CHAPTER III
RESULTS

Part I Transformation of GFP into *O. sativa* KDML105 without selection pressure

3.1.1 Detection of deleted hygromycin pCAMBIA5305*hpt*- vector

Approximately 5 μg of extracted pCAMBIA5305 was digested with *Xho*I to remove the *hpt* gene from the plasmid , resulting in linearized of pCAMBIA5305(9.3 kb) and *hpt* fragment (1.1 kb). The *hpt* fragment was separated by 0.7% agarose electrophoresis and eluted with QIAquick Gel Extraction Kit. The 9.3 kb product was religated and transformed into *E. coli* XL1-Blue. The recombinant plasmid were extracted by alkaline extraction and then digested with *Xho*I to determine the corrected fragment. The size of pCAMBIA5305*hpt*- vector was determined as 9.3 kb (Fig 3.1). The structure and restriction maps of binary vectors pCAMBIA5305 and pCAMBIA5305*hpt*-vector were given in Appendix C.

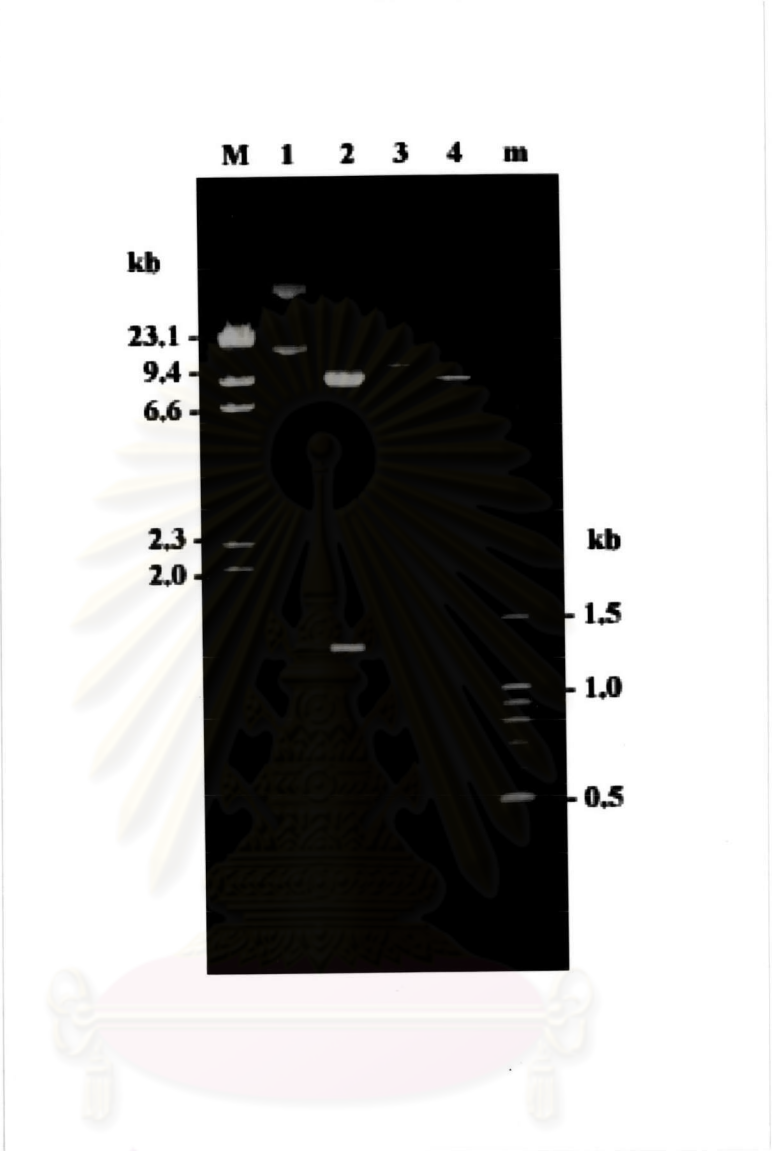


Figure 3.1 Characterization of 9.3 kb linearized fragment of pCAMBIA5305*hpt*-vector

Lane M = λ/ *Hin*dIII standard marker

Lane 1 = undigested pCAMBIA5305 vector

Lane 2 = *Xho*I digested pCAMBIA5305vector

Lane 3 = undigested pCAMBIA5305*hpt*- vector

Lane 4 = *Xho*I digested pCAMBIA5305*hpt*-vector

Lane m = a 100 bp DNA ladder

In all experiments, the calli initiated from scutellum of mature seeds were used as a starting material for *Agrobacterium* - mediated transformation of rice. Mature seeds were cultured on NB medium supplemented with 2 mg/l 2,4D for callus induction. After 4 weeks, the embryogenic calli; odserved as being compact , yellowish and granular ; were separated with sterile scapel and subcultured to fresh medium for 4 days before using in co-cultivation. One embryogenic calli was defined as one line, and the different lines were kept separately during subculturing.

3.1.2 Co-cultivation of rice calli with *A. tumefaciens* EHA105 (pCAMBIA5305*hpt*-vector)

Selection of marker genes are almost always included in transformation systems, but the safety of each marker gene is another potential problem in the plant genetic engineering. In the first study, the main effort was focused on use of *A. tumefaciens* carrying pCAMBIA5305*hpt*-vector (having only GFP in the T-DNA) to infect embryogenic calli without selection pressure. Then we tested that the calli can potentially be regenerated to GFP positive plants. The embryogenic calli were co-cultivated for 3 days and transferred to induction medium (NB-C) supplemented with cefotaxime (500 mg/l) to inhibit *A. tumefaciens*. Analysis of transformed embryogenic calli was performed by fluorescent microscopy with FITC filter for scoring plant transformation. The transformed calli were visualised as GFP-positive spot. No green fluorescence was observed in untransformed control calli. One day after co-cultivation, out of 25 calli cocultivated , 2 GFP fluorescent calli were obtained corresponding to 8% transformation efficiency. After 10 days of co-cultivation, out of 33 calli co-cultivated, 5 GFP fluorescent calli were obtained corresponding to 15% transformation efficiency (Table 3.1, Fig 3.2). At 4 week after co-cultivation, the infected embryogenic calli could not be detected due to the overgrowth of untransformed cells. All GFP-positive calli were then cut into small pieces and transferred to regenerated medium. The sectioned calli did not show continuous growth , turned brown and died in regeneration medium. Additionally, it was contaminated in some pieces.

When the embryogenic calli were co-cultivated with *A.tumefaciens* EHA105 carrying pCAMBIA5305. These plasmids have the *hpt* and *gfp* gene in the T-DNA. The hygromycin (50mg/l) were used as a selectable agent. All of three selection

periods, GFP positive spot was observed in the embryogenic calli under fluorescence microscope. After 1 day on selection medium, out of 25 calli co-cultivated, 2 GFP fluorescent calli were obtained corresponding to 8% transformation efficiency. After 10 and 30 days on selection medium, the transformation efficiency values were 12 and 9%, respectively (Table 3.1). At 30 days selection, bright green fluorescent calli were more clearly visible than those obtained in 10 days selection.

Table 3.1 Expression of GFP in rice callus after co-cultivation with pCAMBIA5305*hpt*- vector for 3 days

vector	Day on selection medium					
	1 day		10 days		30 days	
	No. of co-cultivated calli	Transformation efficiency GFP+ calli	No. of co-cultivated calli	Transformation efficiency GFP+ calli	No. of co-cultivated calli	Transformation efficiency GFP+calli
pCAMBIA5305	25(2)	8	33(4)	12	33(3)	9
pCAMBIA5305 *hpt*- vector	25(2)	8	33(5)	15	--	--

Number in parenthesis indicates GFP-positive calli

Figure 3.2 GFP-positive spot on transgenic callus after 10 days co-cultivation

Part II Detection of stable GFP expression and inheritance in T1 progeny

3.2.1 Co-cultivation of rice calli with *A. tumefaciens* EHA105 (pCAMBIA5305)

A. tumefaciens strain EHA105 harbouring pCAMBIA5305 (*hpt* is a selectable marker and GFP is a reporter gene) was used for co-cultivation of embryogenic calli. Co-cultivation was carried out in the dark at 28°C for 2-3 days. The co-cultivated calli were transferred to the selection medium (NB-CH) containing cefotaxime (500 mg/l) and hygromycin (50 mg/l) to inhibit growth of non–transformed rice cells. Hygromycin resistant calli were obtained after 4 weeks selection. These growing calli were excised and transferred to fresh selection medium and incubated in dark at 28°C for 4 weeks. Continuous selection on hygromycin–containing medium resulted in the appearance of proliferating, apparently resistant, embryogenic calli. The uninoculated control embryogenic calli did not show continuous growth, turned brown and died in selection medium (Fig 3.3). After 8 weeks on selection with hygromycin, bright-green fluorescent calli were observed growing on hygromycin (Fig 3.4). No green fluorescent calli were observed in untransformed control calli. The frequency of transformed calli, based upon hygromycin-resistance and GFP expression, 10 and 16 % were observed from 2 and 3 days co-cultivated calli, respectively (Table 3.2 and 3.3).

