Transformation and Expression of Green Fluorescent Protein and Barley Stress-Induced Embryogenic *hva* 1 Gene in *Indica* Rice KDML 105 by *Agrobacterium tumefaciens* EHA 105

Kanyanee Samiphak and Napa Siwarungson

Agrobacterium tumefaciens EHA 105 habouring pCAMBIA 5305 was used for co-cultivation of embryogenic calli from indica rice KDML 105. Conditions of infection were 10 minutes infected/2 days co-cultivated and 15 minutes infected/3 days co-cultivated. After 8 weeks selection with hygromycin, the efficiency of transformation of calli upon hygromycin resistance and GFP expression was 10 and 16%, respectively. Transgenic rice plants were regenerated from visually selected GFP-positive calli. The frequency of transformation, based upon GFP fluorescence and PCR analysis, ranged from 4 to 5.5%. Expression of GFP and its inheritance was stable in T_1 progeny. The embryogenic calli were co-cultivated with *A. tumefaciens* EHA 105 (pCAMBIA 5305*hva*1). The embryogenic calli were co-cultivated for 3 days. Concentrations of selected medium used were 6, 8 and 10 mg/l glufosinate. The efficiency of transformed calli upon resistance to glufosinate and GFP expression were 1.8, 2.7 and 2.2%, respectively. Of the 12 callus lines produced from the three concentrations, one line from 8 mg/ml glufosinate selection gave rise to transgenic plants. The frequency transformation of plants upon GFP expression and *hva*1(level mRNA), when selected at 8 mg/ml glufosinate, was 0.54%.

Key words: GFP, KDML *Agrobacterium tumefaciens* EHA 105 and *hva*1

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การส่งทอดและการแสดงออกของยีนที่สร้างโปรตีนเรืองแสงสีเขียวและยีน *hva* 1 จากต้นข้าวบาร์เล่ย์อ่อนที่เหนี่ยวนำภายใต้สภาวะความเครียดเข้าสู่ข้าวหอมดอกมะลิ 105 โดยอาศัย *Agrobacterium tumefaciens* EHA105

กัลยาณี สามิภักดิ์ และนภา ศิวรังสรรค์ (2549)

วารสารวิจัยวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย **31(1)**

Agrobacterium tumefaciens strain EHA 105 ที่มี pCAMBIA 5305 เป็นส่วนประกอบได้ถูกนำมาใช้สำหรับการเพาะเลี้ยงร่วมกันของแคลไลจากต้นอ่อนข้าวหอมดอกมะลิ 105 สภาวะของการติดเชื้อได้แก่ 10 นาทีติดเชื้อ / 2 วัน เพาะเลี้ยงร่วมกัน และ 15 นาทีติดเชื้อ / 3 วัน เพาะเลี้ยงร่วมกัน หลังจากการคัดเลือกเป็นเวลา 8 สัปดาห์ด้วย hygromycin พบว่าประสิทธิภาพของการส่งทอดของแคลไลโดยการต้านทานต่อ hygromycin และการแสดงออกของยีน GFP เป็นร้อยละ 10 และ 16 ตามลำดับ ต้นข้าวที่ได้รับการส่งยีนเจริญเป็นต้นใหม่จากการคัดเลือกแคลไลที่เห็นเรืองแสงสีเขียว ความถี่ของการส่งทอดโดยวัดการเรืองแสงของ GFP และการวิเคราะห์ PCR อยู่ในช่วงจากร้อยละ 4 ถึง 5.5 การแสดงออกของ GFP และการถ่ายทอดกรรมพันธุ์มีความเสถียรในรุ่นลูก T_1 แคลไลจากต้นอ่อนเพาะเลี้ยงร่วมกันกับ A. tumefaciens EHA 105 (pCAMBIA 5305*hva*1) เป็นเวลา 3 วัน มีการเปลี่ยนแปลงความเข้มข้นของ glufosinate 6, 8, 10 มิลลิกรัม/ลิตร ประสิทธิภาพของแคลไลที่ส่งทอดโดยความต้านทานต่อ glufosinate และการแสดงออกของ GFP เป็นร้อยละ 1.8, 2.7 และ 2.2 ตามลำดับ จาก 12 แคลไลที่ผลิตจากความเข้มข้น 3 ระดับ ในอาหารที่คัดเลือกพบว่า มี 1 แคลไลที่คัดเลือกจากความเข้มข้นของ glufosinate 8 มิลลิกรัม/ลิตร เกิดเป็นต้นข้าวที่ได้รับการส่งทอดยีน ประสิทธิภาพการส่งทอดของพืชที่มีการแสดงออกของ GFP และยีน *hva* 1 โดยตรวจจากระดับ mRNA ที่คัดเลือกจากความเข้มข้นของ glufosinate 8 มิลลิกรัม/ลิตร มีค่าร้อยละ 0.54

