Agrobacterium-MEDIATED TRANSFORMATION OF *Cry1Ab* GENE INTO *Tectona grandis* L.(TEAK)

Prakay Onwimol[1,2], Sontichai Chanprame[1,2,3] and Sermsiri Chanprame[1,2,4]
[1]Center for Agricultural Biotechnology, Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom, 73140, Thailand and the Center of Excellence on Agricultural Biotechnology (AG-BIO/PERDO-CHE), Bangkok 10900, Thailand
[2]The Center for Advance Studies for Agriculture and Food, Kasetsart University Institute for Advance Studies, Kasetsart University, Bangkok 10900, Thailand (CASAF, NRU-KU, Thailand)
[3]Department of Agronomy, and [4]Department of Horticulture, Faculty of Agriculture at Kamphaeng Saen, Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom, 73140, Thailand
Corresponding author: agrsrc@ku.ac.th

(Received: March 5, 2017; Accepted: May 22, 2017)

ABSTRACT

A binary vector pCAMBIA1302-Ab containing*Cry1Ab* and *hygromycin phosphotransferase* (*hpt*) genes was used in the transformation of *in vitro* teak nodes and multiple shoots clumps. The explants were submerged in suspension culture of *Agrobacterium tumefaciens* strain EHA105 (OD_{600}=1.2) then were sonicated for 3 min in sonicator bath followed by vacuum-infiltration for 5 min. The co-cultivation was performed for 3 days on MS medium containing 100 µM acetosyringone. Two months after selection on 50 mgL^{-1}hygromycin containing medium, calli were formed. Total RNA was extracted from these calli and was subjected to first-stranded RT-PCR analysis of *Cry1Ab* gene. The result indicated the presence of *Cry1ab* transcript in all selected calli. A single shoot developed from a multiple shoots clump cultured on selective medium. The 4-weeks-old putative transformed shoot also showed positive results of *Cry1Ab* and *hpt* genes by PCR analysis. However, chimera tissue was observed in this shoot. The Cry1Ab protein in transgenic tissue was also determined using Bt-Cry1Ab/1Ac ELISA Kit. The expressedCry1Ab protein in putative transformed shoot was confirmed in the leaves that contained *Cry1Ab* and *hpt* genes.

Keywords*:* Genetic transformation, tree, insect resistance gene, *Agrobacterium*

INTRODUCTION

Teak (*Tectona grandis* L.) is one of the highly demanded timber-producing trees of the tropical zone. The demands for teak wood and wood products are continuously increasing throughout the world but its natural resources are rapidly depleting (Pandey and Brown, 2000). Thus, the large scale plantations are promoted beyond its native countries in Asia (i.e. Thailand, Indonesia, Malaysia) and Africa (i.e. Ivory Coast, Congo, Nigeria), and Latin America (i.e. Brazil, Costa Rica, Panama and Honduras) (White, 1991).However, mono-cropping plantation system of teak is confronted with many problems, and the big problem is infestation of various pest species. The teak defoliator (*Hyblaea puera* Cramer) and teak bee-hole borer (*Xyleutes ceramicus* Walker) are serious insect pests in teak plantation. Low growth rate and low quality of wood normally occurred when these two insect pests come into the plantation (Nair *et al*., 1984). There are many approaches that tried to solve these problems such as spraying with *Bacillus thuringiensis (Bt)* on the leaves. The main limitation of this technique, however, is the poor coverage on plant surfaces which cannot reach the teak bee-hole borer

inside the tree trunk. The conventional breeding for insect pest tolerance teak cultivar can be done but still needs long period of time to succeed (Suseno and Wibisono, 2000).

Therefore, genetic transformation in order to create an insect-resistant teak is a possible way to improve pest management in the teak plantation. A group of *Bt* genes has been proven to be effectively used in various crops to control the damaged due to the insects in the order Lepidoptera, Diptera and Coleoptera (Chilcott and Wigley, 1993). The Bt endotoxin can only be digested by the enzymes in the guts of these specific insect species and become toxic to these insects within few days and that prevent the damage done by insects. Thus, Bt toxin is not harmful to human due to the lack of specific enzymes in human body (Knowles, 1993).Thus, BT can reduce the risk of pesticides use and reduce the amount of chemical residues in agricultural produces. However, the big problem of gene transformation in teak is low transformation rate and poor regenerating ability. The introduction of useful agricultural genes into teak or woody plant species has been delayed when compared to other species. Teak transformation has been reported using both biolistic and A*grobacterium tumefaciens*-mediated methods to transform the *gus* reporter gene. Widiyanto *et al.* (2009) reported the success of transient gene expression (94.6%) after transferring *gus* reporter gene into the multiple shoots clumps of teak using *A. tumefaciens*- mediated method. Likewise, Sontikun *et al*. (2013) reported the success of transformation of *gus* reporter genes into the nodal segment tissue of teak by *A. tumefaciens*-mediated method of approximately 58.32%.For the transformation with insect resistance gene, Norwati *et al.* (2011) successfully transferred *Cry1Ab* gene into teak tissue *via* particle bombardment. In this report, we demonstrated the successful *Agrobacterium*-mediated gene transformation of *Cry1Ab* gene into teak using the multiple shoots clump explant.

