ORIGINAL ARTICLE

Transformation of rice (*Oryza sativa* L.) cv. Chainat 1 using chitinase gene

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Abstract

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As prerequisites for generating stable transformed rice (*Oryza sativa* L.) cv. Chainat 1, efforts were made to improve the efficiency of regeneration systems for rice cv. Chainat 1. The suitable medium which resulted in 85.7% callus induction from rice seeds was N_6 medium supplemented with 4.5 μM 2,4-D, 2.5 μM NAA and 500 mg/l casein hydrolysate under light condition. The calli were dehydrated in a petridish for 5 days before being transferred to regeneration medium. The suitable medium for shoot regeneration from the calli of rice cv. Chainat 1 was MS medium supplemented with 9 μM BA, 1 μM NAA and 300 mg/l casein hydrolysate. An experiment was conducted to determine the effect of antibiotics on callus induction. It was found that hygromycin at 20 mg/l was effective for transformant selection. The highest concentration of cefotaxime that the calli could tolerate was 400 mg/l. The genetic transformation of rice cv. Chainat 1 mediated by *Agrobacterium tumefaciens* strain LBA4404, which harbored the plasmid pCAMBIA 1305.1 containing chitinase gene, β-glucuronidase (GUS) and hygromycin resistance (*hptII*), was used in the procedure. The optimal co-cultivation time was 30 minutes. Particle bombardment was also used to transform chitinase, GUS and *hptII* genes, using GUS assay to test transformation efficiency under the proper

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conditions. It was found that particle bombardment at the distance of 9 cm from stopping screen to callus brought about the highest transformation efficiency. PCR method confirmed the integration of chitinase gene, selectable marker and screenable marker in the transformants.

Key words : callus induction, regeneration, transformation, *Agrobacterium*, particle bombardment, chitinase gene

บทคัดย่อ

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การส่งถ่ายยีนไคทิเนสสู่ข้าวพันธุ์ชัยนาท 1

ว. สงขลานครินทร์ วทท. 2548 27(6) : 1151-1162

ในการส่งถ่ายยีนเข้าสู่ข้าวพันธุ์ชัยนาท 1 เพื่อให้ได้ข้าวแปลงพันธุ์ที่มีความเสถียรนั้น จำเป็นต้องปรับปรุงประสิทธิภาพในการชักนำให้แคลลัสข้าวเจริญเป็นต้นอ่อนก่อน สูตรอาหารที่เหมาะสมและสามารถชักนำให้เมล็ดข้าวเกิดแคลลัสได้ 85.7% คือ อาหารสูตร N_6 ที่เติม 2,4 -D ความเข้มข้น 4.5 ไมโครโมลาร์ ร่วมกับ NAA 2.5 ไมโครโมลาร์ และเคซีนไฮโดรไลเซต 500 มก./ล. ภายใต้สภาวะที่มีแสง ในการชักนำแคลลัสข้าวให้เกิดเป็นต้นอ่อน ต้องดึงน้ำออกจากแคลลัสก่อน โดยการวางในจานเพาะเลี้ยงเป็นเวลา 5 วัน และสูตรอาหารที่เหมาะสมในการชักนำให้แคลลัสเกิดยอดได้ดีคือ อาหารสูตร MS ที่เติม BA ความเข้มข้น 9 ไมโครโมลาร์ ร่วมกับ NAA 1 ไมโครโมลาร์ และเคซีนไฮโดรไลเซต 300 มก./ล. ในการศึกษาอิทธิพลของสารปฏิชีวนะ ไฮโกรมัยซินและซีโฟแทกซีมต่อการเจริญของแคลลัส พบว่าไฮโกรมัยซินความเข้มข้น 20 มก./ล. เหมาะสมที่จะใช้คัดเลือกต้นข้าวแปลงพันธุ์ชัยนาท 1 ที่ทนทานต่อไฮโกรมัยซิน และความเข้มข้นสูงสุดของซีโฟแทกซีมที่แคลลัสทนได้คือ 400 มก./ล. การส่งถ่ายยีนเข้าสู่ข้าวโดยใช้ *Agrobacterium tumefaciens* สายพันธุ์ LBA4404 (pCAMBIA 1305.1) ที่มียีน chitinase ยีน GUS (ยีนรายงานผล) และยีน *hptII* (ยีนคัดเลือก) พบว่าระยะเวลาที่เหมาะสมในการบ่มชิ้นส่วนพืชร่วมกับ *Agrobacterium* คือ 30 นาที และได้ทำการศึกษาการส่งถ่ายยีน chitinase ยีน GUS และยีน *hptII* เข้าสู่ข้าวโดยวิธีตรงโดยใช้วิธี particle bombardment จากการตรวจสอบประสิทธิภาพการส่งถ่ายยีนโดยวิธี GUS assay พบว่าในการส่งถ่ายยีนเข้าสู่ข้าวโดยวิธี particle bombardment ซึ่งมีระยะห่างระหว่าง stopping screen กับแคลลัส เท่ากับ 9 ซม. ให้ประสิทธิภาพในการส่งถ่ายยีนสูงสุด ซึ่งเมื่อใช้วิธีตรวจสอบการสอดแทรกของ DNA โดยใช้ปฏิกิริยาลูกโซ่โพลีเมอเรส (PCR) พบว่ามีการสอดแทรกของยีน chitinase ยีน GUS และยีน *hptII* ในข้าวแปลงพันธุ์ชัยนาท 1

