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STUDY ON THE RESPONSE OF VIETNAMESE INDICA RICE VARIETIES TO *Agrobacterium*-MEDIATED TRANSFORMATION BY ASSAY OF TRANSIENT GENE EXPRESSION AND ON THE REGENERATION EFFICIENCY OF EMBRYOGENIC CALLI

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ABSTRACT

The response of rice to Agrobacterium-mediated transformation is variety dependent; therefore it is needed to identify the potential varieties to be used for a successful transformation using Agrobacterium method. In this study, we evaluated the response of 51 Vietnamese indica rice varieties to Agrobacterium-mediated transformation by assay of the transient expression of the gusA gene. The gene expression was detected with the blue spots to emerge on the callus transformed with pTOK233 carrying gusA gene by Agrobacterium tumefaciens. There was a variation on the gene expression among the tested varieties. Based on the level of expression quantified by the percentage of calli showing GUS^+ (blue spots), the tested varieties could be classified in five groups: very high, high, average, low and none expression. The potential varieties for transformation were identified and recommended for use in gene transfer by Agrobacterium. It was also noted that up to 28.07% of the tested varieties showed none expression. In this study, we also evaluated the regeneration ability of the embryogenic calli of seven indica varieties cultured onto four different media; each medium differed from the other in terms of plant hormone combination and a few other components like sugar. The results showed that the medium MSReg3 appeared to have the highest regeneration efficiency across the varieties. This medium contained basic MS medium supplemented with 20 gl^{-1} sucrose, 30 gl^{-1} sorbitol, 0.5 mgl^{-1} NAA and 2 mgl^{-1} BAP. The addition of sorbitol seemed to enhance the regeneration efficiency of indica rice.

Keywords: *Agrobacterium tumefaciens*, plant regeneration, rice, transformation

INTRODUCTION

Rice is the staple food for more than three billion people or over half the world's population (FAO 2004). It is grown in more than one hundred countries worldwide and is the dominant crop in Asia where it covers half of the arable land used for agriculture in many countries (Cantrell and Hettel 2004). The increase of rice production is being a goal both nationally and globally because otherwise the world will face a shortage of food in future due the expansion of population coupled with the shrinkage of natural resources, particularly land and water.

The advent of modern biotechnology advances has offered opportunity to raise further rice production in term of both quantity and quality. It is envisaged that the development and application of transgenic rice will be the core of modern biotechnology contributing to the achievement of the mentioned goal. In 2005, for the first time, transgenic rice has been commercialized in Iran on an area of 4,000 ha (ISAAA 2005). Transgenic rice varieties are also on the threshold of commercial release in some other countries (AgBios 2004, Jia *et al*. 2004). In the same trend, efforts are being made in many parts of the world to heighten the research of developing transgenic rice varieties, particularly transgenic indica rice suitable to the local ecosystems and cultural practices. In Vietnam, transgenic rice lines developed from popular local varieties expressing vitamin A (golden rice) are in the pipeline of selection (Hoa et al. 2003)

Agrobacteium-mediated transformation has been identified as an efficient method of gene transfer to plant. This transformation method

was recently successfully applied to both japonica and indica rice (Ye et al. 2000, Hoa et al. 2003, Bong et al. 2003). Although *Agrobacteium*-mediated transformation applied to japonica varieties among them Taipei was a model variety was well established, the use of this transformation method applied to indica rice needs further refinement because the success in transformation is variety (genotype) dependent and it is difficult to regenerate plants from the transformed indica cells as compared to the transformed japonica ones. For these reasons, in the present study we evaluated the transformation ability of a large number of Vietnamese indica rice varieties by assay of transient expression of the *gusA* gene transferred to these varieties by using *Agrobacterium tumefaciens* and further we evaluated the regeneration efficiency of the embryogenic cells of some indica varieties which showed GUS^+ expression using different regeneration media.

MATERIALS AND METHODS

Bacterial strain and plasmid

Agrobacterium tumefaciens LBA 4404 harboring the "superbinary" plasmid pTOK233

(Hiei et al., 1994) was used for all the experiments. The vector had a *gusA* gene. A single colony of *Agrobacterium* was grown in 3 ml liquid YEB medium containing 50 mgl^{-1} Hygromycine B and 100 µM acetosyringone under continuous shaking at 28^0C. The transformation protocol described by Hoa et al. (1999, 2002) was followed.