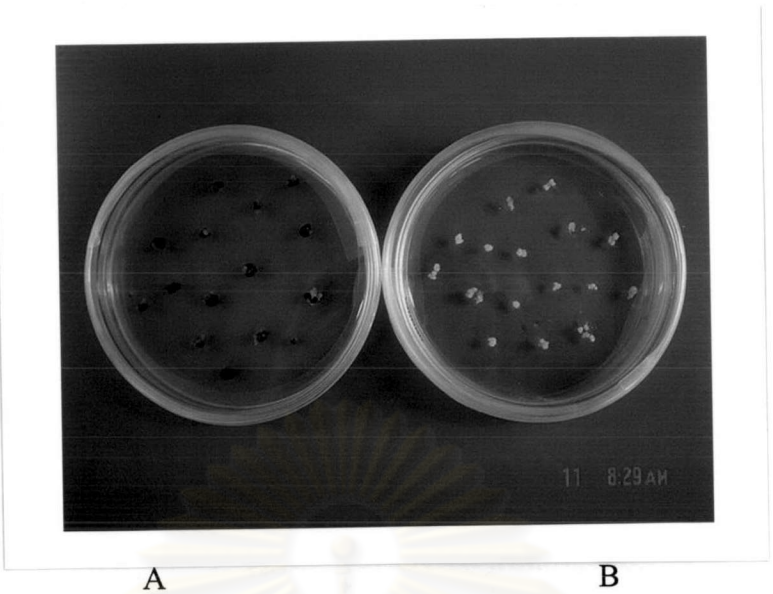
Table 3.2 Efficiency of rice transformation by *A. tumefaciens* EHA105 (pCAMBIA5305) [10 min infection time , 2 days co-cultivation]

No. of co-cultivated calli (a)	Produced HygR and GFP+ calli (b)	Transformation efficiency HygR/GFP+ (b/a)	Produced plants (c)	(%) Regeneration (c/b)	Produced GFP+ Plants (d)	Frequency (d/a%)
90	9	10	5	55.5	5	5.5

Table3.3 Efficiency of rice transformation by *A. tumefaciens* EHA105 (pCAMBIA5305) [15 min infection time , 3 days co-cultivation]

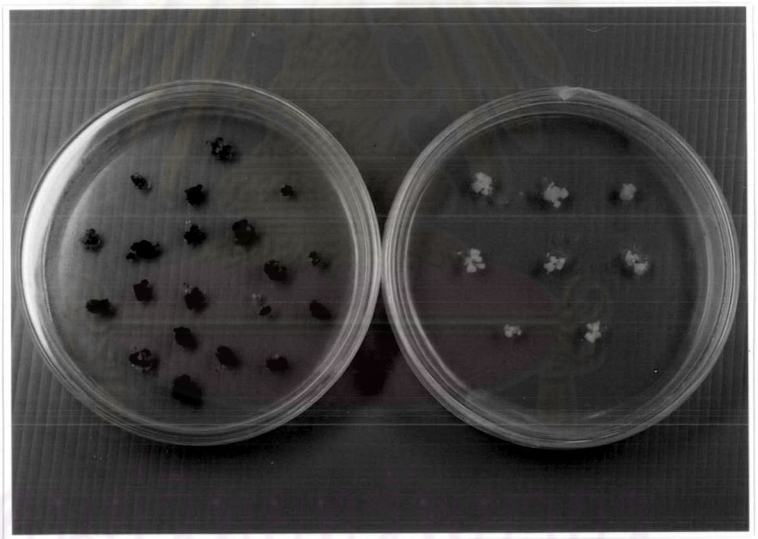
No. of Co-cultivated Calli (a)	Produce HygR and GFP+ calli (b)	Transformation efficiency HygR/GFP+ (b/a)	Produced plants (c)	(%) Regeneration (c/b)	Produced GFP+ Plants (d)	Frequency (d/a%)
100	16	16	4	25	4	4

HygR = hygromycin resistance



A B

Infection for 10 min and co-cultivation for 2 days



A B

Infection for 15 min and co-cultivation for 3 days

Figure 3.3 Proliferation of the infected callus under hygromycin selection (50 mg/l). Rice embryogenic callus transformation mediated by *A. tumefaciens* EHA105 (pCAMBIA5305)

(A) Brown non-transformed embryogenic calli

(B) Resistant embryogenic callus growing on selective hygromycin medium after 9 weeks on selection medium

Figure 3.4 GFP as a vital screenable marker in rice transformation using *A. tumefaciens* EHA105 (pCAMBIA5305). Observations were performed using a fluorescent microscope.

(A1) Transformed embryogenic callus 8 weeks on selection medium observed under the fluorescent microscope using blue filter

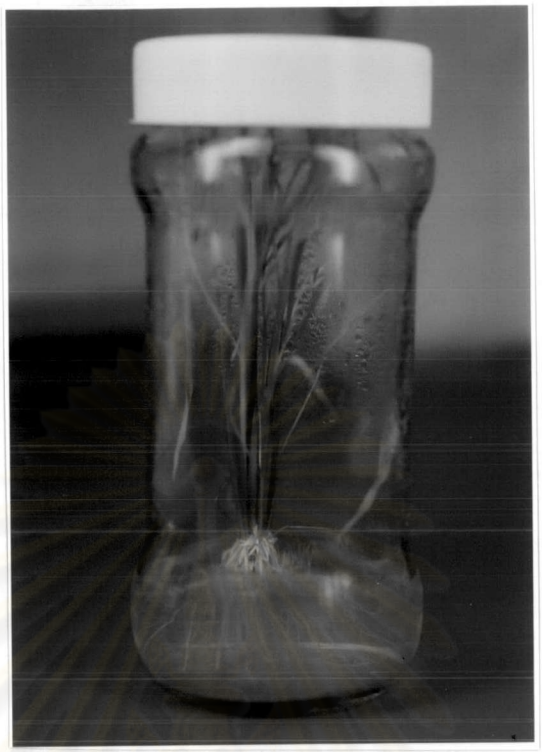
(A2) Non-transformed embryogenic callus 8 weeks on selection medium observed under the fluorescent microscope using blue filter

(B1) The same transformed callus show green fluorescence observed under the fluorescent microscope using fluorescein isothiocyanate (FITC) filter

(B2) The same non-transformed callus observed under the fluorescent microscope using fluorescein isothiocyanate (FITC) filter

3.2.2 Regeneration of putative GFP transformed calli

Transgenic rice plants were regenerated from visually selected GFP-positive calli. Although stringent selection was maintained through callus growth , the hygromycin was not included during regeneration. The formation of green buds/shoots from somatic embryos occurred within 3 weeks of transferring hygromycin resistant calli onto regeneration medium (NB4-RE). Healthy plantlets with extensive root system were established on NB medium without any plant hormone after another 2-3 weeks [Fig. 3.5(A)]. The frequency of regeneration range from 25 to 55.5 % selected colonies (Table 3.2 and Table 3.3). No difference were observed in the morphology between transformed and untransformed plants. Regenerated plant were tested by GFP fluorescence (Fig 3.6) and PCR analysis. A total of 14 transformants could be regenerated from GFP-positive and hygromycin-resistant calli of rice (KDML105). Of these 5 transformants were from 2 days co-cultivated calli and 4 transformants were from 3 days co-cultivated calli. These putative transgenics were subsequently transferred to soil in a growth chamber [Fig. 3.5(B)].