MATERIALS AND METHODS

Plant materials

The *in vitro* teak clone 22c53obtained from The Forest Industry Organization, Thailand was cultured on hormone-free MS medium (Murashige and Skoog, 1962) at 25±2ºC and 16 h day^{-1} in cool light (55µmol.m^{-2}.sec^{-1}) with monthly sub-cultured. Samples of nodal segment and *in vitro* multiples shoot clump of teak, 300 each, were prepared for genetic transformation. Samples of nodal segment of teak were prepared by single node cutting with leaf blade detached. The *in vitro* multiples shoots clump (MSC) was prepared by culture of the single node on solid MS medium supplemented with 6 mgL^{-1} N_6-benzyleadenine (BA) for a month to obtain callus clump with emerging shoots. The MSC was then cut in half prior to be used as an explant for transformation.

Genetic transformation using *Agrobacterium tumefaciens*

Plasmid vector and A. tumefaciens preparations

The binary vector, pCAMBIA1302-Ab, was constructed by inserting 2.5 kb *Cry1Ab* gene under the control of 35SCaMV promoter into the *Nco*I–*Bst*EII site of pCAMBIA1302as a replacement of *mgfp* gene. The carrier plasmid also contained *hygromycin phosphotransferase* (*hpt*) gene as a selectable marker gene. The finished construct of pCAMBIA1302-Ab is shown in Figure 1.The *A. tumefaciens* strain EHA105 was then transformed with this plasmid vector by heat-shock method. After the confirmation of the existing of pCAMBIA1302-Ab, the single colony of *A. tumefaciens* was grown in LB medium containing 50 mg L^{-1} kanamycin and incubated at 28ºC and 120 rpm for 16 h to reach an optical density of 1.2 unit at 600 nm. The aliquot of 200 µL bacterial suspensions was transferred to 50 mL LB medium containing 50 mgL^{-1} kanamycin and 100 µM acetosyringone and incubated at 28ºC at 120 rpm for 16 h. Bacteria cells were then pelleted by centrifugation at 12,100rpm at 4ºC for 10 min then re-suspended in equal volume of MS liquid medium for the latter inoculation procedure.

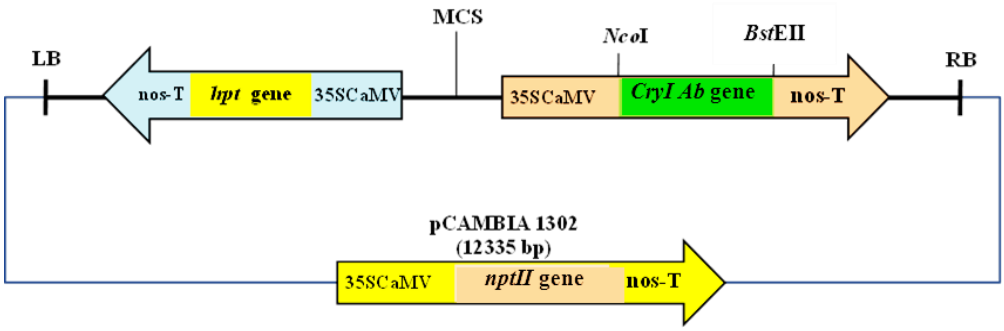

Fig. 1. The structure of the binary vector pCAMBIA1302-Ab

Genetic transformation procedure

For the step of inoculation, the *in vitro* MSC and nodal segments tissues were submerged in suspension culture of *A. tumefaciens* ($O.D._{600}$=1.2) and were sonicated for 3 min using sonicator bath (T460/H, 35kHz, Elma Hans Xchmidbauer, Germany) followed by vacuum infiltration at 600 mmHg for 5 min. Thereafter, inoculation period was allowed to perform for 1h. The MSC and nodal segments tissues were blotted with sterilized filter paper to get rid of the excess bacteria prior to co-cultivated for 3 days on solid MS medium containing 100 µM acetosyringone in dark condition at 25±2ºC. After co-cultivation, explants were washed 3 times with liquid MS medium containing 500 mgL^{-1}cefotaxime and blotted dry on sterilized filter paper. The explants were then transferred to solid MS selective medium containing 300 mgL^{-1} cefotaxime and 50 mgL^{-1} hygromycin and the transferred to the fresh medium every 2 weeks. After 4 weeks on solid selective medium containing cefotaxime, the explants were then transferred to shoot induction medium (MS supplemented with 2 mgL^{-1} BA, and 50 mgL^{-1}hygromycin) for one month. The surviving explants which were putative transformed explants were then cultured on MS medium for further investigation for the existence and expression of *Cry1Ab* gene.

