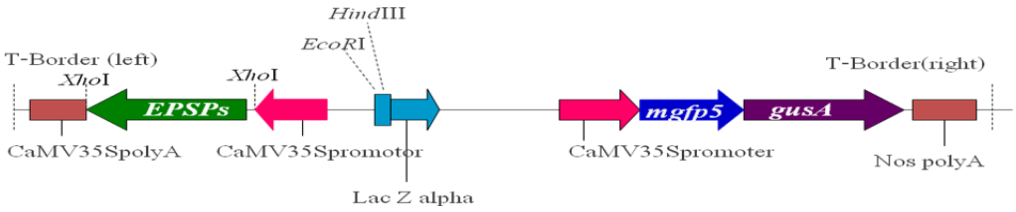

Fig. 1. Schematic map of T-DNA region of the binary vector pCAMBIA1304-EPEPs containing the *gus*A gene containing an intron as reporter genes and the *EPSPs* gene conferring glyphosate (*N*-phosphonomethyl glycine) resistance.

Histochemical GUS assay and transient assessment

GUS assays were carried out using protocols described by Jefferson *et al.* (1987). GUS expression was observed by immersing inoculated explants in X-gluc buffer consisiting of 2 mM X-gluc, 100 mM sodium phosphate buffer pH 7.0, 0.5 mM potassium ferricyanide, and 0.5 mM potassium ferrocyanide. The explants were incubated overnight at 37^{o}C in the dark, and washed with absolute methanol for overnight. The percentage of *gus* expression, in the presence of blue spots, were recorded and scored under stereomicroscope.

Molecular analysis of the transformed plantlets by PCR analysis and Dot blot hybridization

Genomic DNA was isolated from young leaf (0.05 g) of non-transformed and transformed plantlets after 1 months of culturing on selective medium by the CTAB method (Doyle and Doyle, 1990). The *gusA* gene fragment was amplified using forward primer sequence F-primer 5'-CTGCGACGCTCACACCGATAC-3’ and reverse primer sequence R-primer 5’-TCACCGAAGTTCATGCCAGTCCAG-3’. The forward and reverse primer sequences for the *EPSPs* gene amplification were 5’-CCATTCCGCTCGAGATGGCACAAATTAACAACATGGC-3’ and 5’-ATCCACCGCTCGAGCGGTCATCAGGCAGCCTTCG

TAT-3’, respectively. The reaction mixture contained 1 µl of genomic DNA (20 ng), 0.5 µl of each primer (5 pmol), 4 µl of dNTP mix (1 mM each), 2

µl ml of PCR buffer, 0.1 µl of *Taq* DNA polymerase (1 U/ml) and the volume was adjusted to 20 µl with sterile distilled water. The PCR conditions included hot start at 96^{o}C for 2 m, followed by 30 cycles of denaturation (96^{o}C, 20 s), annealing (55^{o}C, 1 min) and extension (72^{o}C, 2 min), with a final extension of 5 min at 72^{o}C. PCR amplified products were resolved in 1.0 % agarose gel with ethidium bromide and visualized by gel documentation at 260 nm of UV.

For Dot blot hybridization, 4 µg of the genomic DNA of non-transformed and transformed plantlets and 2 µL of PCR products were dropped on a nylon membrane (Hybond-N, Amersham). Blotted membrane was dried by incubation at 80 º C for 1 hour. The membrane was pre-hybridized in hybridization solution (5X SSC, 0.1 % N-lauroylsarcosine, 0.02 % SDS and 1X Blocking solution) for 1 hour at 65 ºC. Hybridization was performed with DIG-labeled DNA probe overnight at 65 ºC, which was generated using the PCR DIG Probe Synthesis Kit (Roche Applied Science). Hybridized membrane was washed twice in low stringency buffer (2x SSC, 0.1 % SDS) for 15 min, twice in high stringency buffer (0.1x SSC, 0.1 % SDS) for 15 min and once in washing buffer) 1x Maleic acid buffer, 0.3% Tween 20 (for 10 min. The membrane was blocked in blocking solution (Dilute 10x blocking solution 1: 10 with Maleic acid buffer) for 30 min. After that the anti-digoxigenin conjugate alkaline phosphate was added into blocking solution and incubated for 30 min. The membrane was then transferred to detection buffer) 0.1 M Tris-HCl, 0.1 M NaCl) for 3 min at room temperature. Finally, the membrane was dropped by chemiluminescent substrate (CDP star^{TM}) and exposed to *Kodak BiomaX-Omat* film for autoradiography. The film was washed with developer and fixer solution after exposure in the cassette for 60 min.