คำสำคัญ GFP ข้าวหอมมะลิ *hva*1

INTRODUCTION

Recent advances in transformation technology have resulted in the routine production of transgenic plants for an increasing number of cereal species.(1) Genetic engineering of rice for insect resistance can be obtained by introducing genes conferring insecticidal activities, such as an endotoxin gene from *Bacillus thuringiensis*.(2-4) Studies on developing virus resistance in rice through genetic engineering(5) and improving quality of rice(6,7) have also been made. Several other genes of agronomically important traits such as herbicide resistance,(8,9) salt and water deficit(10,11) and fungal resistance have also been introduced in rice.(12,13) *Agrobacterium*-mediated transformation offers some advantages in comparison with direct transformation methods such as low copy integration, relatively precise mode of DNA transfer, high efficiency of transformation, transfer of relatively large pieces of DNA, absence of a requirement for protoplast culture techniques, less expense and consisting of a single procedure.(14) The progress with foreign gene delivery and gene expression has been more rapid with rice than with any other cereal. The frequency of transformation varies with both genotype and with the transformation system used; for example, *japonica* cultivars of rice in general are more tissue culture-responsive and give higher frequency of transformation than *indica* cultivars, and one finds variations even between different *japonica* cultivars. The objective of this study was to create transformed rice (*Oryza sativa* cv. KDML 105) with green fluorescent protein (GFP) reporter genes. In addition, we transformed *O. sativa* cv. KDML 105 with the barley *hva*1 gene by co-cultivation with *Agrobacterium* and detected expression of this gene. The knowledge obtained can be applied to development of plant genetic engineering.

MATERIALS AND METHODS

Transformation vector

The transformation vector, pCAMBIA 5305 (Figure 1), contains the synthetic green fluorescent protein (sGFP), the hygromycin-resistant gene (*hpt*) with an intron as a plant selectable marker within the left and right border of T-DNA. Each gene was under the control of a 35S promoter from cauliflower mosaic virus (CaMV). The expression vector pBY520,(11) which is a derivative of pBluescript II KS (+) (strategene), consists of two gene expression cassettes. One of the cassette genes, *hva* 1, is regulated by the *act*1 promoter and the potato Pin2 3' region and the other one bar gene, which serves as the selectable marker in rice transformation, is controlled by the CaMV 35S promoter and the nopaline synthase gene 3' region. The vector pBY520 was partially digested with *Xho*I and *EcoR*I. The two gene cassettes were inserted between the *Xho*I and *EcoR*I site of pCAMBIA 5305 to create pCAMBIA 5305*hva*1 (Figure 2).

Production of embryogenic calli from mature embryos

The *indica* rice cultivar (*Oryza sativa* L. KDML 105 was obtained from the Bangkhen rice research center. Mature seeds were dehulled and first sterilized with 70% ethanol for 1 minute and then with 2.5% sodium hypochlorite for 40 minutes with shaking. The seeds were rinsed 3 times with sterile deionized water. These were cultured on NB-1 (Table 1) supplemented with 2 mg/l of 2,4-dinitrophenoxy acetic acid (2,4D) for callus induction. The cultures were incubated in the dark at 28°C for 6 weeks. The embryogenic calli were observed as being compact, yellowish and granular(15) and were separated with a sterile scapel and subcultured on fresh medium.

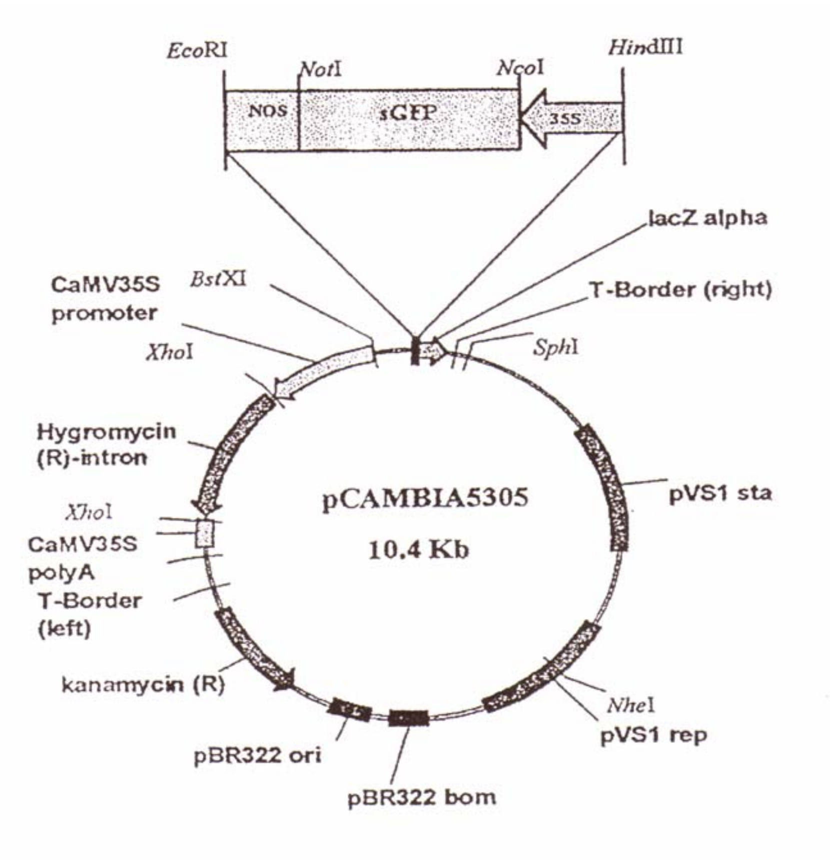

Figure 1. pCAMBIA 5305 vector

sGFP = synthetic green fluorescent protein gene
35S, CaMV35S = 35S promoter from Cauliflower Mosaic Virus
NOS = Nopaline synthetase termination
Hygromycin(R)-intron = Hygromycin resistant gene
Kanamycin (R) = Kanamycin resistant gene
Poly A = Polyadenylation site
pBR322 ori = Origin of replication on pBR322