Verification of *Cry1Ab* gene in putative transformed tissue

DNA level verification

The genomic DNA was isolated from the putative transformed calli and leaves using a method described by Phire Plant Direct PCR Master Mix (Thermo Scientific, Thailand). To confirm the integration of the transferred genes into the genome, the polymerase chain reaction analysis (PCR) was performed based on the amplification of 2500 bp fragment of the*Cry1Ab* gene and 800 bp fragment of the *hpt* gene using specific primers for *Cry1Ab* and *hpt* genes respectively, as following sequences:

Cry1Ab-F	5'-CATGGACAACAACCCAAACATCAACGA-3'
Cry1Ab -R	5-GTCACCTTGCTACCGAAAGTCCTCGTTC-3'
hpt -F	5'-CCTGAACTCACCGCGACG-3'
hpt -R	5'-AAGACCAATGCGGAGCATATA-3'

PCR reaction was conducted in total volume of 20 µL containing100 ng of genomic DNA, 10 µL 2x Phire Plant Direct PCR Master Mix (Thermo Fisher Scientific, Thailand) and 1µM of each primer under the following conditions: one cycle of 95°C for 5 min; 30 cycles of 95°C for 10 s, 60°C for 10 s, 72°C for 1 min; a final extension at 72°C for 1 min. The amplified products were separated by electrophoresis on a 1% (w/v) agarose in TAE gel and visualized by ethidium bromide staining.

RNA level verification

A reverse transcriptase-polymerase chain reaction (RT-PCR) was used for genetic transformation verification in RNA level. The cDNAs were synthesized using the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas). For the first-strand cDNA synthesis from RNA, the amount of 1µg RNA was first added to an oligo-dT primer (0.5 µgµL^{-1}) or a specific primer for*Cry1Ab* gene (15-20 pmol) and adjusted to a final volume of 12.5µL with nuclease-free deionised water. The mixture was then denatured at 65ºC for 5 min and then instantly cooled on ice. Subsequently, other reaction components were added: 4 µL5X reaction buffer; 0.5µLRNAse inhibitor (20 UµL^{-1}), 2 µLmixed dNTPs (10 mM each) and 1 µL RevertAid™ M-MulV reverse transcriptase (200 UµL^{-1}). The reaction mixture was then incubated at 42ºC for 1 h. Subsequently, the reaction was stopped by heating the tubes at 70ºC for 10 min and the products were then directly used for PCR amplification.

Protein level verification

To confirm the expression of *Cry1Ab* gene, the Cry1Ab protein was detected using enzyme-linked immunosorbent assay (ELISA) by following the protocol of Bt-CryIAb/1Ac ELISA Kit (Agdia Company). Putative transgenic, non-transgenic teak leaves and transgenic tobacco leaves were separately ground in 1×PBST wash buffer at a ratio of 1:10 (g of tissue : ml of buffer). The aliquot of 100 µL supernatants were then loaded into ELISA well that was coated with RUB6 enzyme conjugate solution and 100 µL of CryIAb specific antibody. The 100 µL of positive control (Agdia Company) and negative control (deionized water) were loaded into the appropriate test wells. The testing wells were mixed gently by swirling the plate on the bench-top shaker and incubated for 2 h at room temperature. After 2 h, the test wells were washed 7 times with 300 µL of PBS-Tween 20 (PBST) washing buffer prior to blot dry using paper towel. The aliquot of 100 µL 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate solution was added into each well and incubated for 20 min at 37ºC. The result of each test well was evaluated by ELISA reader (Labsystems/Multiskan EX) at 450 nm. The positive result that represented Cry1Ab protein expression must had the value of more than 2 folds of a non-transformed plant.

RESULTS ANDDISCUSSION

Plant transformation and regeneration

The *in vitro* MSC and nodal segments tissue were transformed with *A. tumefaciens* strain EHA105 containing the binary vector pCAMBIA1302Ab carrying*Cry1Ab*and*hpt* genes, both under the controlled of 35SCaMV promoter and Nos-terminator. After 8 weeks on selective medium containing 300 mgL^{-1}cefotaxime and 50 mgL^{-1}hygromycin, two independent putative transformed MSC and nodal segments survived (Figure 2I).The calli were formed from these surviving tissues when they were transferred onto MS medium containing2 mgL^{-1}BA, however, only one shoot regenerated from callus derived from MSC (Fig. 2II B). These calli and shoot tended to be the putatively transformed tissue because the concentration of hygromycin at 50 mgL^{-1} was proven to be the effective concentration to get rid of non-transgenic tissue (Sontikun *et al*., 2013). Therefore, the surviving tissue on this selective medium could be, at least, *hpt* putative transformed lines because they could survive and grew on the selective medium containing hygromycin. However, the molecular level analysis was needed to be performed to confidently confirm the existence and expression of both *hpt* and *Cry1Ab* genes.

For the emerging shoot, chimera may occur due to the fact that it may originate from the multiple cells. Thus, for intensively investigation, the putative transformed shoot was divided into two nodes and one shoot explants prior to subculture onto hormone-free solid MS medium to obtained 3 mature plantlets (Fig. 3). Each plantlet was then separately subjected to further molecular analysis of the transgenes, *Cry1Ab* and *hpt*.