Agrobacterium-mediated transformation procedures

Fifty two rice varieties (Table 2) were used for the initiation of calli derived from mature embryos following the procedures of Hoa et al. (1999, 2002). Briefly, 150 mature seeds of each variety were cultured on MSCI medium (Table 1). After 3 weeks, the calli were selected and transferred onto petri dish (Φ90) containing a fresh MSCI medium (Murashige and Skoog, 1962) and cultured in the dark at 29^0C. Observations were taken at weekly intervals for 3-4 weeks. The embryogenic calli from the plates were selected for further study.

To study the transformation ability of the tested rice varieties, 50 embryogenic calli of each variety were inoculated with *Agrobacterium tumefaciens* LBA 4404 described briefly as follows. The embryogenic calli were cut into small pieces having a size of about 5 mm and cultured on a fresh MSCI medium containing 2.0 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), 30.0 g l^{-1} sucrose, 6.0 g l^{-1} agarose (Sigma), 1.0 g l^{-1} casein hydrolysate at pH 5.8) at 29^0C in dark condition for 3-4 days before used for transformation. Five selected calli were transferred into each petri dish (Φ60) contained co-cultivation medium and 10 µl of bacterial suspension were dropped over one callus.

Histochemical detection of gus A expression

The expression of *gusA* was visualized by transferring the tested calli to a sterilized GUS substrate mixture following the procedure of Jefferson et al. (1987).

Plant regeneration

To study the efficiency of plant regeneration, the embryogenic calli derived from 7 varieties were cultured on 4 different regeneration media (Table 1). Ten selected calli were placed on each petri dish (Φ90) containing the regeneration medium. Each variety was repeated with 3 experiments and each experiment was performed with 100 calli. These cultures were kept at 29^0C in growth chamber with 16h dark and 8h light, and a humidity of 50%.

RESULTS AND DISCUSSION

Rice is the most important food crop in the world. The improvement of rice to be brought about by genetic engineering would certainly help increase rice production in the near feature. Techniques of rice transformation although are well established for japonica rice, they still need further refinement for indica rice, particularly in the case of using *Agrobacterium*-mediated transformation methods. In other words, it is important to study carefully the factors that could enhance the efficiency of *Agrobacterium*-mediated

transformation for indica rice. In this study, therefore, we investigated the response of different Vietnamese indica varieties to *Agrobacterium*-mediated transformation and the effect of different media on plant regeneration. The knowledge on these factors would help improve the effectiveness in research and development of Vietnamese transgenic rice lines.

To identify the response ability of rice varieties to *Agrobacterium*-mediated transformation, we employed the transient gene expression assay, of which the inoculated and uninoculated calli and were stained for GUS activity by placing in GUS histochemical stain (Jefferson et al. 1987). The GUS assay was carried out with all tissues after 3 days of co-cultivation.

Transient gene expression was detectable as early as 2 h after incubation with staining solution and reaches a peak of more than 30 spots per tissue clump after overnight. In our study, we found that the transient gene expression was visible with blue spots emerged on the callus and there was a positive correlation between number of spots per callus with number of calli showed GUS positive for each variety (Fig. 3). No GUS expression in the non-transformed calli (control) during 48 h of incubation (Fig. 3).

It was obvious that the level of gene expression varied widely among 51 varieties tested. Based on the percentage of the calli showing blue spots (GUS^+) per the total inoculated calli, the tested varieties could be classified into five groups as given in Table 2 (a) 1 variety showed very high expression with more than 30% GUS^+ calli- the same level as the check japonica variety Taipei 309, (b) 5 varieties showed high expression with 21-30% GUS^+ calli, (c) 18 varieties showed average expression with 11-20% GUS^+ calli, (d) 11 varieties showed low expression with 1-10% GUS^+ calli and (e) 16 varieties showed none expression with 0% GUS^+ calli. These data revealed that a large number of Vietnamese rice varieties did not respond well to *Agrobacterium*-mediated transformation (21.57% of the tested varieties showing low expression, and 28.07% showing none expression).