A

B

Figure 3.5 Regeneration of transformants

(A) Regenerated transgenic rice plants were transferred to NB medium (rooting medium) for 4 weeks.

(B) Regenerated transgenic rice plants with extensive root system were transferred to soil for further growth

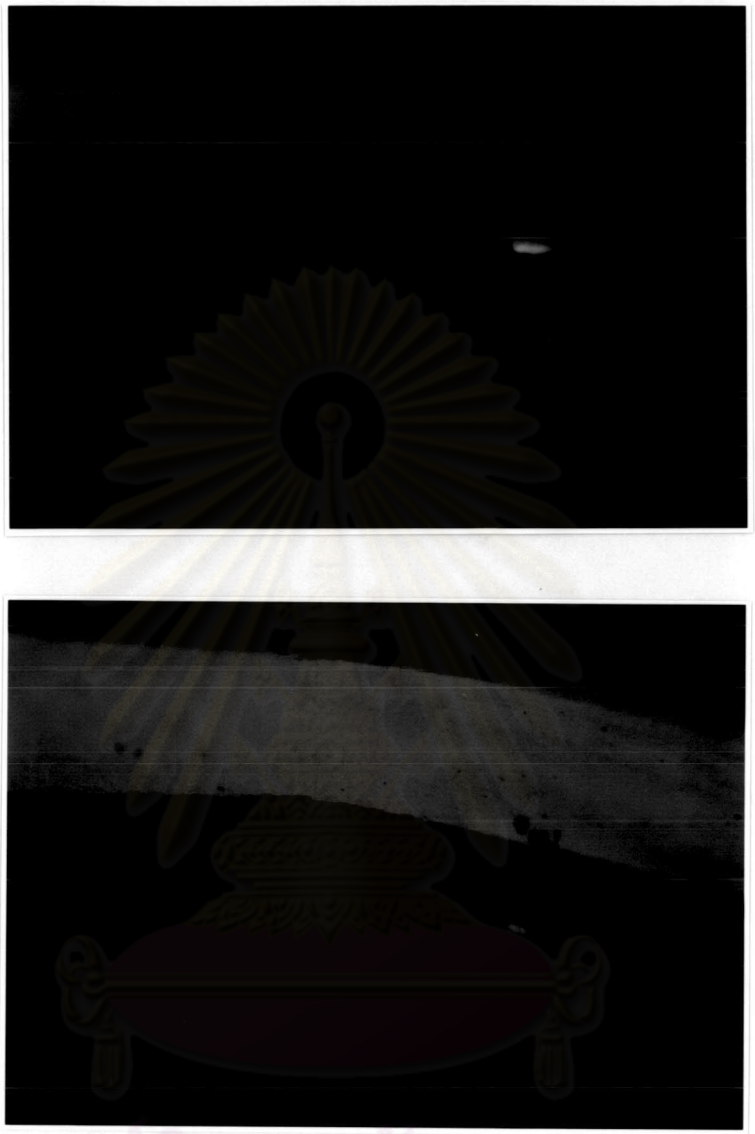


Figure 3.6 GFP expression in primary transformants

(A1) Transformed root primordia 6 weeks after regeneration on NB4-RE regeneration medium observed under the fluorescent microscope using blue filter

(A2) Non-transformed root primordia 6 weeks after regeneration on NB4-RE regeneration medium observed under the fluorescent microscope using blue filter

(B1) The same transformed root primordia show green fluorescence observed under the fluorescent microscope using fluorescein isothiocyanate (FITC) filter

(B2) The same non-transformed root primordia observed under the fluorescent microscope using fluorescein isothiocyanate (FITC) filter

3.2.3 Expression of GFP in primary transformed rice plants

Transgenic rice plants were regenerated from visually selected GFP-positive calli. All of the 9 transformants recovered in both experiments showed GFP expression. Rooted plantlets were then tranferred to pot. After 2 months in field, both root and leaves were cut by cross-section and observed under fluorescence microscope. In young root section, the expression of GFP was found in vascular bundle cylinder , cortex and epidermal cell (Fig 3.7). While expression in leaves of older plants was limited in vascular bundle tissue (Fig 3.8). More fluorescence was prominent in xylem and phloem than in the other cells.

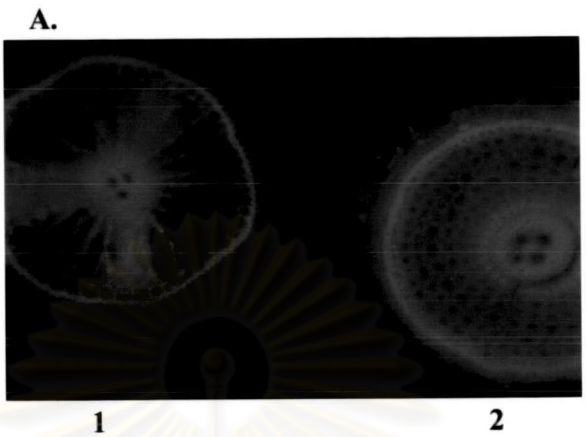


Figure 3.7 Cross-section of root under fluorescent microscope

(A1) Cross section of control root observed under fluorescent microscope using blue filter

(A2) Cross section of transformed root observed under fluorescent microscope using blue filter

(B1) The same control root section observed under fluorescent microscope using FITC filter

(B2) The same transformed root section observed under fluorescent microscope using FITC filter

Figure 3.8 Cross-section of older leaves after 2 months in field

3.2.4 PCR analysis of putative transformants

Molecular analysis by PCR amplification confirmed that the GFP gene was present in the putative transformed rice plant. Genome DNA from rice (KDML 105) putative transformants resulting from infections with *A. tumefaciens* EHA105 (pCAMBIA5305) was amplified with GFP gene primer as well as *npt*II gene primer. One band corresponding to the expected GFP fragments of 720 bp was detected in all the putative tested transformants. No band was detected in case of negative control DNA; DNA of uninfected rice tissues. Since *npt*II gene is present outside the T-DNA in pCAMBIA5305, *npt*II gene primers were used to check the band of 707 bp for the presence of any contaminating *Agrobacterium* cells in the plant tissue, although amplification could also result from T-DNA transfer that extended beyond the T-DNA border. However, none of the plants analysis showed amplification of the *npt*II gene sequence eliminating the possibility of bacterial contamination in the transformed tissue (Fig 3.9). The transformation efficiency determined by transgenic plants that stably expressed GFP fluorescence to the number of pieces of scutellum-derived calli that had been co-cultivated with bacterial cells was 5.5 and 4 % (Table 3.2)