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Rice (*Oryza sativa* L.) is an important economic crop. Loss in rice yield is mostly due to pathogens. Application of broad spectrum pesticides is a primary method used for controlling fungal and insect pests. Such application has resulted reduction of environmental pollution and ecological disruption. As the target pests become less susceptible to the pesticide, there can be a resurgence of original pests, requiring recurrent application of excessive quantities of pesticides to control them. Various strategies for biological control of fungal and insect pests have been done. An attractive technique for pest control is the induction of chitinase gene incorporated into rice for pathogen resistance. Chitinase (EC.3.2.1.14), which degrades chitin, a β-1,4-linked homopolymer of N-acetyl-D-glucosamine (Glu-NAC), is widely distributed in many species of higher plants. Plant chitinase has played an important role in the defense mechanism against pathogenic fungi that

contain chitin in their cell walls. Enhanced chitinase in transgenic plants can reduce the damage caused by pathogens (Broglie *et al*., 1991). The introduction of chitinase gene into plants enhances resistance to fugal diseases in several crops (Chen and Punja, 2002; Kishimoto *et al*., 2002; Nishizawa *et al*., 1999).

Several rice cultivars were transformed with *Agrobacterium* harboring the rice chitinase gene under the control of CaMV 35 S promoter. The transgenic plants were confirmed to possess and express the transformed gene; they showed enhanced resistance to rice blast disease (Nishizawa *et al*., 1999). Lin *et al*. (1995) obtained a certain degree of resistance against sheath blight in transgenic indica rice, Chinsurah BoroII harboring the rice chitinase gene Chi1.1. Recently, Datta *et al*. (2000) introduced the chimeric chitinase gene into rice by using *Agrobacterium*. The transgenic rice exhibited resistance to sheath blight pathogens. The regeneration of rice plantlets from callus grown in tissue culture has been previously reported (Raineri *et al*., 1990; Thadavong *et al*., 2002; Pipatpanukul *et al*., 2004).

This research was carried out to verify a transformation system using *Agrobacterium* and particle bombardment to transfer chitinase gene into indica rice. The rice cultivar used for the transformation was Chainat 1, a photoperiod insensitive rice widely grown in the irrigation zones of Thailand (Rice Research Institute, 2005). This cultivar is susceptible to insect pests and diseases. The successful production of transgenic plants of this cultivar will be useful for study of the effects of chitinase expression in the transgenic rice upon pathogens.

Materials and Methods

1. Callus induction

Dehusked mature seeds of rice cv.Chainat 1 were washed with mild detergent and surface-sterilized by soaking in 70% ethanol for 2 min and in 35% clorox containing a wetting agent "Tween 20" for 60 min. After three washes in sterile water, these seeds were cultured on Nitsch & Nitsch (1969) (N_6) medium (pH 5.7) containing 3.5, 4.5 or 5.5 µM 2,4-dichlorophenoxyacetic acid (2,4-D); 2, 2.5 or 3 µM naphthaleneacetic acid (NAA), 500 mg/l casein hydrolysate, 30 g/l sucrose and 0.8% agar. The explants were divided into two groups; the first group was cultured under light condition (illuminated by 2000 lux of cool-white fluorescent light with 16 h photoperiod) and the second group was maintained under darkness at 25±2ºC. After 4 weeks of culture, the number of seeds forming callus and the size of callus [(width +length)/2] were recorded to determine the optimum concentration of 2,4-D and NAA for callus induction. The experiment was arranged as a completely randomized design with 5 replications.

2. Plant regeneration

Four-week-old calli were cultured on N_6 medium supplemented with 4.5 µM 2,4-D, 2.5 µM NAA and 500 mg/l casein hydrolysate and dehydrated by being placed on the single layer of filter paper in petridishes and sealed with parafilm. The petridishes were kept at 25ºC under light condition for 5 days. Dehydrated calli were then cultured on MS medium (pH 5.7) with or without 15% coconut water, 10 µM kinetin or 9 µM BA in combination with 5 µM IAA or 1 µM NAA, 300 mg/l casein hydrolysate, 2% sucrose, and 0.8% agar. The cultures were incubated at 25ºC under light condition. Six weeks after culture, the number of calli forming shoots and the number of shoots per callus were counted to determine the optimum combination of IAA or NAA and BA or kinetin for plant regeneration from seed-derived calli.

3. Effect of antibiotics

Surface-sterilized seeds of rice cv. Chainat 1 were induced to form calli on N_6 medium (pH 5.7) containing 4.5 µM 2,4-D, 2.5 µM NAA, 500 mg/l casein hydrolysate, 30 g/l sucrose and 0.8% agar. After 10 days, calli were transferred to the callus induction medium supplemented with hygromycin and cefotaxime.

For determining the effect of antibiotics on callus induction,hygromycin and cefotaxime were

added to the callus induction medium. The concentrations tested were 0, 10, 20, 30, 40 and 50 mg/l for hygromycin and 250, 300, 350, 400, 450, 500, 550, 600 and 650 mg/l for cefotaxime. All levels of the two antibiotics were added to the callus induction medium after if was autoclaved. Calli were cultured at 25ºC under 16 h photoperiod. Four weeks after culture, the effectiveness of antibiotics was evaluated.

4. Transformation

A 1.1 Kbp rice chitinase gene (*chi 1.1*) was released from plasmid (pAHG11) by *Hind*III restriction enzyme.