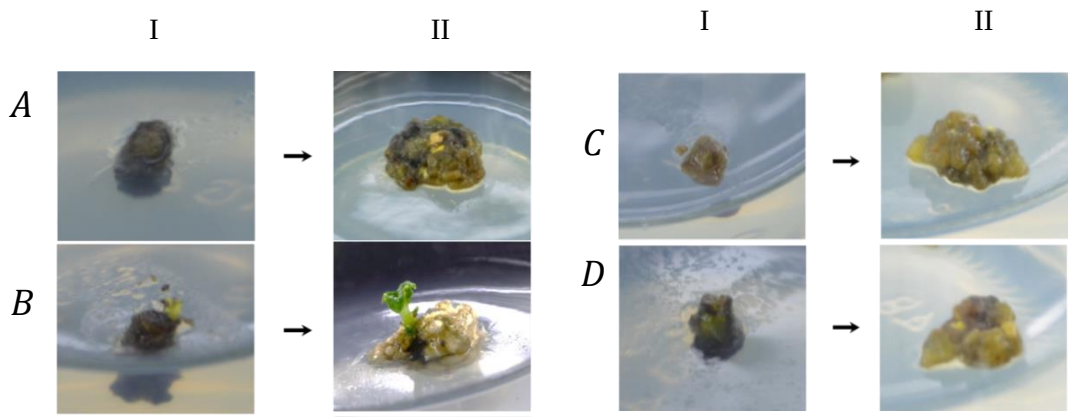

Fig. 2. The putative transformed multiples shoot clumps of teak clone 22c53 (A and B) and nodal segments tissue (C and D) after culture for 4 weeks (I) on MS selective medium containing 300 mgL^{-1}cefotaxime and 50 mgL^{-1} of hygromycin. The survive calli were form after 4 weeks on MS medium containing 2 $mgL^{-1}$of BA (II) and a single shoot emerged from MSC tissue (II B).

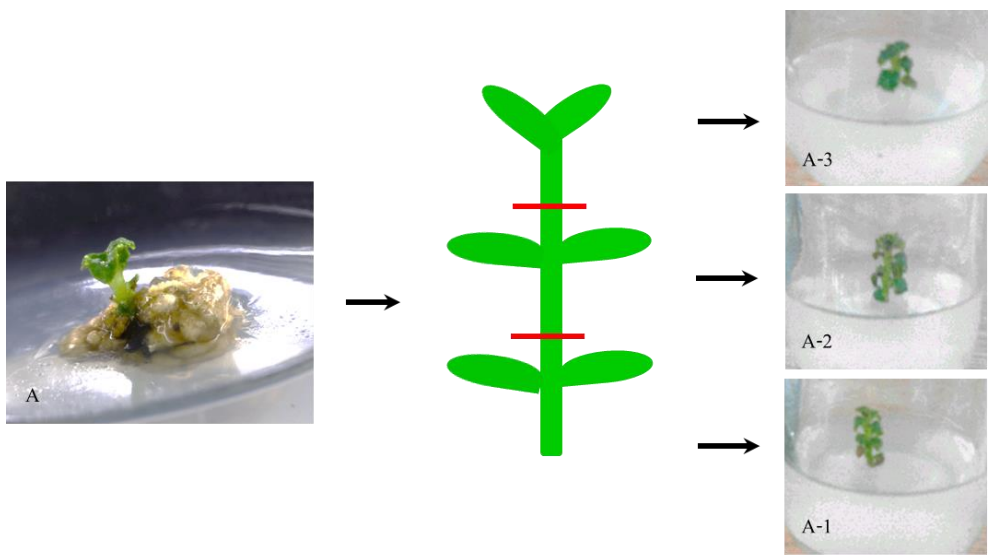

Fig. 3. The putative transformed shoot derived from multiple shoots clump was divided into two nodes and one shoot explants prior to subculture onto hormone-free solid MS medium to obtained 3 mature plantlet lines, A-1, A-2 and A-3.

Verification of the genetic transformation in putative transformed calli

DNA level verification* of *Cry1Abgene in calli

To confirm the presence of *Cry1Ab* gene in the survive calli, genomic DNAs were extracted from each callus and subjected to PCR amplification using *Cry1Ab* gene specific primers. The results demonstrated that genomic DNA of each calli produced the amplified DNA band of 2,500 bp similar to the size of the DNA band produced from pCAMBIA 1302-Abpositive control. For the PCR analysis of the non-transgenic callus (negative control), no DNA band was observed (Fig.4). These results confirmed the existence of*Cry1Ab* gene in all selected putative transformed calli.