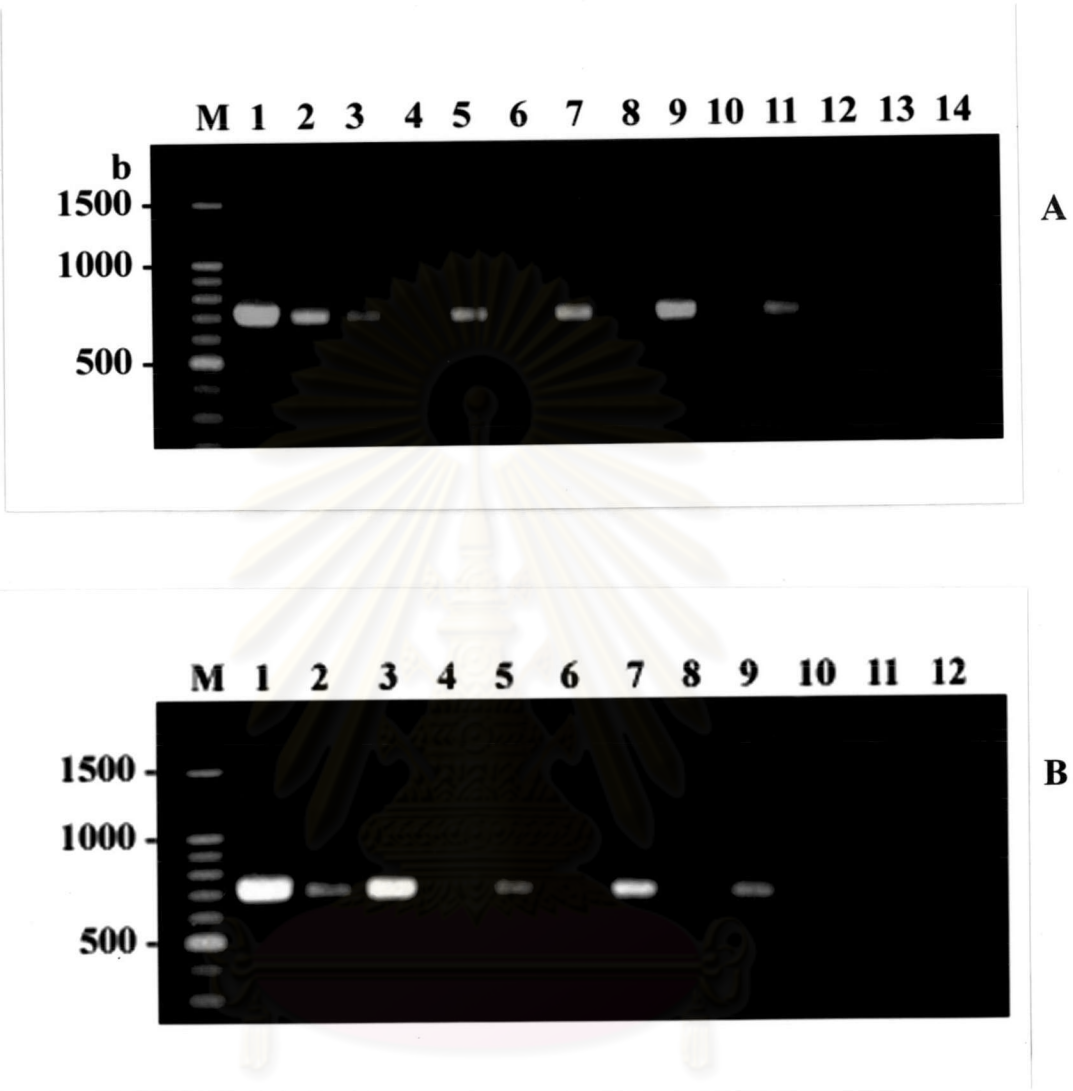


Figure 3.9 PCR analysis of the GFP gene in putative transformed rice plants co-cultivated with *A. tumefaciens* EHA105 (pCAMBIA5305) for 3 days. *Agrobacterium* used was around 0.01 A_{600} unit. Total DNA isolated from whole plants was subjected to PCR amplification with GFP specific primers (lane 3, 5, 7, 9, 11; A and lane 3, 5 ,7, 9; B) showing the presence of an expected 720 bp DNA fragment of GFP gene. Kanamycin specific primers were also used to check the band of 707 bp for the presence of any contaminating *Agrobacterium* cells in plant tissues (lane 4, 6, 8, 10, 12; A and lane 4, 6, 8, 10; B). The GFP gene and the kanamycin gene (positive control) were also amplified from the pCAMBIA5305 vector (lane 1 A, B and 2 A, B; respectively). DNA isolated from non-transformed plants was used as a negative control for GFP and for kanamycin specific primer (lane 13,14 A and 11,12 B)

3.2.5 Progeny analysis

Putative transgenic plants were grown in pots (Fig. 3.10-3.11). After 4-6 weeks, all of plants grew to flower. Control plant was able to set seeds (Fig. 3.12). For transgenic plant, three of nine plant lines were able to set seeds (Fig. 3.13). They had the same seed fertility as compared to control. However, the others reached to flower late and unabled to set seeds (Fig 3.14). After 1 month, kernels developed on the plant and some mature seed was harvested from plant. Approximately 3 to 5 seeds of each line were soaked in water for 3 days. Germinated seedling was approximately 2 to 3 cm and then transferred to soil. The data on the statistic test was summarized in Table 3.4, 3.5, 3.6 and 3.7. The expression of GFP and its inheritance was tested in T_1 progeny by GFP fluorescence (Fig. 3.15-3.16) and PCR analysis (Fig 3.17). Stable GFP expression in T_1 progeny was observed in all 3 lines.

Table 3.4 Germination of seed

Treatment	germinated plants / 5 seeds			Treatment Total	Treatment Mean
Line 1	3	4	3	10	3.3
Line 2	2	2	3	7	2.3
Line 3	2	3	3	8	2.7
Control	3	3	4	10	3.3
Grand total (G)				35	
Grand mean					11.6

Source of Variation	Degree of Freedom	Sum of Squares	Mean Square	Calculated F^b	Tabular *F* 5%	1%
Treatment	3	2.3	0.76	2.24^{ns}	4.07	7.59
Experimental error	8	2.7	0.34			
Total	11	5				

ns = non - significant

Table 3.5 Non-germination of seed

Treatment	non-germinated plants / 5 seeds			Treatment Total	Treatment Mean
Line 1	2	1	2	5	1.6
Line 2	3	3	2	8	2.7
Line 3	3	2	2	7	2.3
Control	2	2	1	5	1.6
Grand total (G)				25	
Grand mean					8.2

Source of Variation	Degree of Freedom	Sum of Squares	Mean Square	Calculated F^b	Tabular *F* 5%	Tabular *F* 1%
Treatment	3	2.33	0.78	2.36^{ns}	4.07	7.59
Experimental error	8	2.67	0.33			
Total	11	5				

ns = non - significant

Table 3.6 GFP-positive germinated seed

Treatment	(GFP +)germinated plants			Treatment Total	Treatment Mean
Line 1	3	4	3	10	3.3
Line 2	2	2	3	7	2.3
Line 3	2	3	3	8	2.7
Control	0	0	0	0	0
Grand total (G)				24	
Grand mean					7.97

Source of Variation	Degree of Freedom	Sum of Squares	Mean Square	Calculated F^b	Tabular *F* 5%	Tabular *F* 1%
Treatment	3	18.67	6.2	36.47**	4.07	7.59
Experimental error	8	1.33	0.17			
Tatal	11	20				

** = significant at 1% level

Table 3.7 GFP positive plants of each line compared to control

Treatment	Mean difference of (GFP +) germinated plants from control
Line 1	3.3**
Line 2	2**
Line 3	2.67**
Control	0

** = significant at 1% level

From above results, we summarised that the different average between control and transgenic seeds germination based on the non-significant. And different average GFP positive between control and transgenic seeds germination based on the highly significant difference.

Figure 3.10 T_0 plants were grown in pot.

Fig 3.11 Comparison of non-transformed rice (left) and transgenic rice(right) after 3 months in pot.

Figure 3.12

Figure 3.13

Figure 3.14

Figure 3.12 Control T_0 plant set seeds in pots.

Figure 3.13 Transgenic T_0 plant set seeds in pots.

Figure 3.14 Transgenic T_0 plant failed to set seeds.