The plant transformation vector, pCAMBIA 1305.1 was linearised with *Hind*III. The chitinase gene was ligated with linearised vector. This plasmid, pCAMBIA 1305.1 containing chitinase gene was mobilized into *A. tumefaciens* host strain, LBA4404 by electroporation. The plasmid pCAMBIA 1305.1 contained rice chitinase, β-glucuronidase (GUS) and hygromycin resistance (*hpt*II) genes, each of which was expressed under CaMV 35S promoter.

4.1 Effect of co-cultivation condition on transformation efficiency

Sterilized mature seeds, 7- and 10-day-old seed-derived calli of rice cv. Chainat 1 were used as the explants for transformation in this experiment. *A. tumefaciens* strain LBA 4404 pCAMBIA 1305.1 was cultured overnight at 28ºC in Luria Broth medium containing 50 m/l kanamycin for 48 h until OD_{600} = 1.0. The explants were soaked in the *Agrobacterium* suspension for 10, 20, 30, 40 and 50 min, then blotted dry on sterilized filter paper and transferred to the induction medium for 3 days. Five days after co-cultivation, the optimal co-cultivation time for transformation efficiency was determined by recording numbers of blue spots on explants.

4.2 *Agrobacterium*- mediated transformation

Seven-day-old seed-derived calli were immersed in the *Agrobacterium* suspension for 30 min, then blotted dry on sterilized filter paper. They were then co-cultivated on the callus induction medium for 3 days. After co-cultivation, the calli were washed thoroughly in sterile distilled water containing cefotaxime. Explants co-cultivated with *A. tumefaciens* were transferred for selection procedure to the same medium supplemented with cefotaxime and hygromycin. The surviving calli were then transferred to the regeneration medium supplemented with the same selective agents. After 60 days of culture, regenerating plants with well developed roots were potted and grown in a greenhouse.

4.3 Particle bombardment

Prior to bombardment, the plasmids containing chimeric DNA were precipitated and adsorbed to M17 tungsten particles following the procedure recommended by Sanford *et al*. (1993). Ten-day-old seed-derived calli of rice cv. Chainat 1 were used as the explants for bombardment. The calli were placed in the center of petridishes containing N_6 medium. These calli were bombarded with the DNA-coated tungsten particles. The distance from the stopping screen to target was 9-12 cm, and the rupture disc strength was 1,100 psi. The calli were then maintained at 25ºC under light condition. Three to five days after bombardment, calli were transferred to the callus induction medium containing hygromycin for selection of antibiotic resistant calli and cultured at 25ºC under light condition. The resistant calli were then transferred to the regeneration medium supplemented with the same selective agent. After 60 days of culture, regenerating plants with well developing roots were potted and grown in a greenhouse.

5. Assay for β-glucuronidase (GUS) activity

The histochemical assay for GUS gene expression was performed according to the method of Jefferson (1987), using 5-bromo-4-choro-3-indolyl glucoronide (X-gluc) as a substrate. An incubation temperature of 37ºC was used.

6. PCR analysis

Total genomic DNA was extracted from the transformed calli and non-transformed (control) calli by the CTAB method (Doyle and Doyle,1987). The primer sequences for PCR were as follows:

35 S forward sequence (F)5'-GCTCCTACAAAT GCCATCA-3', reverse sequence (R) 5'GATAGTG GGATTGTGCGTCA-3' to yield a 195 bp fragment; NOS (F) 5'-GAATCCTGTTGCCGGTCT TG-3', (R) 5'-TTATCCTAGTTTGCGCGCTA-3' to yield a 180 bp fragment; chi (F) 5'-GCTCCT ACAAATGCCATCA-3', (R) 5'-GATAGTGGGA TTGTGCGTCA-3' to yield a 454-bp fragment. The DNA was denatured at 94ºC for 3 min, followed by 40 cycles of amplification [20 sec at 94ºC; 40 sec at 50ºC (35 S and NOS) or 56ºC (chi); 60 sec at 72ºC]. The final incubation at 72ºC was extended to 3 min, and the reaction material was cooled and kept at 4ºC . The PCR products were visualized by running the completed reaction on a 2% agarose gel containing ethidium bromide.

Results

1. Callus induction

The development of rice embryos was distinct at 2-3 days after being cultured on the N_6 medium containing 2,4-D and NAA. The color of scutellum-derived callus was yellow and the callus had compact appearance under light condition while the color of calli cultured in darkness was creamy white and the callus had friable character. It was found that the seeds cultured on the N_6 medium supplemented with 2,4-D and NAA in every concentration, produced calli under light and dark conditions (Table 1). However, seeds cultured under light condition gave higher callus formation percentage (74.0) and larger average size of callus (6.7 mm). The highest callus induction percentage (85.7%) and largest size of callus (7.3 mm) were obtained in the N_6 medium containing 4.5 µM 2,4-D and 2.5 µM NAA (Figures 1A and 1B).

2. Plant regeneration

Dehydrated calli cultured on MS medium with or without coconut water containing either kinetin or BA and IAA or NAA, formed green spots after being transferred to modified MS medium for 1 week. However, low frequencies of shoots derived from green spot calli were observed. The dehydrated calli cultured on MS medium supple-

Table 1. Callus induction of rice cv. Chainat 1 on the N_6 medium supplemented with different concentrations of 2,4-D and NAA under light and dark conditions.