Statistical analysis

Data were analyzed using completely randomized design (CRD) and the differences among means were separated using Duncan's multiple range test (DMRT). All statistical analyses were performed at the level 5% using statistically analysis system (SAS).

Results

Types of explants and inoculation time

Infection time is the most important step in the transformation process. However, conditions for inoculation may considerably affect the efficiency of the transformation process. In this study, the transformation efficiency was compared under different inoculation times and types of explants (shoot tip and

cotyledonary node). Between the two explants immersed in *A. tumefaciens* suspension at optical density of 600 nm at various times and co-cultivated for 3 days, it revealed that shoot tip explants gave the better results than nodal explants in all parameters tested. Transient GUS activity of shoot tip explants increased with inoculation time, reaching 72.11% GUS expression for 120 min, significant difference with the other time of inoculation (Table1, Figure 2). However, longer periods of inoculation affected survival rate of explants. The highest survival rate of shoots at 77.78% was obtained from 30 minute inoculation as shown in Table 2 and Figure 3. Unfortunately, all nodal explants died after one month of culture on selection medium.

Table 1. Effect of inoculation times on percentage of GUS activities after 2 weeks of culture

Inoculation time	Percentage of blue spots from		$Average_{time}$
	Shoot tip explants	Nodal explants	
15 min	28.89c	0.00d	14.44C
30 min	46.66b	0.00d	23.33BC
45 min	51.00b	0.00d	25.50B
120 min	72.11a	0.00d	36.05A
$Average_{source}$	49.66A	0.00B	**

** significant difference ($p< 0.01$)

Mean with different capital letter indicate significant differences among treatments and means with different small letter indicate significant differences among treatment combinations according to DMRT.

Table 2. Effect of inoculation time on percentage of glyphosate-resistant shoot tip and nodal explants of rubber tree after 4 weeks of culture

Inoculation time	Percentage of glyphosate-resistance explants		$Average_{time}$
	Shoot tip explants	Nodal explants	
15 min	55.56ab	0.00d	27.78AB
30 min	77.78a	0.00d	38.89A
45 min	33.33bc	0.00d	16.67B
120 min	22.22cd	0.00d	11.11B
$Average_{source}$	47.22A	0.00B	*

** significant difference ($p< 0.01$)

Mean with different capital letter indicate significant differences among treatments and means with different small letter indicate significant differences among treatment combinations according to DMRT.

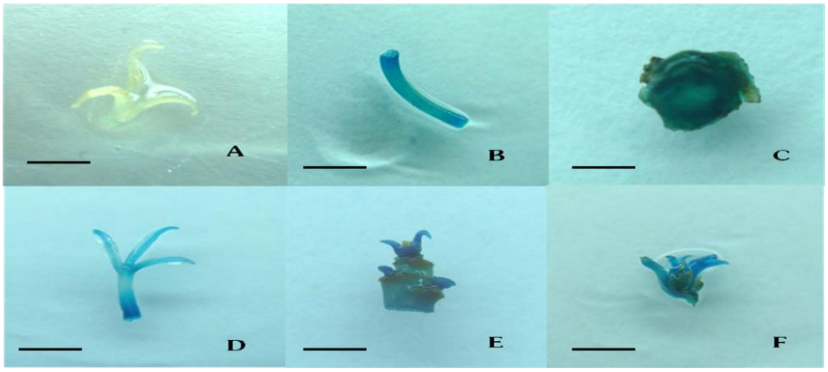

Fig. 2. Histochemical assay of β-glucuronidase (GUS) activity in transgenic explants transformed by *Agrobacterium* harboring pCAMBIA1304-EPSPs containing the *gus*A gene and the *EPSPs* gene conferring glyphosate (*N*-phosphonomethyl glycine) resistance (bars= 5 mm).