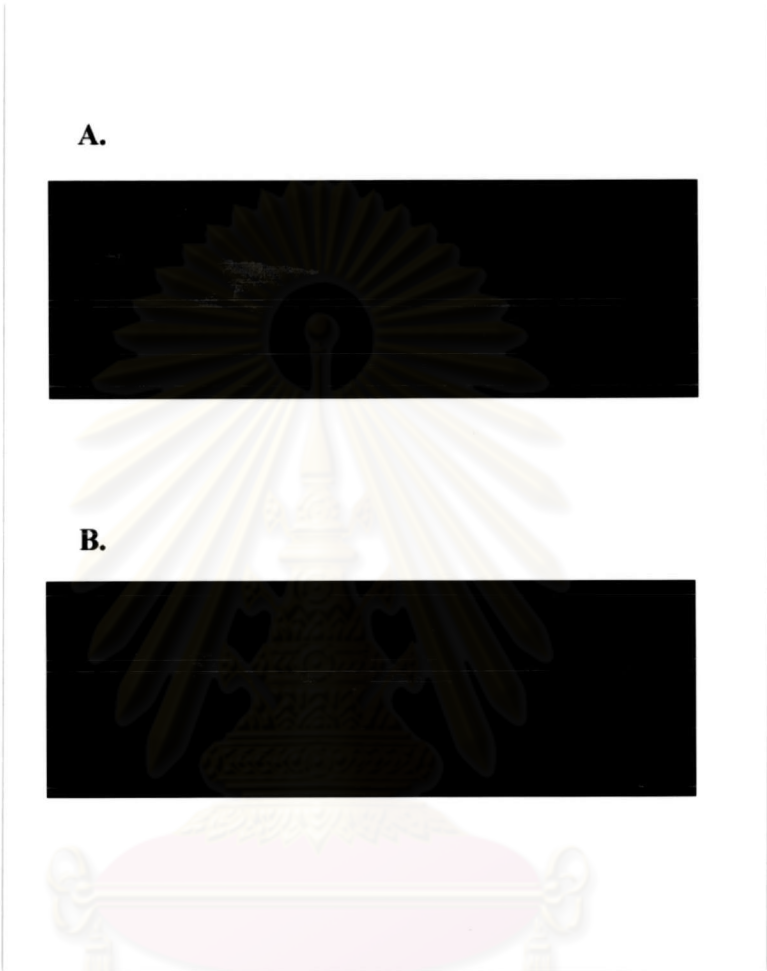


Figure 3.15 Expression of GFP in young flower observed under fluorescence microscope

(A) Transformed young flower observed under fluorescence microscope using blue filter

(B) Transformed young flower observed under fluorescence microscope using FITC filter

A B

Figure 3.16 GFP expression in T_1 transformants observed under fluorescent microscope

(A) GFP-expressing leave of T_1 transformant observed under fluorescence microscope using FITC filter

(B) Autofluorescence of non-transformed leave in T_1 plant observed under fluorescence microscope using FITC filter

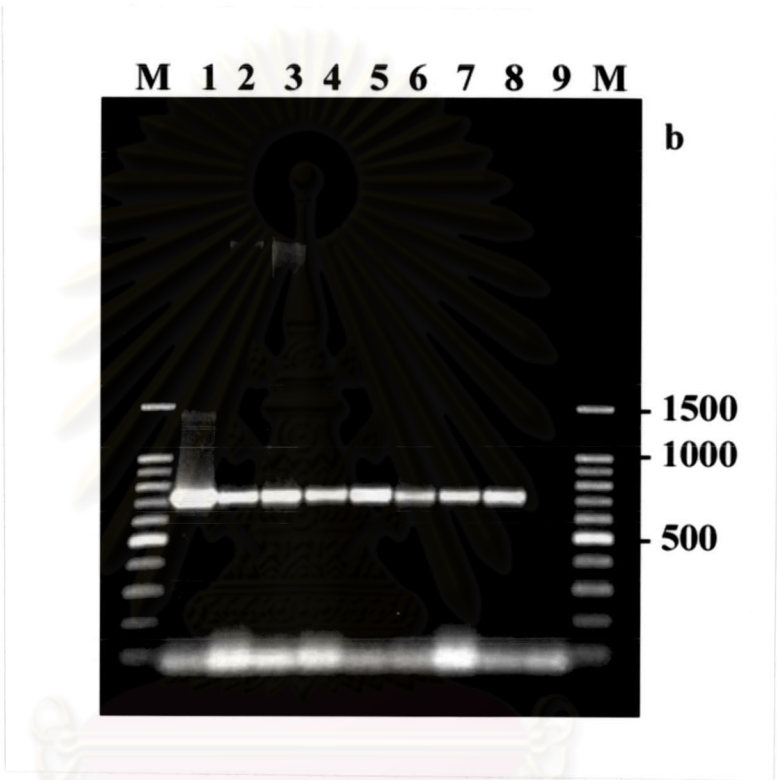


Figure 3.17 PCR analysis of the GFP in T1 transformed rice plants co-cultivated with *A. tumefaciens* EHA105 (pCAMBIA5305). Total DNA isolated from whole plants was subjected to PCR amplification with GFP specific primers (lane 2, 3, 4, 5, 6, 7, 8) showing the presence of an expected 720 bp DNA fragment of GFP gene. The GFP gene were also amplified from the pCAMBIA5305 vector (lane 1). DNA isolated from non-transformed plants was used as a negative control for GFP(lane 9)

PartIII Transformation of *hva* 1 in *O. sativa* KDML105

3.3.1 Detection of pCAMBIA5305*hva*1

Approximately 5 μg of extracted pBY520 was partial digested with both *Xho*I and *EcoRI*, resulting in linearized of pBluscriptIIKS(+) (3 kb) and fragment of inserted DNA (6kb). After separation in 1% agarose gel, the 6 kb fragment was eluted with QIAquick Gel extraction Kit and ligated into XhoI/EcoRI site of pCAMBIA 5305. The plasmid were extracted the recombinant clone by alkaline extraction and then digested with *Xho*I and *EcoRI* to determine the corrected insert fragment. As shown in Fig 3.18, pCAMBIA5305*hva*1 carried 4 DNA fragments with estimated sizes of 8.3, 4.2, 1.1 and 0.4 kb (lane 4). The 8.3 kb fragment corresponded to the linearized pCAMBIA5305. The others showed the same restriction pattern identical to those of pBY520 (6kb).

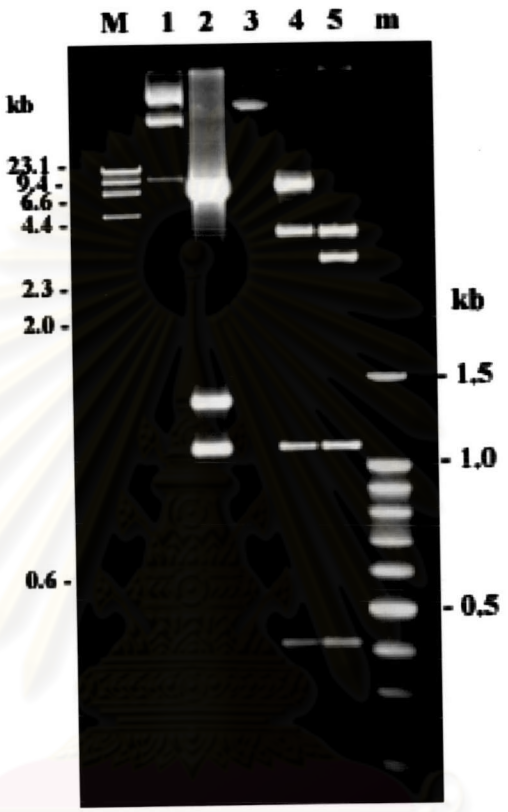


Figure 3.18 A restriction pattern of *Xho*I / *Eco*RI digested pCAMBIA5305, pBY520 and pCAMBIA5305*hva*1

Lane M = λ/ *Hin*dIII standard marker

Lane 1 = undigested pCAMBIA5305 vector

Lane 2 = pCAMBIA5305 digested with *Xho*I and *Eco*RI

Lane 3 = undigested pCAMBIA5305*hva*1 vector

Lane 4 = pCAMBIA5305*hva*1 digested with *Xho*I and *Eco*RI

Lane 5 = pBY520 digested with *Xho*I and *Eco*RI

Lane m = a 100 bp DNA ladder

3.3.2 Co-cultivation of rice calli with *A. tumefaciens* (pCAMBIA5305 *hva*1)

We constructed vector, pCAMBIA5305*hva*1, containing the *Act*1-*hva*1-*nos* gene in the T-DNA region. The embryogenic calli were co-cultivated for 3 days and GFP fluorescence was examined after 3 days co-cultivation. The embryogenic calli showed GFP-positive green spot. Out of 35 calli co-cultivated, 6 GFP positive calli were obtained. The transformation efficiency, defined as the number of independent transformants divided by the number of explants inoculated, as a percentage, was 20.5% (Table 3.8). Control treatment have never yielded GFP-positive green spot.