2,4-D + NAA (µM)	Light		Dark	
	% Seed forming callus	Size of callus[1/] (mm)	% Seed forming callus	Size of callus[1/] (mm)
3.5+2.0	77.0	6.3 b	71.0	4.0 ab
3.5+2.5	78.0	6.5 ab	65.0	3.6 b
3.5+3.0	70.0	6.0 b	70.0	3.5 b
4.5+2.0	67.0	6.2 b	63.0	3.7 b
4.5+2.5	**85.7**	**7.3 a**	75.0	4.7 a
4.5+3.0	83.3	7.2 a	71.3	4.5 a
5.5+2.0	63.0	6.7 ab	62.0	4.7 a
5.5+2.5	79.0	7.2 ab	68.0	4.8 a
5.5+3.0	63.0	6.5 ab	70.0	4.8 a
Average	**74.0**	**6.7**	68.3	4.3
F-test		*		*
CV(%)		8.51		10.26

*** Significant at p<0.05.[1/] Means within a column not sharing a common letter differ significantlyby $LSD_{0.05}$.**

mented with 9 µM BA, 1 µM NAA and 300 mg/l casein hydrolysate had the highest percentage of shoot regeneration (40%) and the largest number of shoots per callus (6) (Figures 1C and 2).

3. Effect of antibiotics

Antibiotics used in the study strongly reduced callus induction of rice. In the presence of 10-15 and 50-250 mg/l hygromycin and cefotaxime, a slight inhibition effect was observed. The lowest dose of hygromycin that completely inhibited callus growth was 20 mg/l (Figure 3A). All of the calli turned brown and eventually died in three weeks after they were transferred to the selective medium. The highest dose of cefotaxime that yielded surviving calli was 400 mg/l (Figure 3B and 4).

4. Transformation

β-Glucuronidase enzyme activity was used to monitor transformation efficiency. Blue staining was observed as early as 5 days after cocultivation. The highest number of blue spots was observed from 7-day-old seed-derived calli cocultivated in *Agrobacterium* suspension for 30 min (Figure 5).

Rice transformation via particle bombardment at the distance of 9 cm from stopping screen to callus yielded a higher percent expression (100%), compared to using *Agrobacterium* (61%) and particle bombardment at the distance of 12 cm (35.5%) (Figure 6). DNA integration of the transformed calli of rice cv. Chainat 1 was investigated using PCR method. It was found that the sizes of amplified fragments were 195 bp, 180 bp and 464 bp for the 35S, NOS and chitinase gene, respectively, whereas non-transformed control rice did not show any expected band size (Figure 7A,B and C).

Discussion

The presence of plant growth regulators is generally essential to growth promotion. They are required either singly or more commonly in combination. The most suitable auxin for callus induction of rice in tissue culture is 2,4-D at various concentrations, depending on the explant source and genotype of rice (Raina,1989). Although 2,4-D alone induced callus formation from rice embryos, some organic substances such as casein hydrolysate, auxin (NAA,IAA) added to the callus induction medium containing 2,4-D could enhance the efficiency of callus formation (Sripichitt and Cheewasestatham, 1994; Vajrabhaya *et al*., 1986). We indicated that 2,4-D, NAA, and casein hydrolysate at 4.5 µM, 2.5 µM and 500 mg/l, respectively, were suitable for callus production from rice seeds. This result was in accordance with many previous reports (Pipatpanukul *et al*., 2004; Raina *et al*., 1987; Sripichitt and Cheewasestatham, 1994; Thadavong *et al*., 2002). Light condition seemed to have stimulative effects on callus proliferation from embryos of rice. This is in agreement with the previous works of Pipatpanukul *et al*. (2004), Thadavong *et al*. (2002) and Toki *et al*. (1997).

Dehydration of calli for 5 days under light condition before transferring to regeneration medium promoted plant regeneration capacity. The effect of dehydration on plant regeneration from rice callus has been manifested in several previous studies (Sripichitt and Cheewasestatham, 1994; Thadavong *et al*., 2002; Tsukahara and Hirosawa, 1992). Gray (1987) suggested that the cells of callus which lost water reabsorbed water and nutrients when they were transferred to the regeneration medium, and this consequently resulted in higher capacity of plant regeneration. The suitable medium for plant regeneration from dehydrated calli under light condition in this experiment was MS medium supplemented with 9 µM BA, 1 µM NAA and 300 mg/l casein hydrolysate. Increasing the concentration of BA in the medium enhanced the frequency of shoot regeneration. Kinetin is a kind of cytokinin; however, it induced less shoot regeneration than BA in this study. There were several reports demonstrating that casein hydrolysate induced higher percentage of calli forming green spot and shoot than no supplement (Raina *et al*., 1987; Thadavong *et al*., 2002).