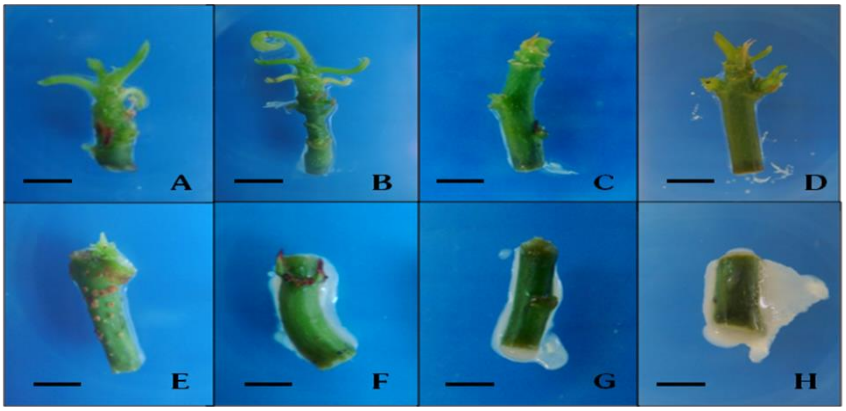

Fig. 3. Morphological appearance of shoot tip and nodal explants inoculated with *Agrobacterium* harboring pCAMBIA subsequent to culture on co-cultivation medium for 3 days (bars = 5 mm).

Molecular analysis of the transformed plantlets by PCR analysis and dot blot hybridization

To confirm the presence of the *gus* gene in transformed rubber tree, PCR analysis was conducted to evaluate putative transformants, along with non-transgenic plant (negative control). Specific primers for the *gus* gene were designed to amplify a 919 bp DNA fragment. The result showed the presence of *gus* gene from plasmid as positive control and all four putative-transgenic shoot tips (Figure 4), whereas the corresponding band was not detected in the non-transgenic tissues after 1 month of transformation. In case of dot blot hybridization using *gus* gene probe, the genomic DNA of 4 samples showed dark color dots indicating the success of gene transfer into plant genome (Figure 5).

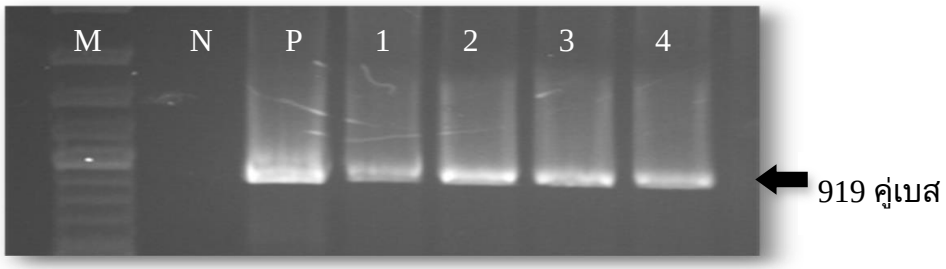

Fig. 4. PCR analysis showed the presence of *gus* gene at 919 bps from different plant genomes. (M: marker, N: negative control, P: positive DNA control, 1-4: transformed shoots)

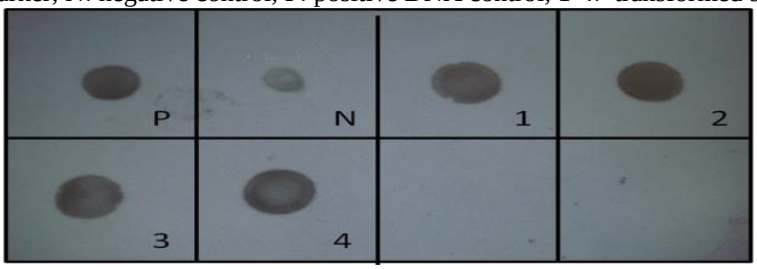

Fig. 5. Dot blot hybridization using *gus* gene probe showed dark color dots into plant genome. (M: marker, N: negative control, P: positive DNA control, 1-4: transformed plantlets obtained from shoot tip explants)