3.3.3 Investigation of the role of LEA3 accumulation in *Oryza sativa* KDML105

To recover the *hva*1 transformed calli, the embryogenic calli were co-cultivated with *A. tumefaciens* EHA105 (pCAMBIA5305*hva*1). The embryogenic calli were co-cultivated for 3 days. Concentrations of the herbicide selection agent was varied at 6 , 8 and 10 mg/l glufosinate. After 6-7 weeks on the first selection medium (NB-0-0-0; NB without glutamine, casein hydrolysate and proline), the GFP positive calli were selected through a fluorescence microscope. The transformed calli were visualised as small cluster of GFP-expressing cells. No green fluorescence was observed in untransformed control calli. On 6 mg/l glufosinate selection , 3 GFP resistant calli were obtained corresponding to 1.8% transformation efficiency. And 8,10 mg/l glufosinate selection, the value were 2.7 and 2.2% respectively (Table 3.9). The transformed calli appeared to greatly reduce the number of surviving untransformed cell. However, above selection medium used during the callus induction stage did not effectively inhibit non-transformed calli. Therefore, the second selection was transferred to medium (NB-0-0; NB without casein hydrolysate and glutamine), individual callus pieces were broken down into several small pieces and separately maintained. Non infected control treatments did not show continuous growth and turned brown in selective callus induction medium (Fig 3.19). During the subsequent two selection passages (each selection were 14 days) for 4 weeks at 8 mg/l glufosinate. Some pieces of callus observed as being compact and grew vigorously; and in some case callus became soft and watery. Analysis of transformed embryogenic calli was performed by fluorescent microscopy. The 8 mg/l glufosinate resistance calli were observed GFP expression under fluorescent microscope whereas

no green fluorescence we observed in untransformed control calli (Fig 3.20). The frequency transformation of plant, upon GFP expression and *hva*1 (level mRNA) ,which selected on 8 mg/l glufosinate, was 0.54% (Table 3.9).

3.3.4 Regeneration of transformed calli

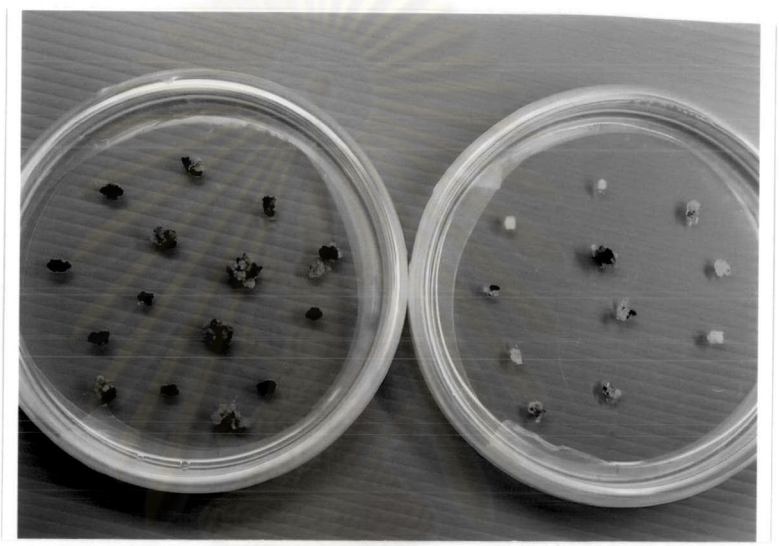
Most of callus lines were not morphogenic and did not produce any shoots after regeneration onto medium. Of the 12 callus lines produced from three concentration selection, 1 line from each glufosinate selection 8,10 mg/l gave rise to plants (Fig 3.19). Transformed calli were transferred into regeneration medium (NB4-RE) supplemented with 6 mg/l glufosinate to induce shoot formation. Shoot emerged from resistant calli on selective regeneration medium within 2-3 weeks (Fig. 3.21). One line from 8 mg/l glufosinate had normal morphology. No differences were observed in the morphology transformed (Fig 3.22) and untransformed plants. Another line from 10 mg/l glufosinate gave rise to albino plants (Fig 3.23). Subsequently, both lines were then transferred into rooting medium supplemented with 3 mg/l glufosinate to induce root medium. The frequency of regeneration was range from 20 to 25% of selected calli (Table 3.9).

Table3.8 Expression of GFP in rice callus after co-cultivation with pCAMBIA5305*hva*1

Glufosinate mg/l	No. of co-cultivated calli	Produced GFP+ calli	Transformation efficiency GFP+ calli
8	34	7	20.5

Table3.9 Efficiency of rice transformation by *A. tumefaciens* EHA105 (pCAMBIA5305*hva*1)

Glufosinate mg/l	No of co-cultivated calli (a)	Produce glufosinateR and GFP+ callli (b)	Transformation efficiency glufosinateR and GFP+ (b/a)	Produced plants (c)	(%) Regeneration (c/b)	Produced GFP+ and hva1+ plants (d)	Frequency (d/a%)
6	164	3	1.8	--	--	--	--
8	186	5	2.7	1	20	1	0.54
10	178	4	2.2	1	25	0	--



A B

Figure 3.19 Proliferation of the infected callus under the second glufosinate selection (8 mg/l glufosinate). Rice embryogenic callus transformation mediated *by A. tumefaciens* EHA105 (pCAMBIA5305*hv*a1)

(A) Brown non-transformed embryogenic calli

(B) Resistant embryogenic callus growing on selective hygromycin medium after 4 weeks on the second selection medium

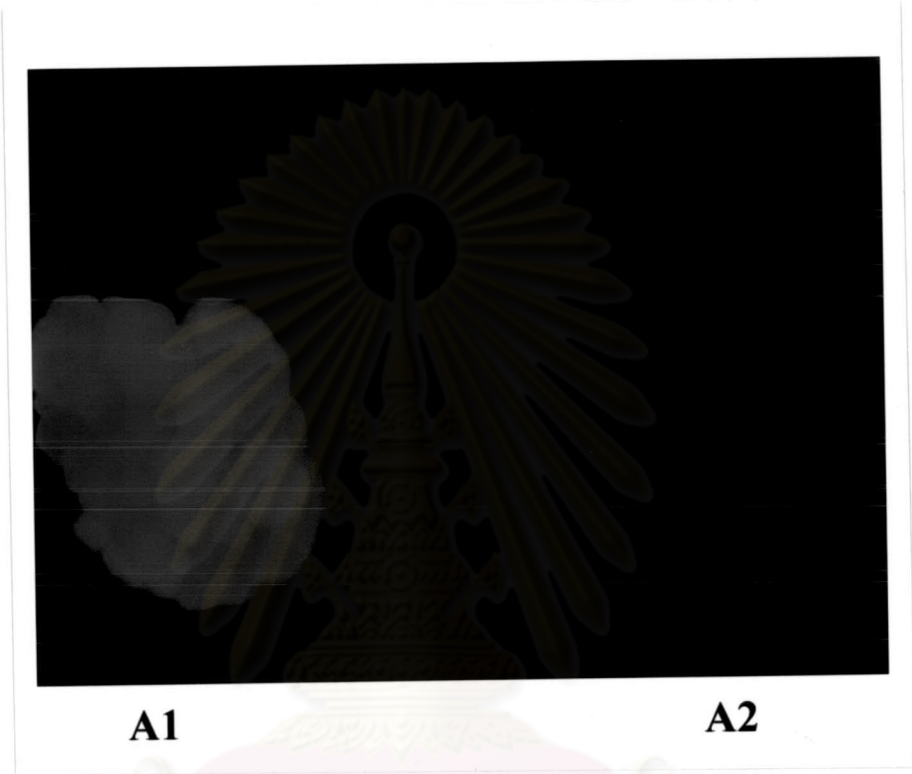


Figure 3.20 GFP as a vital screenable marker in rice transformation using *A. tumefaciens* EHA105 (pCAMBIA5305). Observations was performed using a fluorescent microscope

(A1) Transformed embryogenic callus 4 weeks on the second selection medium observed under the fluorescent microscope using (FITC) filter.

(A2) Non-transformed embryogenic callus 4 weeks on the selection medium observed under the fluorescent microscope using (FITC) filter.