A selective agent is crucial for selection of

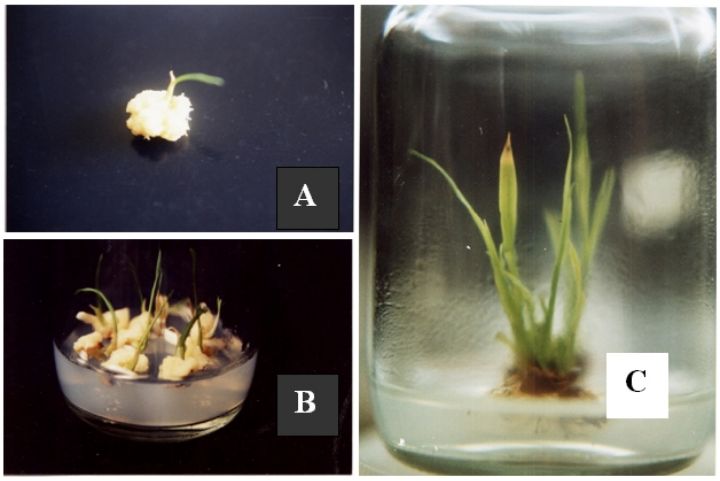


Figure 1. (A,B) Callus formation of mature embryos cultured on the N_6 medium containing 500 mg/l casein hydrolysate, 4.5 µM 2,4-D and 2.5 µM NAA under light condition (C) Shoot regeneration from dehydrated calli cultured on MS medium containing 300 mg/l casein hydrolysate, 9 µM BA and 1 µM NAA under light condition.

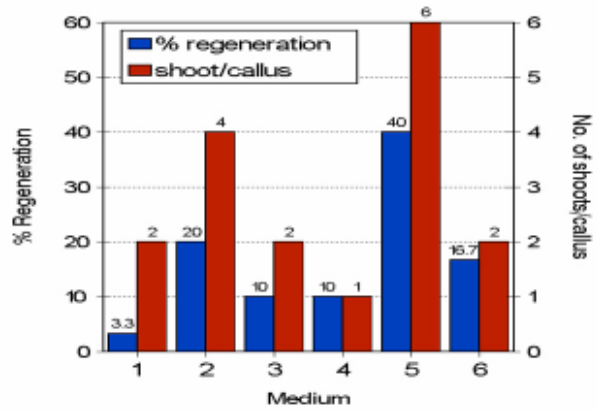
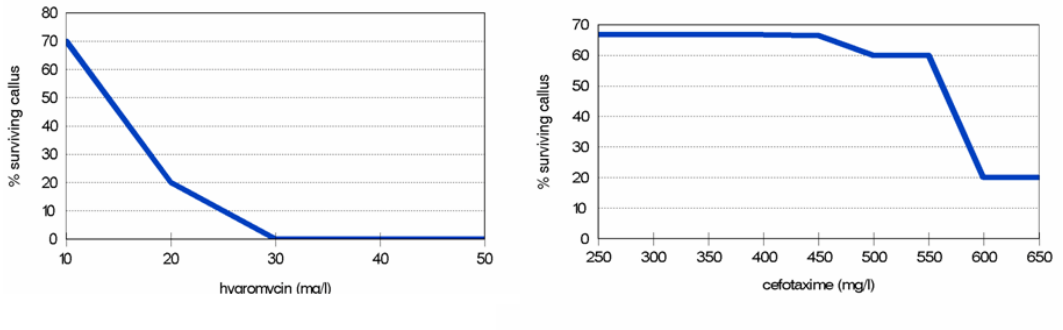


Figure 2. Plant regeneration from dehydrated calli of rice cv. Chainat 1 on MS medium supplemented with growth regulators: media 1 to 3 contained 15% coconut water and 4 to 6 did not contain coconut water.
There are combinations with;
1 and 4 : 10 µM kinetin and 1 µM NAA
2 and 5 : 9 µM BA and 1 µM NAA
3 and 6 : 9 µM BA and 5 µM NAA

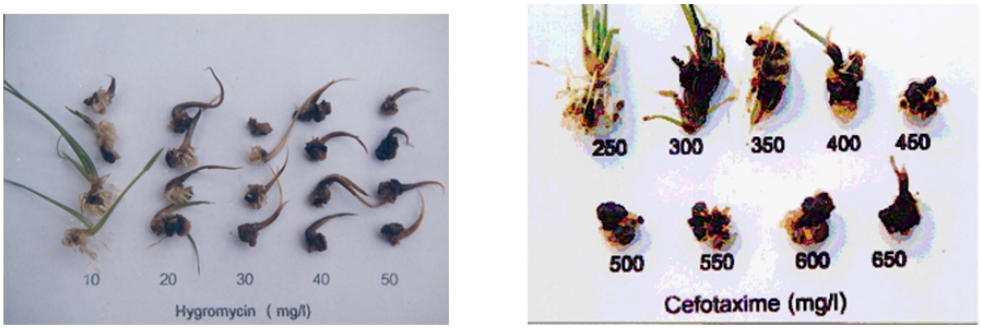
the transformants in distinguishing medium. Datta (1999) suggested that the antibiotic hygromycin is an excellent selectable agent for rice and needs to be optimized for each rice cultivar. We found that hygromycin at 20 mg/l was the effective concentration for selection of the transformants. This is in agreement with Pipatpanukul *et al*. (2004), who used hygromycin at 20 mg/l as a selective agent of rice cv. RD6.

In the transformation of plants by co-cultivation explants with an *Agrobacterium* culture, elimination of the *Agrobacterium* with antibiotics



A **B**

Figure 3. Effect of different concentrations of hygromycin (A) and cefotaxime (B) on callus induction in rice cv. Chainat 1.