Discussion

Types of explants and Inoculation time

In this report, shoot tip immersed in *A. tumefaciens* suspension at optical density of 600 nm at 0.6 for 30 min gave the better results than nodal explants in GUS expression. Transient expression of GUS activity obtained from shoot tip explants increased with increase in inoculation time and reach to 72.11% after inoculation for 120 min. The periods of inoculation seem to be effective for the efficient transfer of the T-DNA to plant cells (Kondo *et al.*, 2000). However, longer periods of inoculation gave negative effect on survival rate of explants. According to this result, the inoculation period was critical for transformation. The highest survival rate of shoots was obtained from 30 minutes inoculation (77.78%). Generally, inoculation time applied in transformation procedures varied from species to species. It took about 30 min for immature embryo of oil palm (Abdulah *et al.*, 2005) and for alfafa calli (Zhang *et al.*, 2010), 40 min for embryogenic callus of *Parthenocissus tricuspidata* (Yang *et al.*, 2010), and up to 2 h for tobacco leaf ring (Vinad Kumar *et al.*, 2004). Interestingly, Blanc *et al.* (2006) reported that successful in transformation process of *H. brasiliensis* took only one second submerging integument calli in *A. tumefaciens* solution. Contrary results were obtained

from the present study. Fisrtly, different explants were used. In the present study, shoot tip explants were applied. Organised tissues seem to resist to *A. tumefaciens* solution better than callus, thus, time required for inoculation is longer. Secondly, regenerability of those explants was far different. Callus was reported to be very sensitive to all stimulants applied *in vitro*, e.g. toxin, colchicine (Te-chato *et al.*, 1995). Plantlet regeneration from callus just after treating with those chemicals was not reported. In case of nodal explants, they were died after 1 month of culture on selection medium, which was SIM medium containing 0.5 mM glyphosate due to the long inoculation period (120 min). This evident caused an overgrowth of *Agrobacterium* and decreased the survival rate (22.22%) of plant tissues. A similar result has also been reported in many plant species, such as *Parthenocissus tricuspidat* (Yang *et al.*, 2010) and alfafa (Zhang *et al.*, 2010). A longer period of inoculation decreased the percentage of survival rate of explants co-cultured with *Agrobacterium*.

Molecular analysis of the transformed plantlets by PCR analysis and dot blot hybridization

PCR analysis confirmed the presence of *gus* gene at size of 919 bps from transformed plantlets. The presence of *gus* genes was confirmed in 4 transgenic plants and in the plasmid DNA, whereas the corresponding band was not detected in the non-transgenic control (Fig. 5a). Dot blot hybridization confirmed the positive signals of *gus* gene in the genomic DNA of transformed plantlets of 4 samples. The positive transgenic plant samples developed dark black spots as well as the positive control sample indicating the success of gene transfer into plant genome (Fig. 1E). However, negative control showed slightly pale spots without a clear signal. This might be an error due to the procedure of washing the membrane with low concentration of low and high stringency buffers. These two buffers consist of SSC solution (sodium chloride and sodium citrate) and SDS solution that might affect specific integration between *gus* probe and membrane.

Conclusion

Shoot tip explants inoculated with *A. tumefaciens* carrying plasmid pCAMBIA 1304, harboring *gus* as screenable marker genes and *EPEPS* gene for 30 minutes gave the highest survival rate of the explants. GUS histochemical assay of transformed shoot tips revealed positive results. Similar results were also detected by PCR technique and dot blot hybridization.

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References

Abdullah, R., Zainal, A., Heng, W. Y., Li, L. C., Beng, Y. C., Phing, L. M., Sirajuddin, S. A., Ping, W. Y. S., Joseph, J. L., Jusoh, S. A., Muad, M. R. and Huey, Y. L. (2005). Immature embryo: A useful tool for oil palm (*Elaeis guineensis* Jacq.) genetic transformation studies. Journal biotechnology 8:24-34.

Arokiaraj, P., Rueker, F., Obermayr, E., Shamsul Bahri, A. R., Hafsah, J., Carter, D. C. and Yeang, H.Y. (2002). Expression of human serum albumin in transgenic *Hevea brasiliensis*. Journal of rubber research 5:157–166.

Blanc, G., Baptiste, C., Oliver, G., Martin, F. and Montoro, P. (2006). Efficient *Agrobacterium tumefaciens*-mediated transformation of embryogenic calli and regeneration of *Hevea brasiliensis* Mull Arg. plants. Plant cell reports 24:724–733.

Bimboim, H. C. and Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic acids research 7:1513-1523.

Boase, M. R., Harriman, R. W., Smith, F. D. and Deroles, S. C. (2012). Herbicide-resistant, transgenic zonal pelargoniums produced by step-down glyphosate selection and plantlet recovery in the present of aromatic amino acids. In vitro cellular and developmental biology-plant 48:313-323.