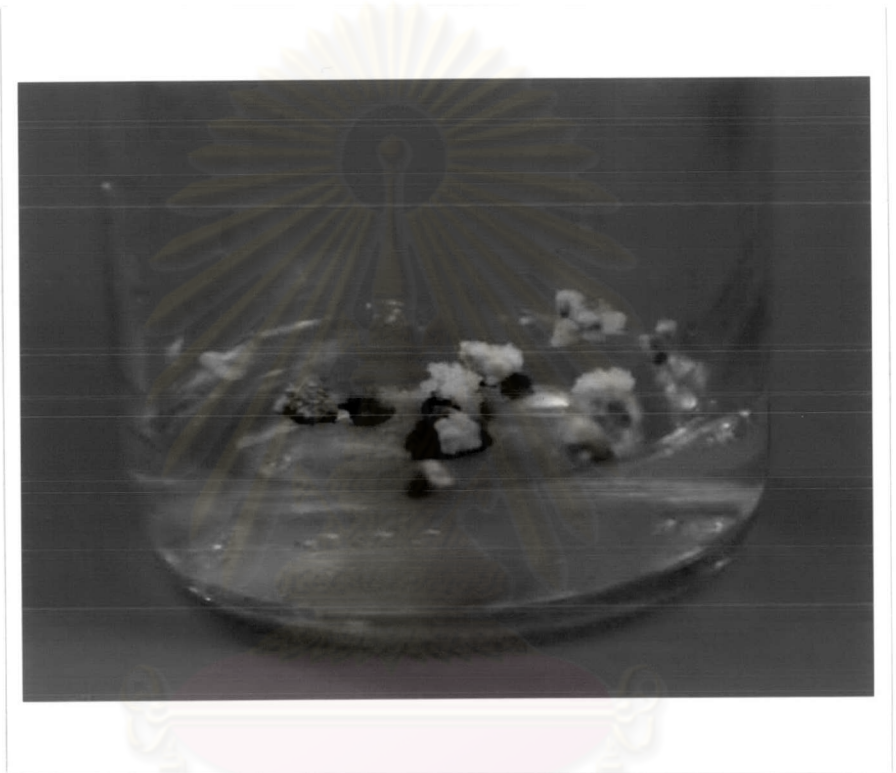


Figure 3.21 Embryogenic callus produced green spots after 3 weeks on regeneration medium supplemented with 6 mg/l glufosinate

Figure 3.22 Comparison of regenerated non-transformed rice (left) and regenerated transgenic rice(right) after transfer to rooting medium at 6 mg/l glufosinate for 3 weeks.

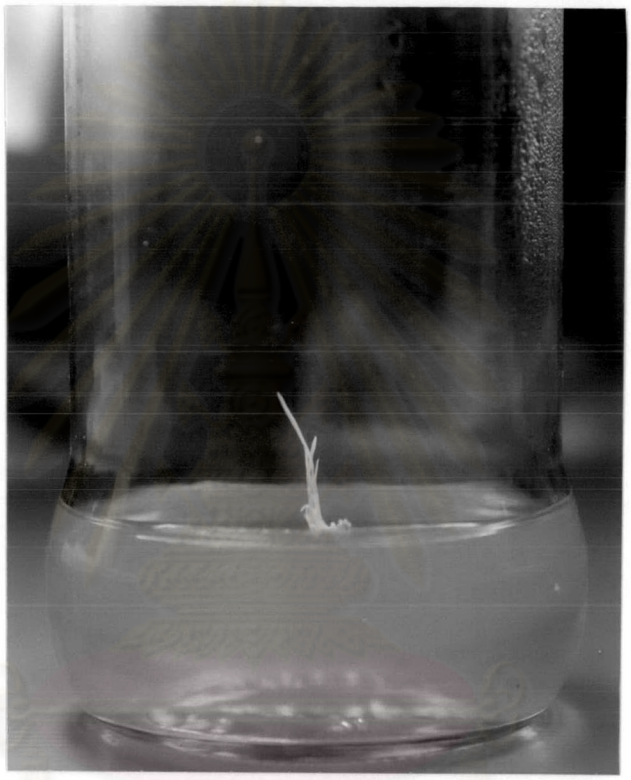


Figure 3.23 Embryogenic callus produced albino shoot after 3 weeks on rooting medium supplemented with 3 mg/l glufosinate

3.3.5 PCR analysis of *hva*1 transformed rice plants

Molecular analysis by PCR amplification confirmed that the *hva*1 gene was present in the putative transformed rice plants. A pair of primers from *hva*1 gene was designed. Genomic DNA from KDML 105 putative transformants was amplified with *hva*1 gene primers. Of the 2 lines from 8, 10 mg/l glufosinate, one band corresponding to the expected *hva*1 fragment of 652 bp was detected in line that was selected on 8 mg/l glufosinate (Fig 3.24). No band was detected in case of negative control and albino plant which selected on 10 mg/l glufosinate.

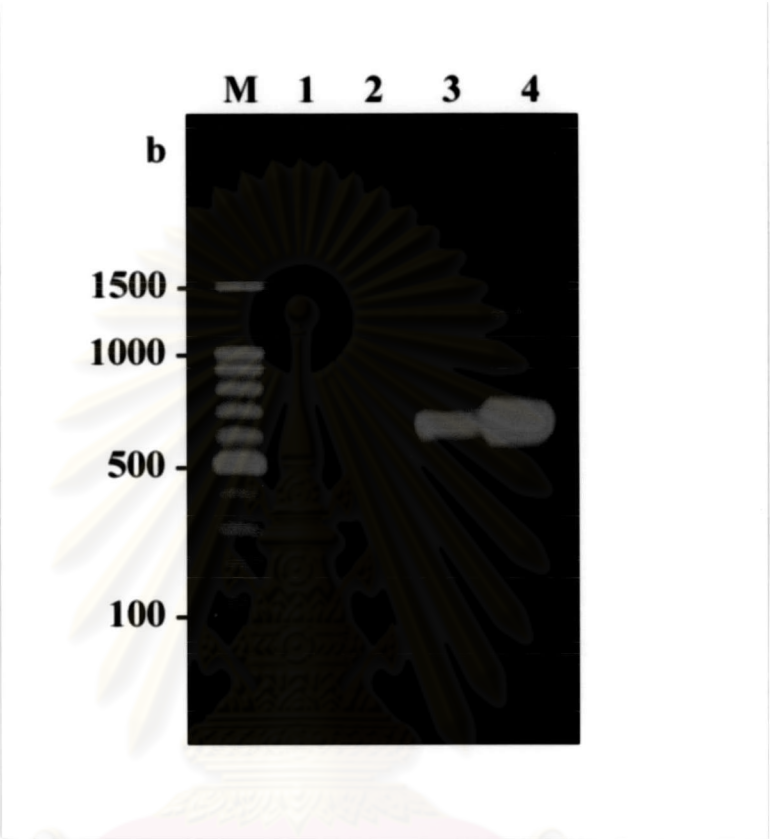


Figure 3.24 PCR analysis of the GFP in putative transformed rice plants co-cutivated with *A. tumefaciens* EHA 105 (pCAMBIA5305*hva*1)

Lane M = 100 bp DNA ladder

Lane 1 = PCR product of non-transformant plant with GFP specific primer

Lane 2 = PCR product of albino transformant plant selected on 10 mg/l glufosinate with GFP specific primer

Lane 3 = PCR product of transformant plant selected on 8 mg/l glufosinate with GFP specific primer

Lane 4 = PCR product of pCAMBIA5305*hva*1 with GFP specific primer

3.3.6 Expression of *hva*1 gene and GFP gene

RT-PCR was used to detect the expression of *hva*1 gene in transformants. The Expected 652 bp band was obtained from line that was selected on 8 mg/l glufosinate (Fig. 3.25). The negative control and albino plant, which was selected on 8 mg/l glufosinate, did not have this band. To sure that the amplified bands were not from tract genomic DNA in the DNA solution, the RNA solution was used without reverse transcription for *hva*1 fragment amplification. No band was produced from this sample, indicating the RT-PCR band from transformant was produced from the mRNA of *hva*1.