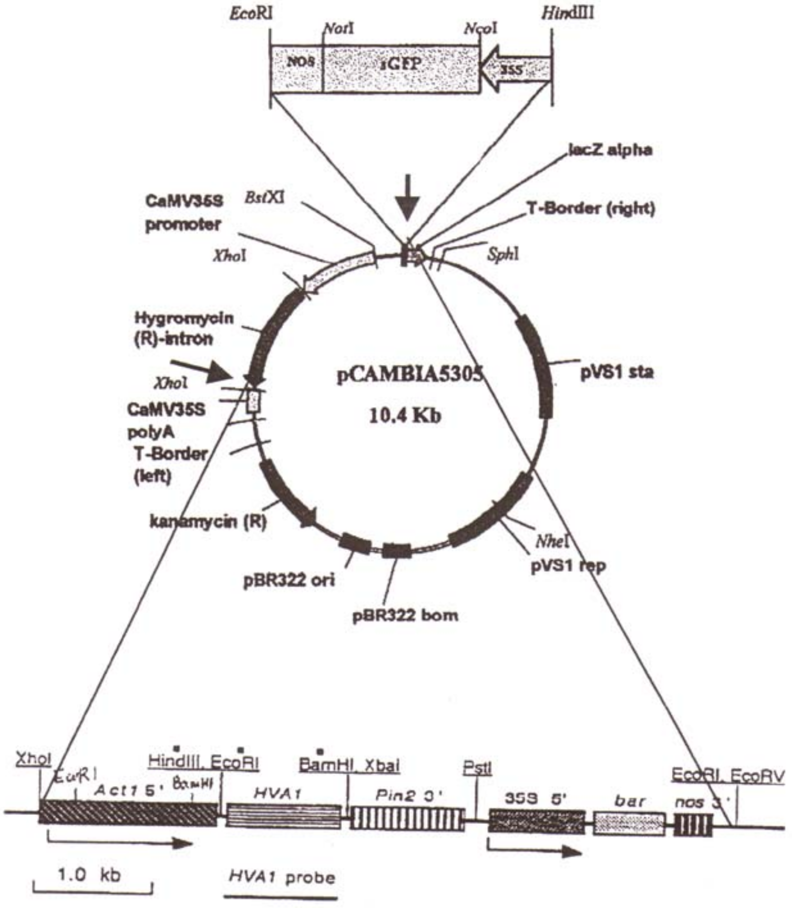

Figure 2. pCAMBIA 5305*hva*1

HVA1 = *hva1* gene
Act1 = Actin 1 gene
Pin2 = Termination signal
bar = Bialphos resistant gene for glufosinate selection

Table 1. Media used for tissue culture and transformation.

Medium	Composition
AB	(Chilton *et al*., 1974)(16)
AAM	AA salts and amino acids (Toriyama and Hinata, 1985),(17) MS vitamins (Murashige and Skoog, 1962),(18) 500 mg/l casamino acids, 68.5 g/l sucrose, 35 g/l glucose, 100µM acetosyringone, pH 5.2
NB-1	NB (Li et al., 1993),(19) 0.3 g/l casein hydrolysate, 0.5 g/l L-proline, 0.5 g/l L-glutamine, 30 g/l sucrose, 2 mg/l 2,4D, 4 g/l phyta gel
NB-1AS	NB-1 plus 100 µM acetosyringone
NB-1CH	NB-1 plus 500 mg/l cefotaxime and 50 mg/l hygromycin B
NB-2	NB, 30 g/l sucrose, 2 mg/l 2,4D, 4 g/l phyta gel
NB-2C	NB-2 plus 500 mg/l cefotaxime
NB-3	NB, 0.5 g/l L-proline, 30 g/l sucrose, 2 mg/l 2, 4D, 4 g/l phyta gel
NB-3CG	NB-3 plus 500 mg/l cefotaxime and 8 mg/l glufosinate
NB-RE1	NB, 0.3 g/l casein hydrolysate, 0.1 mg/l yeast extract, 0.5 g/l L-proline, 0.5 g/l L-glutamine, 30 g/l sucrose, 4 mg/l BAP, 6 g/l phyta gel
NB-RE2	NB, 0.1 mg/l yeast extract, 0.5 g/l L-proline, 30g/l sucrose, 4 mg/l BAP, 6 g/l phyta gel and 6 mg/l glufosinate