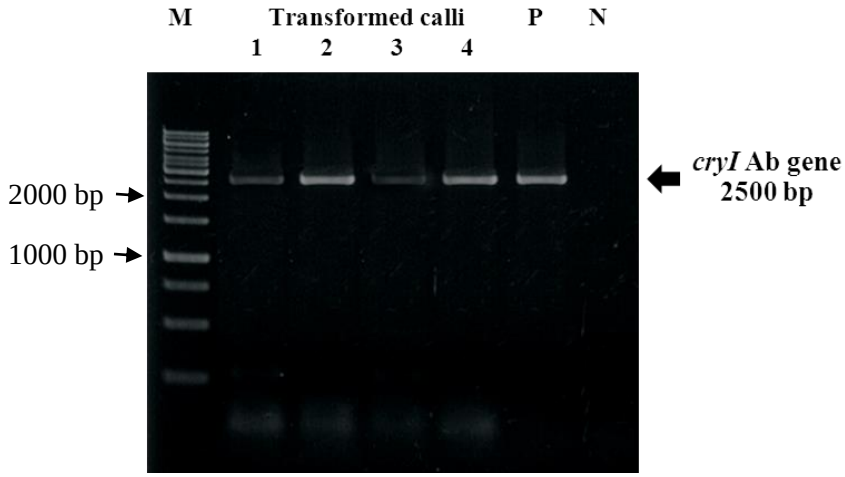

Fig. 4. PCR analysis of *Cry1Ab* gene in putative transformed calli using genomic DNA as DNA template.

M = 1 kb ladder molecular weight marker (Fermentas)
1-4 = genomic DNA from 4 putative transformed calli
P = PCR product of *Cry1Ab* gene from positive control (pCAMBIA 1302-Ab)
N = PCR product of negative control (non-transformed callus)

RNA level expression of Cry1Ab gene in putative transformed calli

The RNA level expression in the putative transformed calli was also conducted using first-strand cDNA analysis. For this technique, the first strand cDNA was generated from total RNA and then it was used as a template for PCR analysis with primers specific to the gene of interest. The positive band presented the specific cDNA band of the novel gene. The results revealed the presence of specific bands of 260 bp of *Cry1Ab* gene only in the samples of putative transformed calli (Fig. 5) but not found in the negative control (non-transformed line).This confirms the presence of *Cry1Ab* gene and at least the intermediate step of expression to RNA level in the putative transformed calli.

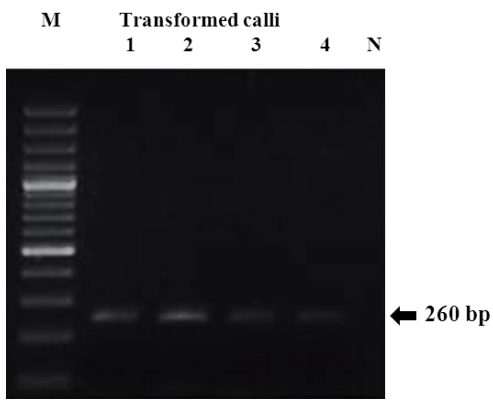

Fig. 5. PCR analysis of *Cry1Ab* gene in putative transformed calli using first-strand cDNA as DNA template.

M = 100 bp plus molecular weight marker (Fermentas)
1-4 = cDNA from each putative transformed callus
N = negative control (non-transformed callus)

The results revealed the difference in level of expression of *Cry1Ab* gene in transformed calli as the quality of the bands were observed. The initial quantity of mRNA is very important in success in the investigation of gene expression by RT-PCR technique. Unfortunately, mRNA was rapidly degraded during extraction process. This may lead to the low visualization of the amplified bands. However, this result was comparable with the previous research work of Norwati *et al.* (2011) in which Southern blot of *Cry1Ab* gene detected in the transgenic teak transformed by particle bombardment demonstrated the positive result but the low level expression of *Cry1Ab* gene was revealed by RT-PCR. Thus, beside the technical difficulties of RNA handling, the *Cry1Ab* gene may poorly express by itself in some plant species. The possibility may due to the A/T rich in 3' poly adenine tail is short and could accelerate the rate of destruction of *Cry*-mRNA, thus, quantity of *Cry*-mRNA is therefore present in small amounts (Rocher *et al*., 1998). Beside, the chimeras should be also accounted for the present of small amounts of *Cry*-mRNA in the calli as well.

Verification of the genetic transformation in putative transformed shoots

DNA level verification

The transformed shoot derived from transformed callus, was cut and sub-cultured to new hormone-free MS medium for 4 weeks to generate 3 new putative transformed shoots (A1, A2 and A3). They were analyzed to confirm the presence of *Cry1Ab*and *hpt* genes. The genomic DNA was extracted from each leaf of the plantlets and subjected to PCR analysis. The non-transgenic teak was used as a negative control, while transgenic tobacco containing *Cry1Ab* and *hpt* genes was used as positive control in this PCR analysis.