Chen, S., Peng, S., Huang, G., Wu, K., Fu, X. and Chen, Z. (2003). Association of decreased expression of a Myb transcription factor with the TPD (tapping panel dryness) syndrome in *Hevea brasiliensis*. Plant molecular biology 51:51–58.

Doyle, J. J. and Doyle, J. L. (1990). Isolation of plant DNA from fresh tissue. Focus 12:13-15.

Howe, A. R., Gasser, C. S., Brown, S. M., Padgette, S. R., Hart, J., Parker, G. B., Fromm, M. E. and Armstrong, C. L. (2002). Glyphosate as a selective agent for the production of fertile transgenic maize (*Zea mays* L.). Plants. Molecular Breeding 10:153–164.

Hua, Y. W., Huang, T. D. and Huang, H. S. (2010). Micropropagation of self-rooting juvenile clones by secondary somatic embryogenesis in *Hevea brasiliensis*. Plant breeding 129:202-207.

Jefferon, R. A., Kananagh, T. A. and Bevan, M. W. (1987). GUS fusion: ß-glucuronidase as a sensitive and versatile gene fusion marker in higher plant. EMBO journal 6: 3301-3306.

Kondo T., Hasegawa, H. and Suzuki, M. (2000). Transformation and regeneration of garlic (*Allium sativum* L.) by A*grobacterium* mediated gene transfer. Plant cell reports 19:989–993.

Lardet, L., Leclercq, J., Benistan, E., Dessailly, F., Oliver, G., Martin, F. and Montoro, P. (2011) Variation in GUS activity in vegetatively propagated *Hevea brasiliensis* transgenic plants. Plant cell reports 30:1847-1856.

Leclercq, J., L. Lardert, F. Martin, T. Chapuset, G. O. and Montoro P. (2010). The green flurescent protein as an efficient selection marker for *Agrobacterium tumefaciens*-mediated transformation in *Hevea brasiliensis* (Muell. Arg.). Plant cell reports 29:513-522.

Montoro, P., Rattana, W., Renaud, V. P., Ferri, N. M., Monkolsook, Y., Kanthapura, R. and Adunsadthapong, S. (2003). Production of *Hevea brasiliensis* transgenic embryogenic callus lines by *Agrobacterium tumefaciens*: roles of calcium. Plant cell reports 21:1095-1102.

Priya, P., Venkatachalam, P. and Thulaseedharan, A. (2006). Molecular cloning and characterization of the rubber elongation factor gene and its promoter sequence from rubber tree (*Hevea brasiliensis*): A gene involved in rubber biosynthesis. Plant science 171:470-480.

Sookmark, U., Pujade-Renaud, V., Chrestin, H., Lacotem, R., Naiyanetr, C. and Seguin, M. (2002). Characterization of polypeptides accumulated in the latex cytosol of rubber trees affected by the tapping panel dryness syndrome. Plant and cell physiology 43:1323–1333.

Te-chato, S. and Muangkaewngam, A. (1992). Tissue culture of rubber *In vitro* micropropagation of rubber. Songklanakarin Journal of science and technology 14:123-132.

Te-chato, S., Suranilpong, P. and Chuenjit, S. (1995). Screening of rubber callus resistant to phytophthora leaf fall agent. Journal of Science and Technology 17:7-16.

Venkatachalam, P., Thulaseedharan, A. and Raghothama, K. (2009). Molecular identification and characterization of a gene associated with the onset of tapping panel dryness (TPD) syndrome in rubber tree (*Hevea brasiliensis* Muell.) by mRNA differential display. Molecular biotechnology 41:42–52.

Vinod Kumar, S. and Rajam, M. V. (2004). Polyamines enhance *Agrobacterium tumefaciens vir* gene induction and T-DNA transfer. Plant science 168:475-480.

Yang, Y., Bao, M. and Liu, G. (2010). Factors affecting *Agrobacterium*-mediated genetic transformation of embryogenic callus of *Parthenocissus tricuspidata* Planch. Plant cell, tissue and organ culture 102:373–380.

Zhang, H., Huang, Q. M. and SU, J. (2010). Development of alfalfa (*Medicago sativa* L.) regeneration system and *Agrobacterium*-mediated genetic transformation. Agricultural Sciences 9:170-178.

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