Co-cultivation of embryogenic calli

A. tumefaciens strain EHA105 habouring pCAMBIA5305 and *A. tumefaciens* EHA105 containing pCAMBIA5305*hva*1 were streaked on solid AB medium supplemented with 50 mg/l kanamycin. The bacteria were incubated at 28°C for 2 days. The bacteria were resuspended in AAM medium containing 100 µM of acetosyringone with vigorous shaking for a minute. The optical density of the bacterial suspension was adjusted to an OD_{600} of 0.01 by diluting with AAM medium. The four-day incubated embryogenic calli were immersed in bacterial suspension for 15 min with occasional shaking. The excess bacteria was removed by decanting the liquid, and the calli were blotted dry on sterile filter papers (Whatman No.1). The calli were then transferred to the co-cultivation medium (NB-1AS) (Table 1) and incubated in the dark at 22°C for three days. After the co-cultivation, the calli were blotted dry on sterile filter papers.

Selection and regeneration of transformed calli

For co-cultivation by *A.tumefaciens* carrying pCAMBIA5305, the calli were transferred to the selection medium (NB-1CH) (Table 1) and incubated at 28°C for 4 weeks. The hygromycin resistant calli obtained after the first round of selection was subcultured for two cycles onto fresh NB-1CH medium every two weeks. The hygromycin resistant calli were then transferred to regeneration medium without any antibiotics (NB-RE1) (Table 1) and incubated at 28°C under 16 hour light photoperiods for 3-4 weeks. Green shoots were observed after 4 weeks. When the transformed shoots became 2-3 cm in length they were transferred to hormone-free Nb-1 medium for stimulation of the roots and stem elongation for 4 weeks and transferred to soil for further growth.

For co-cultivation by *A.tumefaciens* carrying pCAMBIA5305*hva*1, the calli were transferred to the first selection medium (NB-1CH) (Table 1), supplemented with various concentrations of selection agent (6, 8, 10 mg/l ammonium glufosinate). All tissues were nonselectively subcultured without breaking every 12 days for 7 weeks. After the first subculture, the tissue was partially flattened on the medium to promote direct contact with glufosinate. Seven weeks later, all GFP positive calli from 3 concentrations were selected and subcultured on the second selection medium (NB-3CG) (Table 1). Concentration was fixed at 8 mg/l ammonium glufosinate for 4 weeks. Ammonium glufosinate-resistant calli were then transferred to regenerate medium (NB-RE2) (Table 1) supplemented with 6 mg/l glufosinate and incubated at 28°C under 16 hour light photoperiods for 3-4 weeks. Green buds/shoots were observed after 4 weeks. When the transformed shoots became 2-3 cm in length they were transferred to hormone-free rooting medium (NB-3) (Table 1) supplemented with 3 mg/l ammonium glufosinate for 4 weeks.

Detection of GFP expression by fluorescent microscopy

GFP fluorescence in leaves, roots and resistant calli was examined using an Olympus BX-50 fluorescent microscope fitted with a fluorescein isothiocyanate (FITC) filter set comprised of an excitation filter 450-490 nm, dichroic mirror 510 nm and barrier filter LP520 nm. The light source was provided by a HBO 50w high-pressure mercury bulb. Photographs were taken using an Olympus automatic exposure photomicrographic system with Kodak Ektachrome 400 film.

DNA isolation and polymerase chain reaction

Genomic DNA was extracted from control and putative transformed plants using the modified CTAB method. The presence of the GFP, *npt*II and *hva*1 gene in putative transformed plants was assessed by PCR. The amplification reactions were performed in a 25 µl reaction volume containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 100 µM each of dATP, dCTP, dGTP and dTTP, 0.4 µM of each primer and 1.0 unit of Amplitaq DNA polymerase (Promega Corporation Medison, Wisconsin). The concentration of $MgCl_2$ was 2, 1 and 0.5 mM, respectively. The GFP primer sequences were forward primer 5'-ATG GTG AGC AAG GGC GAG GAG C-3' and reverse primer 5'-TTA CTT GTA CAG CTC GTC CAT GCC-3'. The PCR program consisted of one cycle at 94°C for 2 min, then 30 cycles of denaturation at 94°C for 1 min, annealing at 68°C for 1 min and extension at 72°C for 1 min. After the last cycle, extension at 72°C for 5 min was performed to fulfill polymerization. The PCR products were stored at 4°C at the end of the PCR reaction.

The *npt*II primer sequences were forward primer 5'-AAA ACT GAT CGA AAA ATA CCG CTG C-3' and reverse primer 5'-TCC CCA GTA AGT CAA AAA ATA GCT C-3'. The PCR program consisted of one cycle at 94°C for 2 min, then 30 cycles of denaturation at 94°C for 1 min, annealing at 53°C for 30 sec and extension at 72°C for 1 min. The final extension was carried out at 72°C for 5 min. At the end of the reaction, the PCR products were stored at 4°C.