The results showed that specific bands of *Cry1Ab* (2,500 bp) and *hpt* (800 bp) genes wereobserved in the positive control sample (transgenic tobacco), but none was founded in negative control one (non-transgenic teak). For the putative transgenic samples, the results were varied (Fig. 6A and B) and were summarized in Table 1. The plantlets were chimera since some leaves in the same plantlet did not have *Cry1Ab* and/or *hpt* genes. For those leaves that failed to detect *hpt* gene, *Cry1Ab* band also absented (N, L1, 2L6, 3L2, 3L3, 3L4). However, not all of *hpt* gene was co-transformed with *Cry1Ab* gene as the results demonstrated that in 5 out of 14 leave samples (L3, L4, L7, L9, and 2L5), the *hpt* gene was detected but not *Cry1Ab* gene. On the other hand, no *Cry1Ab* gene detected without the presence of *hpt* gene. The event of non co-transformation of these 2 genes may be resulted from the event of the transfer process of *Agrobacterium* that the genes franked by LB and RB are cut and transported into the host cells. During these processes, the transferred DNA may be sheared and that cause the insertion of truncated piece (Sheng and Citovsky, 1996). However, in this experiment, the selective medium used was the medium containing hygromycin, thus at least the cells contained *hpt* gene would survive selection. Nonetheless, the escape event also may be occurred due to the large size of target tissue. In this report, in the A1 plantlet which originated from the bottom part of the emerging shoot, 9 leaves were analyzed and only one leaf demonstrated of non-transformed event, but the rests were transformed in which half of those (4 leaves, 44%) were co-transformed with both *Cry1Ab* and *hpt* genes and another half (4 leaves, 44%) were transformed with only *hpt* gene. The plantlets that obtained from the upper part (A2 and A3) demonstrated even less in the percentage of transformation. In A2 plantlet, 4 leaves out of 6 were transformed. Among these, 3 leaves (66%) contained both *Cry1Ab* and *hpt* genes while 1 leaf (16%) only *hpt* gene was detected. In the top part, A3 plantlet, 5 leaved were analyzed and only 1 contained both *Cry1Ab* and *hpt* genes while in the other 4 leaves none of the transgenes were detected.

The results indicate the chimera event of the transformed shoots in this experiment may be due to the target tissue that used was the multiple shoots clump which was a large tissue. The callus obtained at the first step may also be chimera that consisted of a mixed of transgenic and non transgenic cells but the PCR detection could not be able to distinguish that event. However, the shoot might emerge from a group of cells instead of a single cell origin, some were transformed but some

were non-transformed. Thus, the analysis results in one individual leaf tissue clearly demonstrated the chimera in all of the transformed shoots. The previous research work also reported the chimeric tissue as well. Lachance *et al.* (2007) reported the chimeric plant produced from the transformation of *Cry1Ab* in hypocotyl tissue of *Perilla frutescens* using *A. tumefaciens*. Similarly, the chimera plants also obtained in the transformation of cotyledon and hypocotyl explants of *Eucalyptus tereticornis* Sm.(Lee *et al.*, 2005) and somatic embryo of white spruce (*Picea glauca*) (Prakash and Gurumurthi, 2009) with *Cry1Ab* gene *via Agrobacterium*-mediated genetic transformation. To be able to obtain a solid transformed plantlet from these chimeric plants, the number of sub-cultures and selection on hygromycin containing medium should be performed for several generations to get rid of the non-transformed cells.

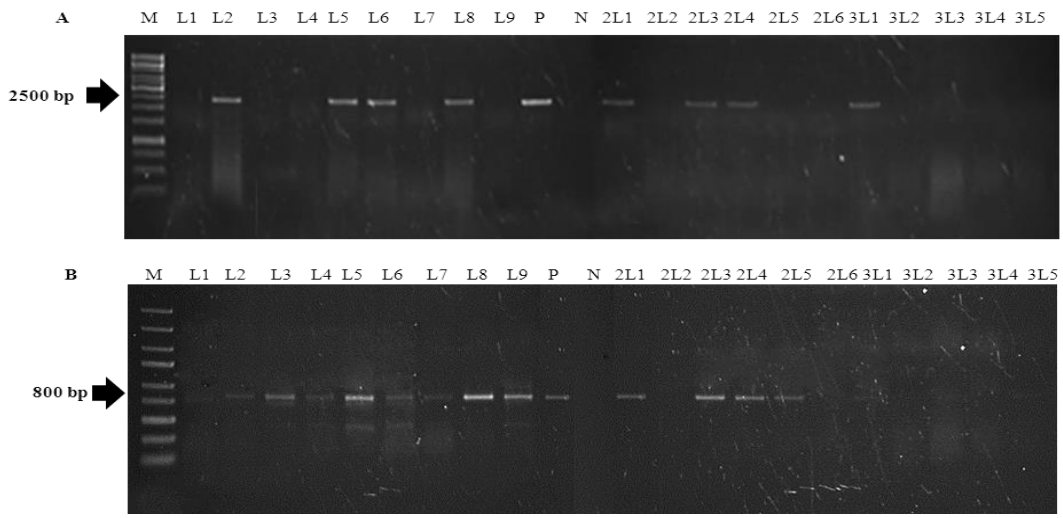

Fig. 6. PCR analysis for the presence of *Cry1Ab* gene (A: 2500 bp) and *hpt* gene (B: 800 bp) in genomic DNA of putative transformed teak leaves from plantlets A-1, A-2 and A-3.