As given in Table 2, the variety showing very high transient gene expression was OM2514, and the varieties showed high expression included OM2490, OM4875, OM1643, Huong thom 1 and OM2822. These varieties should be of choice for genetic transformation using *A. tumefaciens* method. On the other hand, the varieties showing none transient gene expression (Table 2) should not be used for transformation.

Among 51 tested varieties, 31 were high yielding varieties and 20 were aromatic or traditional varieties. As given in Table 3, the percentage of the varieties showing GUS^+ were 75.0% (15/20) in the aromatic/ traditional group and 64.5% (20/31) in the high yielding group. It may indicate that aromatic/traditional varieties were more responsive to *Agrobacterium*-mediated transformation than the high-yielding varieties.

Plant regeneration is an important step in the transformation procedures. For indica rice, the response of the transformed cell to regenerate plants is generally low. Therefore, the optimization of conditions for an efficient regeneration of indica rice cell/tissue is essentially needed. In this study, we focused on comparing the effect of four regeneration media on plant regeneration of embryogenic cells derived from seven varieties. Four media were used for study (Table 1), each medium differed from the other in terms of plant hormone combination and a few other components.

The regeneration ability expressed in term of the percentage of calli producing green plants to the total calli cultured onto the regeneration medium of each variety under four media was given in Table 4. Among the tested varieties, Mot bui do had highest regeneration efficiency (74.3%) across the media, while OM3556 had the poorest regeneration ability.

The medium MSReg3 appeared to be the most efficient regeneration efficiency across the varieties varying from 44.0 to 83.0%, its efficiency was significantly higher than the other three media (Table 4). The MSReg3 medium contained 20 gl^{-1} sucrose, 30 gl^{-1} sorbitol, 0.5 mgl^{-1} NAA and 2 mgl^{-1} BAP. The MSReg2 had a similar composition as the

MSReg3 except it did not contain sorbitol. The MSReg2 had lower regeneration efficiency that the MSReg3, so perhaps sorbitol had a role to enhance the regeneration efficiency in indica rice. The MSReg4 which had a hormone combination of 1 mgl^{-1} Zeatin and 0.5 mgl^{-1} IAA did give high regeneration efficiency in indica rice, but it gave a good response to the regeneration of Taipei 309- a japonica variety (data not shown). The MSReg1 has high concentration of kinetin (2.5 mgl^{-1}) was a good medium for Taipei 309 but not for indica rice (data not shown).

The present investigation showed that the regeneration media MSReg 3 was suitable medium for regeneration. The number of shoots and plantlets produced in MSReg 3 was significantly higher than that of the other 3 media in 7 tested varieties (Table.4).

In conclusion, from the present study we have grouped a large number of the Vietnamese indica varieties based on their ability to respond to *Agrobacterium*-mediated transformation. The varieties which showed high transient gene expression could be employed for gene transfer using *Agrobacterium* transformation method. In addition, we identified a suitable regeneration medium for indica rice. These findings would be useful towards the development of Vietnamese transgenic rice varieties using *Agrobacterium* transformation method.

Table 1. Media used in *Agrobacterium*-mediated transformation and regeneration of indica rice varieties.

Medium	ABG	PIM2	MSCI	MSCo	MSReg1	MSReg2	MSReg3	MSReg4
AB salts (Chilton et al. 1974)	1x	1x	-	-	-	-	-	-
AB (KHPO4) buffer (Chilton et al. 1974)	1x	-	-	-	-	-	-	-
$NaPO_4$ buffer *	-	1x	-	-	-	-	-	-
MES	-	75 mM	-	-	-	-	-	
R2 salts (Ohira et al. 1973)								1x
MS salts (Murashige & Skoog 1962)	-	-	1x	1x	1x	1x	1x	-
MS vitamin (Murashige & Skoog 1962)	-	-	1x	1x	1x	1x	1x	1x
Glucose	5 g/l	10 g/l	-	10 g/l	-	-	-	-
Sucrose	-		30 g/l	30 g/l	30 g/l	30g/l	20 g/l	20 g/l
Sorbitol	-	-	-	-	-	-	30 g/l	30 g/l
Casein hydrolysate	-	-	1 g/l	-	1 g/l	1 g/l	1 g/l	-
Casamino acid	-	-	-	1 g/l	-	-	-	-
Tryptophane	-	-	-	-	50 mg/l	50 mg/l	50 mg/l	-
Agarose	-	-	6 g/l	6g/l	6 g/l	6 g/l	6 g/l	6 g/l
Granular agar	18 g/l	-	-	-	-	-	-	
2,4-dichlorophenoxy acetic acid (2,4-D)	-	-	2 mg/l	2 mg/l	-	-	-	-
Naphthalene acetic acid (NAA)	-	-	-	-	-	0.5 mg/l	0.5 mg/l	-
Benzylamino purine (BAP)	-	-	-	-	-	2.0 mg/l	2.0 mg/l	-
Kinetin	-	-	-	-	2.5 mg/l	-	-	-