A **B**

Figure 4. Calli of rice cv. Chainat 1 on the callus induction medium containing different concentrations of hygromycin (A) and cefotaxime (B)

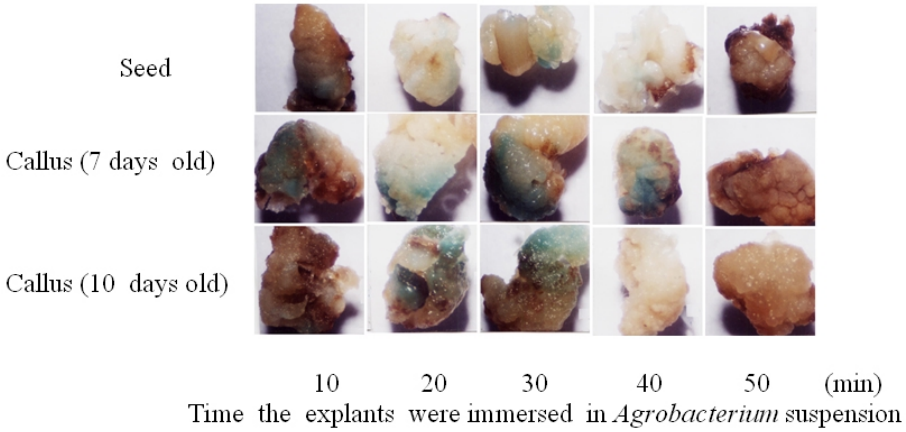


Figure 5. GUS activity of rice calli co-cultivated with *A. tumefaciens* LBA 4404 (pCAMBIA 1305.1 containing chitinase gene)

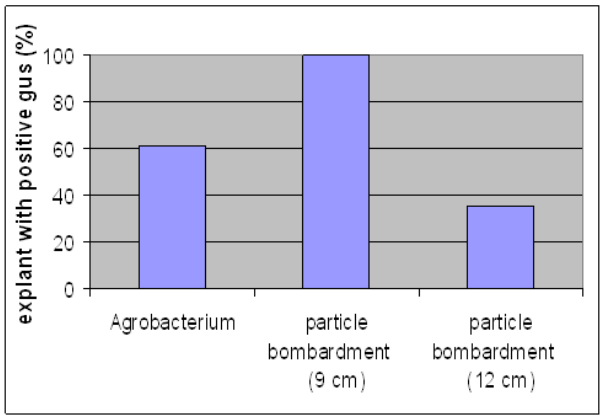


Figure 6. Effect of transformation systems on GUS expression in calli of rice.

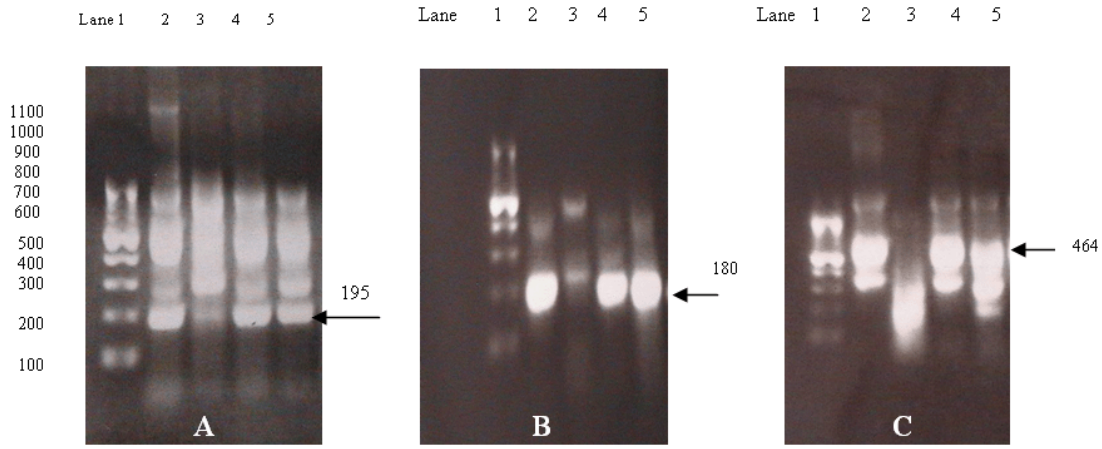


Figure 7. PCR analysis of transformed calli of rice cv. Chainat 1 using primers to detect the 35 S (A), NOS (B) and chitinase gene (C); lane 1 : 100 bp ladder, lane 2: pCAMBIA 1305.1 containing chitinase gene, lane 3 : nontransformed control calli, lanes 4 and 5 : hygromycin resistant calli. The sizes of the amplified fragments are 195 bp, 180 bp and 464 bp for the 35 S, NOS and chitinase genes, respectively.

is required. Several examples of the uses of carbenicillin at 200-500 mg/l (Orlikowska, 1995; Rachmawati *et al*., 2004; Toki, 1997) and more recently cefotaxime at 200-500 mg/l (Gama *et al*., 1996; Pipatpanukul *et al*., 2004) have been published. Barrett *et al.* (1997) reported that levels of antibiotics (carbenicillin, tricarbicillin and cefotaxime) higher than 500 mg/l would be necessary to eliminate *Agrobacterium*, but the problem of phytotoxicity arose. Therefore, they could not be used at higher concentrations. Gama *et al*. (1996) reported that the culture medium containing 500 mg/l cefotaxime decreased embryo production of sweet potato, while embryos cultured on basal medium with 250 mg/l of cefotaxime developed to morphological normal plants. However, Barrett *et al*. (1997) suggested that at least 4 times of the MIC (minimum inhibitory concentration) of the antibiotics should be incorporated to the tissue culture medium for successful bacterium elimination. This value would be much higher than the cefotaxime concentrations used in the study in which higher ones also yielded no surviving calli and may therefore explain the

failure to eliminate *Agrobacterium*. The possibility of using combinations of antibiotics may prove to be a better alternative.