M = 1 kb ladder molecular weight marker (Fermentas)
L1-L9 = Leaves from A-1 plantlet
2L1-2L6 = Leaves from A-2 plantlet
3L1-3L5 = Leaves from A-3 plantlet
P = positive control (transgenic tobacco containing *Cry1Ab* and *hpt* genes)
N = negative control (non-transformed teak)

Table 1. The summary of the results of PCR analysis for *Cry1Ab*and *hpt* genes in leaves of A-1, A-2 and A-3 plantlets (from Figure 6) as + present / - absent.

Genes	Leaves of A-1 plantlet										
	L1	L2	L3	L4	L5	L6	L7	L8	L9	P	N
Cry1Ab	-	+	-	-	+	+	-	+	-	+	-
hpt	-	+	+	+	+	+	+	+	+	+	-
	Leaves of A-2 plantlet										
	2L1	2L2	2L3	2L4	2L5	2L6					
Cry1Ab	+	-	+	+	-	-					
hpt	+	-	+	+	+	-					
	Leaves of A-3 plantlet										
	3L1	3L2	3L3	3L4	3L5						
Cry1Ab	+	-	-	-	-						
hpt	+	-	-	-	-						

Protein level verification of Cry1Ab in transformed plantlets

The expression of *Cry1Ab* gene into Cry1Ab protein was determined by Bt-Cry1Ab/1Ac ELISA Kit, a commercial ELISA test kit specific for Cry1Ab protein, in five selected leaf samples (L2, L8, 2L1, 2L3 and3L1) that were confirmed with both *Cry1Ab* and *hph* genes as described in Table 1. This ELISA test kit visualized yellow solution after tested with standard protein (Cry1Abprotein, Agdia®) in which the OD-450 could be read by the ELISA reader. In this study, the standard positive sample (Cry1Abprotein, Agdia®) had the OD value read at 0.290 and the OD of negative sample was 0.07 (Fig. 7). The value that determined positive result should had at least two fold higher than the value of negative sample, hence, the samples that showed positive in this experiment should have OD higher than 0.14. The results from ELISA reader illustrated that the Cry1Ab protein was detected in the all of transformed leaves tested as they demonstrated the value of OD-450 in the range of 0.163-0.180 and the tobacco positive control had OD value of 0.194 which prove the expression of *Cry1Ab* gene to Cry1Ab protein. Among the transformed leaves, the leaf sample 2L1 had the highest OD value of 0.180 while the other samples had OD in the range of 0.163-0.169. The low level expression of *Cry1Ab* gene may be due to several reasons. The position effect was one of the reasons as the insertion site of the gene affects the level of transcription. The copy number of the inserted gene was another main reason involve with gene expression. However, to address this low level expression problem, several supporting molecular analysis such as Southern blot, iPCR, TAIL PCR and etc. needed to be performed.

The evidence of low OD value of the transgenic leaves was coincided with the finding of the chimera in these plantlets. The variation in OD value possibly accounted for the degree of chimera of each tissue tested. The results confirmed the study of Lachance *et al*. (2007) which transformed *Cry1Ab* gene into white spruce (*Picea glauca*) and expression of *Cry1Ab*gene to Cry1Ab protein in this species was detected by ELISA. However, a relatively high level of protein (389 ng mg^{-1}leaf fresh weigh) was later determined and the insect bioassay with eastern spruce budworm (*Choristoneura fumiferana)* also demonstrated that90% of the insect died after infestation of the transformed white spruce leaves. Therefore, the transformed teak resulted from this experiment should provide the possibility of genetic transformation with the aim to create the insect-resistant teak for the improve pest management in the teak plantation. In the insect pest management of teak plantation, more than 70% of teak was reported to be damaged by teak defoliator and bee-hole borer (Nair *et. al.,* 1984; Royal Forest Department of Thailand, 2009), thus, any figure less than that should be beneficial. On the other hand, the chimera *Bt* transgenic plant also offer an ideal integrated pest management as it allowed some percentage of refuges that concur the less stress to the insect that, in turn, leads to the fact of resistance insects.

ELISA visualization								
Sample	N	P	tobacco	L2	L8	2L1	2L3	3L1
OD-450	0.07	0.290	0.194	0.163	0.169	0.180	0.167	0.163
Analysis result		+	+	+	+	+	+	+

Fig. 7. The detection of Cry1Ab protein by Bt-Cry I Ab/1Ac ELISA Kit® in transgenic teak leaves that confirmed to contained *Cry1Ab* and *hpt* genes. The evaluation is considered positive when the absorbance is higher than two-fold of negative control.