The *hva*1 primer sequences were forward primer 5'-AGA CGA AGA TGG CCT CC-3' and reverse primer 5'-GTC TAG TGA TTC CTG GT-3'. The PCR program consisted of one cycle at 94°C for 2 min, then 30 cycles of denaturation at 94°C for 1 min, annealing at 53°C for 30 sec and extension at 72°C for 1 min. The final extension was carried out at 72°C for 5 min. At the end of the reaction, the PCR products were stored at 4°C.

RNA analysis

Total RNA was extracted with TRI_{zol} (GIBC-BRL). First stand cDNA templates for PCR were prepared by using Omniscript Reverse Transcriptase (Qiagen, Germany). Two microliters of RT product was then used in 25 µl of PCR reaction solution. The reaction conditions were the same as described above for amplification of the *hva*1 fragment.

RESULTS

In the first experiment, hygromycin (50 mg/l) was used as a selectable agent. The infecting conditions varied from 10 min infection/2 days co-cultivation to 15 min infection/3 days co-cultivation. Hygromycin resistant calli were obtained after 4 weeks of treatment. These growing calli were excised and transferred to fresh selection medium and incubated in darkness 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 the selection medium. After 8 weeks on selection with hygromycin, bright-green fluorescent calli were observed growing on hygromycin. No green fluorescent calli were observed in untransformed control calli. Transformation efficiency of 3 days co-cultivated calli was approximately two fold higher GFP positive calli than 2 days co-cultivation. 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 occurred within 3 weeks of transferring hygromycin resistant calli onto regeneration medium (NB-RE1). Healthy plantlets with extensive root system were established on NB-1 medium without any plant hormone after another 2-3 weeks. No difference was observed in the morphology between transformed and untransformed plants. Regenerated plants were tested by GFP fluorescence and PCR analysis. green buds/shoots from somatic embryos A total of 9 transformants could be regenerated from GFP-positive and hygromycin-resistant calli of rice (KDML105). Of these, 5 transformants were from 2 day 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.

Table 2. Efficiency of rice transformation by *A. tumefaciens* EHA 105.

No. of co-cultivated calli	Produced HygR and GFP+ calli	Transformation efficiency HygR/GFP+	Produced plants	(%) regeneration	Produced GFP+ plants	Frequency
(a)	(b)	(b/a)	©	(c/b)	(d)	(d/a%)
[A] 90	9	10	5	55.5	5	5.5
[B] 100	16	16	4	25	4	4

HygR = hygromycin resistance
[A] = 10 min infection, 2 days co-cultivation
[B] = 15 min infection, 3 days co-cultivation

Expression of GFP in primary transformed rice plants

Analysis of embryogenic calli and plant transformation were performed by fluorescent microscopy with FITC filter. Green fluorescence was observed in calli and whole plants. Both roots and leaves were cross-sectioned and observed under fluorescent microscope. In young root sections, the expression of GFP was found in vascular bundle cylinders, the cortex and epidermal cells (Figure 3). The expression in leaves of older plants was limited to vascular bundle tissues (Figure 4) with fluorescence more prominent in the xylem and phloem than in the other cells.

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) transformants resulting from infections with *A. tumefaciens* EHA 105 (pCAMBIA 5305) was amplified with GFP gene primer as well as *npt*II gene primer (Figure 5). One band corresponding to the expected GFP fragments of 720 bp was detected in all the tested transformants. No band was detected in the 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. None of the plants analyzed showed amplification of the *npt*II gene sequence thus eliminating the possibility of bacterial contamination in the transformed tissue.

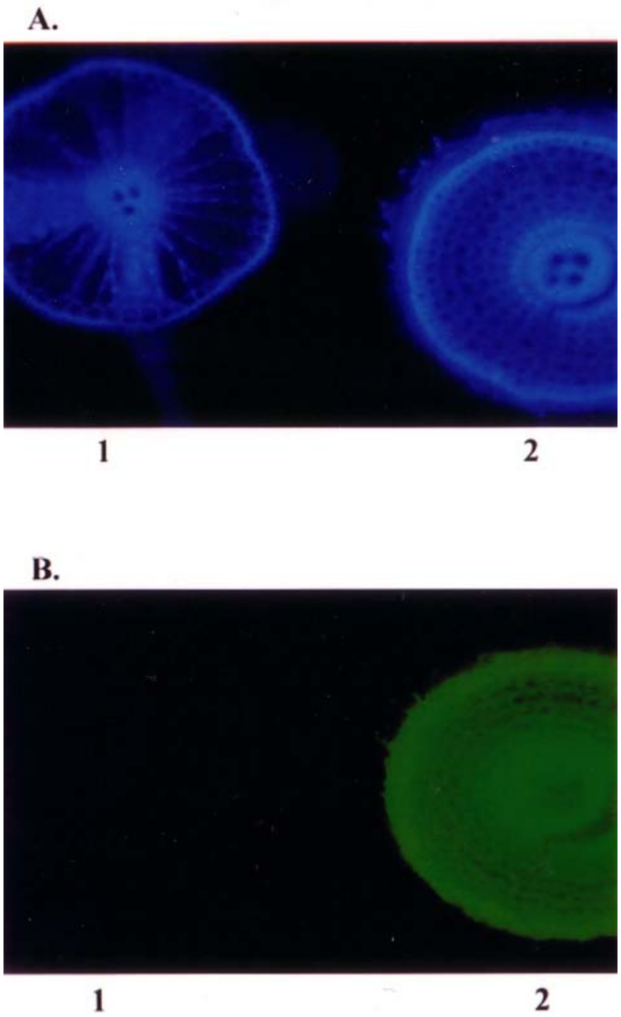

Figure 3. Cross-section of root under fluorescent microscope.