Agrobacterium transformation used organized explants with high regenerative potential as targets for *Agrobacterium* infection. The co-cultivation time with *Agrobacterium* ranges from 1-2 minutes to 3 days depending on plant species (Mendel and Hansch, 1995). Rachmawati *et al*. (2004) transformed japonica rice by immersing rice callus in culture of *Agrobacterium* for 5 minutes followed by co-cultivation on C-modified medium. Pipatpanukul *et al*. (2004) soaked calli of indica rice cv. RD6 in *Agrobacterium* suspension for 30 minutes and then co-cultivated them on the callus induction medium for 3 days. This is similar to our results. The optimum co-cultivation for rice cv. Chainat 1 was soaking 7-day-old derived calli in *Agrobacterium* suspension for 30 min and then co-cultivating them on the callus induction medium for 3 days. These transformed calli gave high intensity of blue stain that indicated high GUS expression. Furthermore, some factors such as strain of *A. tumefaciens* and preincubation with acetosyringone affected transformation efficiency (Rachmawati *et al*., 2004).

In recent years, the successful transformation of rice by *Agrobacterium* and particle bombardment has been reported (Datta *et al*., 1999). *Agrobacterium*-mediated transformation is a simple, low-cost and high-efficient gene delivery method. Another approach of direct DNA delivery was introduced in 1987. DNA is transferred by high-velocity microprojectiles (particle bombardment). This method uses minute metal balls coated with DNA that are shot directly into plant cells (Sanford, 1993). Christou *et al*. (1988) demonstrated that this process could be used to deliver biologically active DNA into living cells and produced stable transformants. Datta *et al*. (1999) suggested that particle bombardment method can help reducing the time and labor used to maintain rice tissue culture. In addition, particle bombardment offers several advantages over *Agrobacterium*-mediated transformation such as simple plasmid construction and less demanding transformation protocols, since complex bacteria/plant interrelationships are eliminated (Gray and Finer, 1993). Our study was performed in an attempt to genetically transform rice using an *Agrobacterium*-based approach, in comparison with using particle bombardment. The results indicated that the best transformation system was particle bombardment at the distance of 9 cm. Regarding bombardment physical parameters, which might affect transient expression, the distance between stopping screen and plant tissue also influenced the number of blue spots on GUS expression. Nine cm microprojectile flying distance showed more spots, compared with the 12 cm, especially with 1,100 psi rupture disks. Filho *et al*. (2003) indicated that 6 cm microprojectile flying distance showed more spots, compared with 9 cm in citrus explants and reported that tungsten particles (M17 and M-25) resulted in more blue spots than gold particles (1.5-3.0 mm) that could have caused large physical trauma on bombarded explants.

The presence of transient gene in putatively transformed plants was confirmed by PCR method. Mayer *et al*. (2000) optimized the PCR method using three primer pairs in one reaction for detecting transformed tissue. By this multiplex PCR analysis, they were able to detect *hpt*-gene, a GUS gene fragment and a fragment derived from the single copy of gene *Gos*5. The latter was chosen as an internal control for the quality of the PCR analysis. In addition, they suggested simple and quick methods to optimize PCR and extract DNA for screening of large number of samples like calli or parts of rice plants.

In summary, our results were successful in the part of tissue culture. We have established an efficient transformation system for indica rice cv. Chainat 1. Therefore, the transformation either by *Agrobacterium* or by particle bombardment might be used to develop transgenic rice cv. Chainat 1 with chitinase gene for developing rice resistant to pathogens .

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References

Barrett, C., Cobb, E., McNicol, R. and Lyon, G. 1997. A risk assessment study of plant genetic transformation using *Agrobacterium* and implications for analysis of transgenic plants. Plant Cell Tissue and Organ Cult. 47: 135-144.

Broglie, K., Chet, I., Holliday, M., Cressman, R., Biddle, P., Knowlton, S., Mavvais, C.J. and Broglie, R. 1991. Transgenic plants with enhanced resistance to the fungal pathogen *Rhizoctonia solani*. Science. 254: 1194-1196.

Chen, W.P. and Punja, Z.K. 2002. *Agrobacterium*-mediated transformation of American ginseng with rice chitinase gene. Plant Cell Rep. 20: 1039-1045.

Christou, P., McCabe, D.E. and Swain, W.F. 1988. Stable transformation of soybean callus by DNA-coated gold particles. Plant Physiology 87: 871-874.

Datta, K., Velazhahan, R. and Oliva, N. 1999. Over-expression of the cloned rice thaumatin-like protein (PR-5) gene in transgenic rice plants enhanced environmental friendly resistance to *Rhizoctonia solani* causing sheath blight disease. Theor. Appl. Genet. 98: 1138-1145.

Datta, K., Koukolikova, N., Baisakh, N., Oliva, N. and Datta, S.K. 2000. *Agrobacterium*-mediated engineering for sheath bright resistance of indica rice cultivars from different ecosystems. Theor. Appl. Genet. 100: 832-839.