N = negative control (protein solution from non-transformed teak leaves)
P = positive control (Cry1Abprotein, Agdia®)
tobacco = protein solution from tobacco leaf transformed with *Cry1Ab* gene
L2, L8 = protein solution from teak leaves of A-1 plantlet
2L1, 2L3 = protein solution from teak leaves of A-2 plantlet
3L1 = protein solution from teak leaf of A-3 plantlet

CONCLUSION

This report demonstrated the success of introduction of *Cry1Ab* and *hpt* genes into teak tissue by *Agrobacterium*-mediated transformation method using the *A. tumefacience* strain EHA105 containing the binary vector pCAMBIA1302Ab carrying *Cry1Ab* and *hpt* genes. The *Cry1Ab* and *hpt* genes were detected in teak tissue by PCR. The expression of *Cry1Ab*gene in leaves tissue of putative transgenic plants was confirmed by RT-PCR and ELISA. However, the plantlets obtained were chimera. Thus, to avoid the chimera problem, the optimization on tissue culture technique should be intensively explored for other plant tissue types such as somatic embryogenesis. Nonetheless, for the chimera transgenic plants obtained from this experiment, the *in vitro* screening technique can be employed in order to obtain the solid transgenic plant.

ACKNOWLEDGEMENT

This research was partially supported by the Center of Advanced Studies for Agriculture and Food, Institute for Advanced Studies, Kasetsart University under the Higher Education Research Promotion and national Research University Project of Thailand. The partially supported by the Center of Excellence on Agricultural Biotechnology, Office of Higher Education Commission (AG-BIO/PERDO-CHE), Ministry of Education, Thailand, was also grateful.

REFERENCES

Chilcott, C.N. and P.J. Wigley. 1993. Insecticidal activity of *Bacillus thuringiensis* crystal protein. Program and abstracts, The Second Canberra *Bacillus thuringiensis* Meeting, 21-23 September 1993, CSIRO Division of Entomology, Australia.45 p.

Knowles, B. H. 1993. The mode of action of Bacillus thuringiensis delta-endotoxin. Programme and Abstracts, The Second Canberra *Bacillus thuringiensis* Meeting, 21-23 September 1993, CSIRO Division of Entomology, Australia. 45 p.

Lachance, D., L.-P. Hamel, F. Pelletier, J. Valéro, M. Bernier-Cardou, K. Chapman, K.V. Frankenhuyzen and A. Séguin. 2007. Expression of a *Bacillus thuringiensis Cry1Ab* gene in transgenic white spruce and its efficacy against the spruce budworm (*Choristoneura fumiferana*). Tree Genet. Genomes. 3: 153–167

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, Tiss. Organ Cult. 83: 51-58.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue. Physiol. Plant. 15: 473-479.

Nair, P.K.R., E.C.M. Fernandes and P.N. Wambugu. 1984. Multipurpose leguminous trees and shrubs for agroforestry. Agroforest. Sys. 2(3): 145-163.

Norwati, A., B. Norlia, H.M. Rosli, M. Norwati and R. Abdullah. 2011. Development of transgenic teak (*Tectona grandis*) expressing a *Cry1Ab* gene for control of the skeletoniser. AsPac. J. Mol. Biol. Biotechnol 19(4): 149-156.

Pandey, D. and C. Brown. 2000. Teak: A global overview. Unasylva 201(51): 3-13.

Prakash, M.G. and K. Gurumurthi. 2009. Genetic transformation and regeneration of transgenic plants from precultured cotyledon and hypocotyl explants of *Eucalyptus tereticornis* Sm. using *Agrobacterium tumefaciens.* In Vitro Cell.Dev.Biol.-Plant 45: 429–434.

Rocher, E.J.D., T.C. Vargo-gogola, S.H. Diehn and P.J. Green. 1998. Direct evidence for rapid degradation of *Bacillus thuringiensis* toxin mRNA as a cause of poor expression in plants. Plant Physiol. 117: 1445–1461.

Royal Forest Department. 2009. Forestry in Thailand.Royal Forest Department, Ministry of Natural Resources and Environment, Bangkok.

Sheng, J, and V. Citovsky. 1996. *Agrobacterium-plant* cell DNA transport: Have virulence protein, will travel. Plant Cell 8:1699-1710.

Sontikun, Y., S. Chanprame, P. Srinives and S. Chanprame. 2013. Optimization of transient *β*-glucuronidase (gus) gene expression in teak (*Tectona grandis* L.f.) by *Agrobacterium tumefaciens*-mediated transformation system. J. ISSAAS 19(2): 49-57.

Suseno, O.H. and M.G. Wibisono. 2000. The history of teak silviculture in Indonesia, p. *in* The 3rd Regional Seminar on Teak. Yogyakarta, Indonesia.

White, K.J. 1991. Teak : Some Aspects of Research and Development. FAO Regional Office for Asia and the Pacific (RAPA), Bangkok.

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. Afr. J. Biotechnol. 8 (10): 2143-2150.

William, C.A. and A. Jeffrey. 1995. Use of PCR to quantitate relative differences in gene expression, p. *in* W. D. Carl and S. D. Gabriela, eds. PCR primer – A Laboratory Manual. New York.