(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

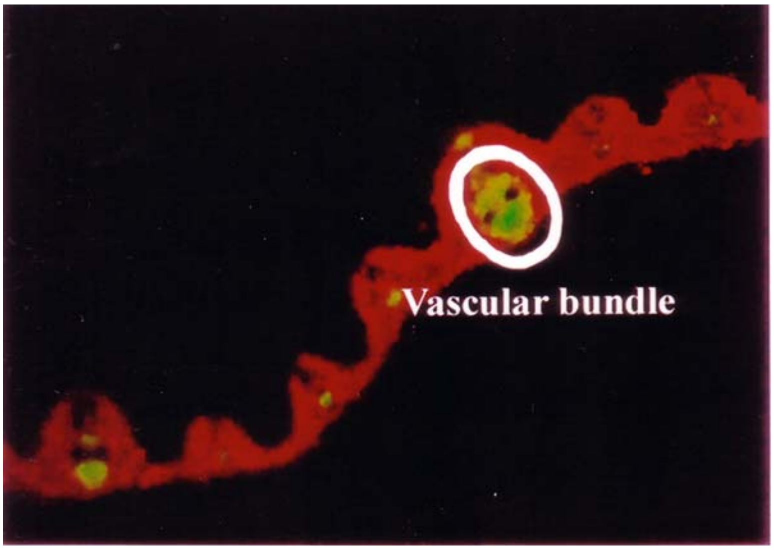

Figure 4. Cross-section of older leaves after 2 months in field. The green fluorescence appeared in vascular bundles only.

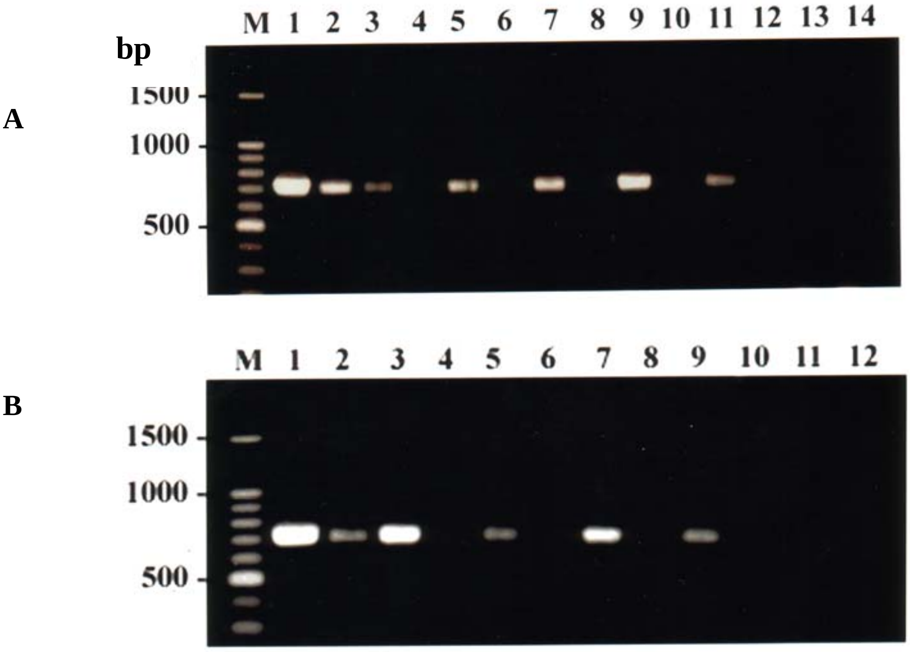

Figure 5. PCR analysis of the GFP gene in transformed rice plants co-cultivated with *A. tumefaciens* EHA 105 (pCAMBIA5305) for 2 and 3 days (Figure 5A and 5B respectively). *Agrobacterium* was used at around 0.01 A_{600} unit. Total DNA isolated from whole plants was subjected to PCR amplification with 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).

Progeny analysis

Transgenic plants were grown in pots. After 4-6 weeks, all plants grew to flower. Some transgenic plantlets flowered late and were unable to set seeds. Of the 9 plant lines, 3 lines were able to set seed. After 1 month, kernels developed on the plant and some mature seed was harvested from the plant (5 seeds per lines : 3 repeat). Germinated seeds of approximately 2 to 3 cm were then transferred to soil. The expression of GFP and its inheritance was tested in T_1 progeny by GFP fluorescence and PCR analysis. The data is summarized in Table 3. We found that GFP fluorescence and PCR were present in all seedlings.

Table 3. 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
$p<0.05$

Table 4. Efficiency of rice transformation by *A. tumefaciens* EHA105 (pCAMBIA5305*hva*1).