Filho, J.C.B., Kobayashi, A.K., Pereira, L.F.P., Galvao, R.M. and Vieira, L.G.E.2003. Transient gene expression of β-glucuronidase in citrus thin epicotyl transversal section using particle bombardment. Braz. Arch. Biol. Technol. 46(1): 1-9.

Gama, M.I.C.S., Leite Jr, R.P., Cordeiro, A.R. and Canttiffe, D.J.1996. Transgenic sweet potato Plants obtained by *Agrobacterium tumefaciens*-mediated transformation. Plant Cell Tissue and Organ Cult. 46: 237-244.

Gray, D.J. 1987. Quiescenec in monocotylenous dicotyledonous somatic embryos induced by dehydration. Hort Science. 22: 810-814.

Gray, D.J. and Finer, J.J. 1993. Development and operation of five particle guns for introduction of DNA into plant cells. Plant Cell Tissue and Organ Cult. 33: 219.

Jefferson, R.A. 1987. Assaying chimeric genes in plants: the GUS gene fusion system. Plant Mol Biol Rep. 5: 387-405.

Kishimoto, K., Nishizawa, Y., Tabei, Y., Hibi, T., Nakajima, M. and Akutsu, K. 2002. Detailed analysis of rice chitinase gene expression in transgenic cucumber plants showing different level disease resistance to gray mold (*Botrytis cinerea*). Plant Science 162: 655-662.

Lin, W., Anuratha, C.S., Datta, K., Potrykus, I., Muthukrishnan, S. and Datta, S.K. 1995. Genetic engineering of rice for resistance to sheath blight. Bio/Technology 13: 686-691.

Mayer, A., Zondag, G.B. and Hensgens, L.A.M. 2000. A simple screening method for transgenic rice tissue base on PCR [Online]. Available from: http://genome.cornell.edu/rice/documents/newsletters/rgn8/v8p161.html.

Mendel, R.R. and Hansch, R.M. 1995. Gene transfer to higher plants. **In**: Molecular Methods in Plant Pathology. Singh, R.P. and Singh, U.S. (Eds.). pp.189-200. CRC Press, Florida.

Nishizawa, Y., Nishio, Z., Nakazono, K., Zoma, M., Nakajima, E., Ugaki, M. and Hibi, T. 1999. Enhanced resistance to blast (*Magnaporthe grisea*) in transgenic japonica rice by constitutive expression of rice chitinase. Theor. Appl. Genet. 99: 383-390.

Nitsch, J.P. and Nitsch, C. 1969. Haploid plant from pollen grain. Science 163: 85-87.

Orlikowska, T.K., Cranston, H.J. and Dyer, W.E. 1995. Factors influencing *Agrobacterium tumefaciens*-mediated transformation and regeneration of the safflower cultivars 'centennial'. Plant Cell Tissue and Organ Cult. 40: 85-91.

Rice Research Institute. 2005. DOA Plant Knowledge Base [Online]. Available from: http:// www.doa.

go.th/data-agri/RICE/3 var/var 01.html.

Pipatpanukul, T., Bunnag, S., Theerakulpisut, P. and Kosittrakul, M. 2004.Transformation of indica rice (*Oryza sativa* L.) cv. RD6 mediated by *Agrobacterium tumefaciens*. Songklanakarin J. Sci. Technol. 26(1): 1-13.

Rachmawati, D., Hosaka, T., Inoue, E. and Anzai, H. 2004. *Agrobacterium tumefaciens*-mediated transformation of japonica rice cv. Rojolele. Biosci. Biotechno. Biochem. 68(6):1193-1200.

Raina, S.K. 1989. Tissue culture in rice improvement: status and potential. Adv. Agron. 42: 339-398.

Raina, S.K., Sathish, P. and Sarma, K.S. 1987. Plant regeneration form *in vitro* culture of anthers and mature seeds of rice (*Oryza zativa* L.) cv. Basmati-370. Plant Cell Rep. 6: 43-45.

Raineri, D.M., Bottino, P., Gordon, M.P. and Nester, E.W. 1990. *Agrobacterium tumefaciens*-mediated transformation of rice (*Oryza sativa* L.). Biotech. 8: 33-38.

Sanford, J.C., Smith, F.D. and Russel, J.A. 1993. Optimizing the biolistic process for different biological applications. Meth. Enymol. 217: 483-504.

Sripichitt, P. and Cheewasestathum, P. 1994. Plant regeneration from embryo derived callus of aromatic rice (*Oryza sativa* L.) variety Khao Dawk Mali 105. Kasetsart J. (Nat. Sci.) 28: 27-37.

Thadavong, S., Sripichitt, P., Wongyai, W. and Jompuk, P. 2002. Callus induction and plant regeneration from mature embryos of glutinous rice (*Oryza sativa* L.) cultivar TDK1. Kasetsart J. (Nat. Sci.) 36: 334-344.

Toki, S. 1997. Rapid and efficient *Agrobacterium*-mediated transformation of rice. Plant Molecular Biology Reporter 15(1): 16-21.

Tsukahara, M. and Hirosawa, T. 1992. Simple dehydration treatment promote plantlet regeneration of rice (*Oryza sativa* L.) callus. Plant Cell Rep. 11: 550-553.

Vajrabhaya, M., Tunvachkul, O. and Vajrabhaya, T. 1986. Effect of auxin and cytokinin on plant regeneration from rice callus. Sci. Res. Chula. Univ. 11: 113-115.