The expression of *gfp* gene was also investigated at the transcription level by RT-PCR of the corresponding mRNA. The first strand cDNA which synthesized from transformants were subjected to PCR amplification with GFP specific primers. The result showed that the expression of *gfp* gene was only obtained from the 8 mg/l glufosinate selected line similar to that demonstrated in the expression of *hva*1 gene (Fig. 3.26). In addition, the expression of *gfp* gene was confirmed by GFP fluorescence. The emerging primary shoots showed uniformed GFP fluorescence under fluorescent microscope using FITC filter (Fig 3.27). No GFP fluorescence was detected in the corresponding parts of non– transformed plants.

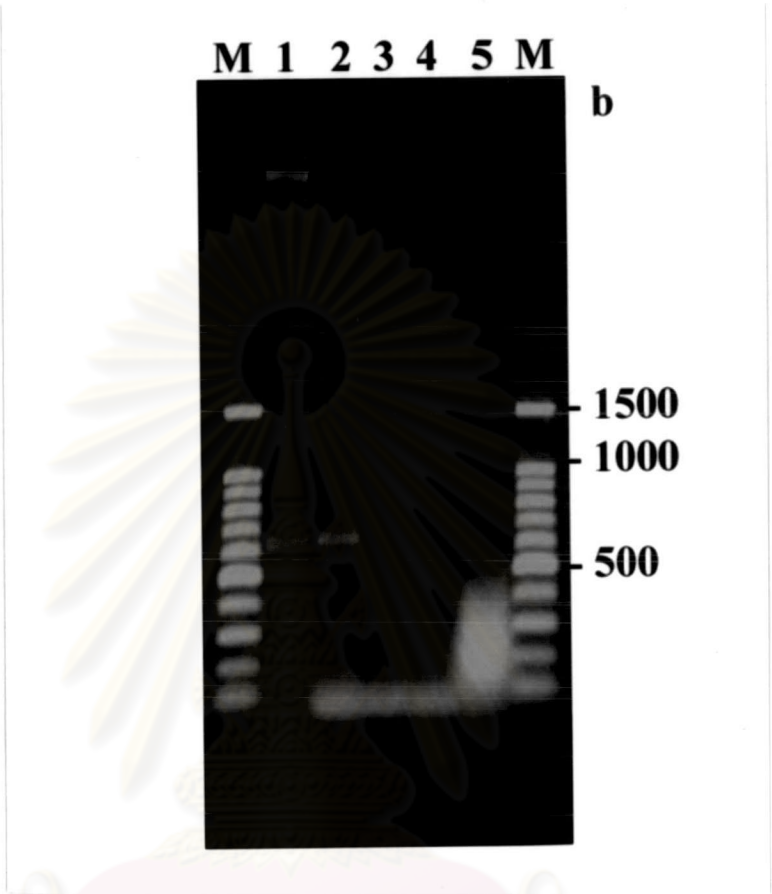


Figure 3.25 RT-PCR analysis of the expression of *hva*1 in transgenic lines

Lane M = 100 bp ladder

Lane 1 = Amplification using DNA templates from pCAMBIA5305*hva*1

Lane2,3 = Amplification using templates from reaction of reverse transcription of RNA extraction from plant which regenerated on 8 and 10 mg/l glufosinate ,respectively.

Lane 4 = Amplification using templates from reaction of reverse transcription of RNA extraction from non-transfomed plant.

Lane 5 = Amplification using RNA solution from plant which regenerated on 8 mg/l glufosinate.

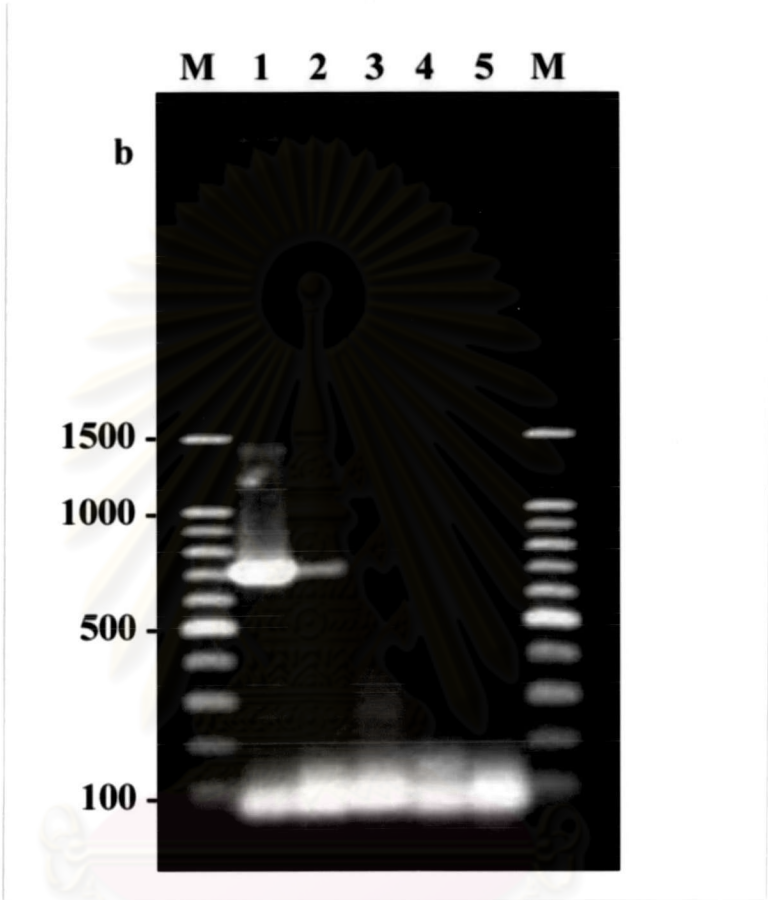


Figure 3.26 RT-PCR analysis of the expression of GFP in transgenic lines

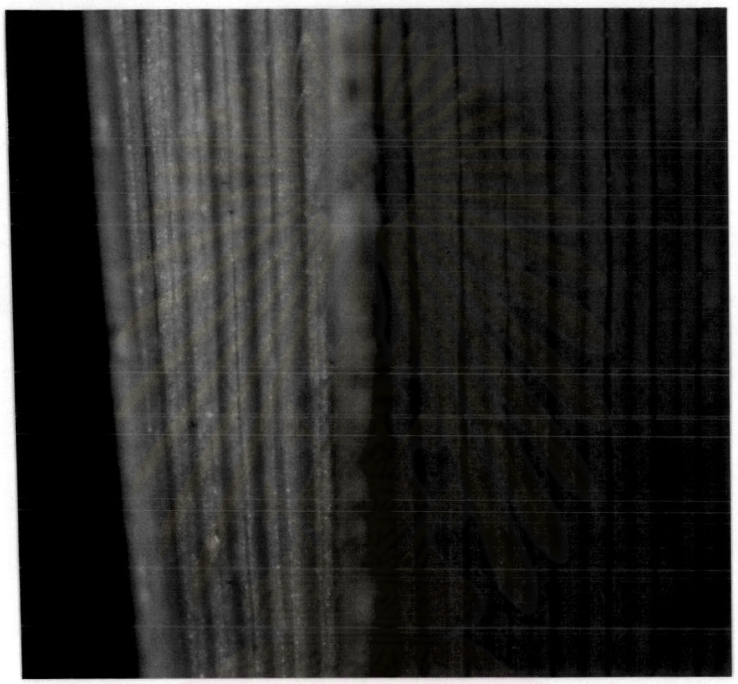
Lane M = 100 bp ladder

Lane 1 = Amplification using DNA templates from pCAMBIA5305*hva*1

Lane2,3 = Amplification using templates from reaction of reverse transcription of RNA extraction from plant which regenerated on 8 and 10 mg/l glufosinate ,respectively.

Lane 4 = Amplification using templates from reaction of reverse transcription of RNA extraction from non-transfomed plant.

Lane 5 = Amplification using RNA solution from plant which regenerated on 8 mg/l glufosinate.



A1 A2

Figure 3.27 Detection of GFP in the leaves of transgenic rice plant (8 mg/l glufosinate selection) 6 weeks after regeneration.

(A1) GFP-expressing leaves observed under fluorescent microscope using FITC filter.

(A2) Autofluorescence of non-transformed leaves under fluorescent microscope using FITC filter.