No of co-cultivated calli	Produce glufosinate R and GFP+ calli	Transformation efficiency glufosinate R and GFP+	Produced plants	% Regeneration	Produced GFP+ and *hva*1 plants	Frequency
(a)	(b)	(b/a)	(c)	(c/b)	(d)	(d/a%)
186	5	2.7	1	20	1	0.54

Glufosinate R = glufosinate resistance
GFP+ = expression of GFP

Expression of *hva*1

Molecular analysis by PCR amplification confirmed that the GFP gene was present in the transformed rice plant (Figure 6). RT-PCR was used to detect the expression of *hva*1 gene in transformants. The expected 652 bp band was obtained from the line that was selected with 8 mg/l glufosinate (Figure7). The negative control did not have this band. To ensure 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 the transformant was produced from the mRNA of *hva*1.

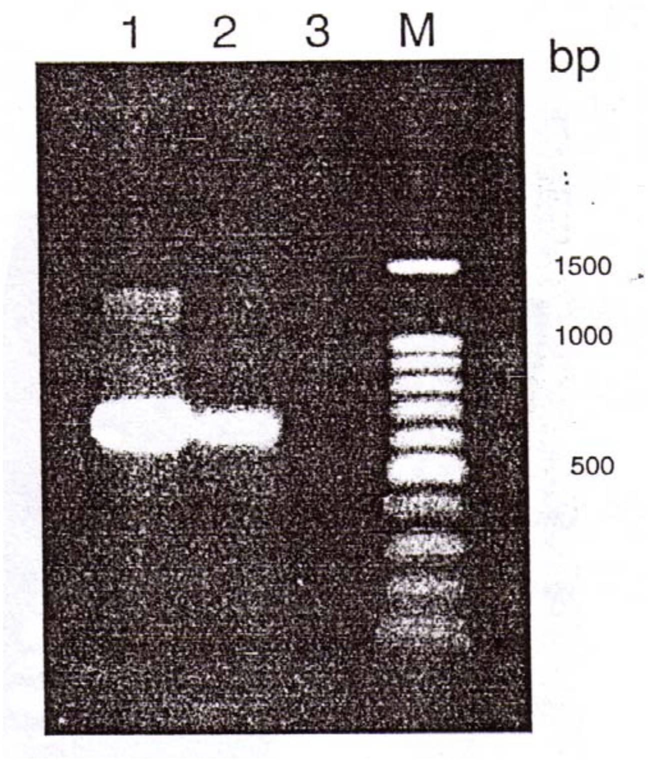

Figure 6. PCR analysis of the *hva*1 in putative transformed rice plants co-cultivated with *A. tumefaciens* EHA 105 (pCAMBIA5305*hva*1).

Lane M = 100 bp DNA ladder
Lane 1 = PCR product of pCAMBIA5305*hva*1 with *hva*1 specific primer
Lane 2 = PCR product of transformant plant selected on 8 mg/l glufosinate with *hva*1 specific primer
Lane 3 = PCR product of non-transformant plant with *hva*1 specific primer

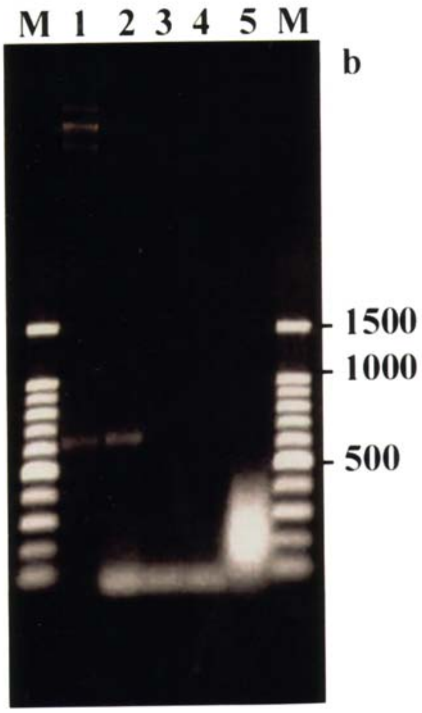

Figure 7. RT-PCR analysis of expression of *hva*1 in transgenic lines

Lane M = 100 bp ladder
Lane 1 = Amplification using DNA templates from pCAMBIA 5305*hva*1
Lane 2 = Amplification using templates from reaction of reverse transcription of RNA extraction from plant regenerated on 8 mg/l glufosinate.
Lane 3 = Amplification using templates from reaction of reverse transcription of RNA extraction from non-transformed plant.
Lane 4 = Amplification using RNA solution from plant regenerated on 8 mg/l glufosinate.

DISCUSSION

The calli initiated from scutella of mature seeds were used as starting material for the *Agrobacterium*-mediated transformation of rice. Some earlier studies used calli initiated from immature embryos(20) due to their better regeneration potential. The seeds were cultured on NB medium supplemented with 2 mg/l of 2,4D for 6 weeks. Actively growing embryogenic calli was used for plant regeneration and transformation experiments. The use of young embryogenic calli as target explants eliminated a long, tedious culture procedure and further problems with somaclonal variation.(21) Preculture of embryogenic calli for 4 days to co-cultivation has been found to enhance the rate of cell division(22) and thereby make the tissue more competent for *Agrobacterium* infection.(23) Hygromycin (50 mg/l) was used as a selectable agent. The infected conditions were varied from 10 min infection / 2 days co-cultivation to 15 min infection / 3 days co-cultivation. Transformation efficiency was different between the 2 conditions. Three day co-cultivation gave approximately 1.6 higher GFP positive calli than 2 day co-cultivation. In contrast, transgenic calli at 3 days co-cultivation reduced 4 fold regeneration frequency compared to 2 fold of 2 day co-cultivation. This study suggested that the excessive growth of bacteria on the calli caused them to brown and resulted in reduced callus initiation. Plants were obtained in only five to six months after transformation. The majority of plants were free of morphological aberrations, probably as a result of the fact that the cells were maintained in culture *in vitro* for a short time. In addition, the important of *hva*1 on the expression of *hva*1 under the provided promoter may affect the survival rate of transgenic rice.

The expression of transgenic plantlets was observed under fluorescent microscope. Both leaves and roots showed GFP expression. They were then cross-sectioned. In young root sections, the expression of GFP was found in vascular bundle cylinders, cortex and epidermal cell. The expression in leaves of older plants was limited to vascular bundle tissue(24) previously reported that the CaMV 35S promoter was more strongly expressed in cells of the vascular bundle.

The healthy transgenic plantlets were transferred to pots in December. During the next 2-3 months all plants grew to flower. Of the 9 month lines, only 3 lines were able to set seed. This observation suggested that rice (KDML105) was photoperiod sensitive. In general, rice seedlings were grown to flower for 3 months. Mature rice set seed in the appropriate photoperiod. Some transgenic plantlets were late to grow. The flowers of immature plants were unable to set seed. Moreover, ear of rice had a low yield.

After harvesting, the germinated seedling was tested for the expression of *gfp* and its inheritance by molecular analysis. We found that GFP fluorescence and PCR in all seedlings. These results suggested that two copies of the *gfp* gene are inserted on homologous chromosomes of the plant. The transgene is able to pass to the progeny though both male and female gametes. The result of segregation is a dominant expression of *gfp* in all progeny, if a single copy *gfp* gene is inserted on heterozygous chromosome of such a plant. The Mendelian segregation for dominant *gfp* expression to recessive no *gfp* expression will be a 3 : 1 ratio, but the number of seeds in each line was low in number. Perhaps the recessive was not obtained in this experiment.

The pCAMBIA5305*hva*1 was constructed by replacing the hygromycin resistance gene with two cassettes DNA of *hva*1 gene and *bar* gene. The direction of these cassettes DNA was reversed to the direction of 35S promoter which controlled the *gfp* gene. We found that three genes within the left and right border of T-DNA showed the expression. Moreover, the rice actin 1 gene showed that an efficient promoter for regulation of the *hva*1 gene in rice KDML 105 was shown.

Maintenance of the embryogenesis status of calli on the selection medium was important for the efficient recovery of regenerants. To have effective phosphinothricin selection, it was important to omit not only glutamine from the selective medium, but also several other amino acids. Addition of 10 mM glutamic acid, 25 mM proline, 10 mM arginine, or 25 mM proline in combination with 10 mM arginine to the medium with 20 mg/l phosphinothricin restored the growth rate from 9% to 31%, 49%, 65% or 80%, respectively, compared to the growth on medium without selection.(25) For this reason, we excluded both proline, casein hydrolysate and glutamine from the first selection medium during the transformation experiments.

We transformed the barley *hva*1 gene into embryogenic calli by co-cultivation with *A. tumefaciens* EHA105 carrying pCAMBIA5305 *hva*1. After 3 days on 8 mg/l glufosinate selection, the transformation efficiency was 20.5%. When the embryogenic calli were selected for 2 months, the transformation efficiency was 2.7%. Moreover, the transformation efficiency was 1.8 and 2.2%, respectively. Of the 12 callus lines produced from three concentration selections, 1 line from each 8 and 10 mg/l glufosinate selection gave rise to plants. This observation suggested that the loss of transformed calli, regeneration capacity and an increase of albinism were usually associated with in the length of time in culture on the selection medium. These results were similar to the study of Witrzens *et al.*(26)

The relationship of the transformation by *Agrobacterium*-mediated method using the two plasmids: pCAMBIA5305 and pCAMBIA5305 *hva*1 in respect to using hygromycin in comparison with glufosinate as the transformation efficiency is 10-16% as compared to 2.7%. The frequency is very different 4-5.5%, as compared to 0.5% due to the bigger size of plasmid having lower transformation efficiency and resulting in the lower frequency as well. *Hva*1 was involved in the survival rate of transgenic rice under the stress response.

In conclusion, the transformation and expression of green fluorescent protein and barley stress-induced embryogenic *hva*1 gene by *Agrobacterium tumefaciens* strain EHA 105 in indica rice KDML 105 was successful and we look forward to applying the modification to the stress response of rice in a field test.

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Received: January 21, 2005
Accepted: January 12, 2006