Zeatin	-	-	-	-	-	-	-	1 mg/l
Indol acetic acid (IAA)	-	-	-	-	-	-	-	0.5 mg/l
pH	7.0	5.6	5.8	5.2	5.8	5.8	5.8	5.8
Acetosyringone (AS)**	-	0.2 mM	-	0.3 mM	-	-	-	

*: $NaPO_4$ buffer: 0.28 g/l Na_2HPO_4, 0.27 g/l $NaH_2PO_4.H_2O$; **: AS was added after autoclaving when medium cooled down 45-50^oC

Table 2. Transient expression of the GUS reporter gene in rice embryogenic calli transformed with pTOK233 using *Agrobacterium tumefaciens.*

No.	Code	Designation	Variety group	Level of GUS expression*
0	V0	Taipei 309	Model check	++++
1	V20	OM2514	High yielding	++++
2	V25	OM2490	High yielding	+++
3	V39	OM4875	High yielding	+++
4	V40	OM1643	High yielding	+++
5	V36	Huong thom 1	Traditional	+++
6	V15	OM2822	Aroma	+++
7	V48	Khao 39	Aroma	++
8	V1	DS20	Aroma	++
9	V22	Nam thom	Aroma	++
10	V12	OM2517	High yielding	++
11	V13	OM567	High yielding	++
12	V35	M12	Aroma	++
13	V14	OM3923	High yielding	++
14	V44	MTL250	High yielding	++
15	V45	OM2031	High yielding	++
16	V47	IR64	High yielding	++
17	V50	OM4410	High yielding	++
18	V51	CM16-27	High yielding	++
19	V23	Mot Bui Do	Traditional	++
20	V26	Te Tep	Traditional	++
21	V34	Tam xoan Hai hau	Traditional	++
22	V46	Tai nguyen	Traditional	++
23	V37	OM3797	High yielding	++
24	V29	Khong luang	Traditional	++
25	V30	Moc Tuyen	Traditional	+
26	V2	OMCS2000	High yielding	+
27	V3	Khang Dan	High yielding	+
28	V21	Jasmine 85	Aroma	+
29	V10	OM3536	Aroma	+
30	V5	CH-2	High yielding	+
31	V7	OM2718	High yielding	+
32	V19	IR68144	High yielding	+
33	V24	Nang thom Cho Dao	Traditional	+
34	V9	OM1490	High yielding	+
35	V11	CH1-2	High yielding	+
36	V18	OM2008	Glutinous	-

37	V8	AS996	High yielding	-
38	V4	OM4926	High yielding	-
39	V6	OM2717	High yielding	-
40	V16	OM3556	High yielding	-
41	V17	OM3566-34	High yielding	-
42	V31	Kham duc	High yielding	-
43	V41	OM2516	High yielding	-
44	V43	IR68	High yielding	-
45	V49	OM4414	High yielding	-
46	V32	C70	High yielding	-
47	V38	OM3566-71	High yielding	-
48	V27	Khau lech	Traditional	-
49	V28	Huong thom so 7	Traditional	-
50	V42	Doc Phung	Traditional	-
51	V33	Ptb33	Traditional	-

**gusA* expression:
+ + + +: more than 30% inoculated calli had GUS^+
+ + +: 21-30 % GUS^+
+ +: 11-20 % GUS^+
+: 1-10 % GUS^+
-: 0% GUS^+

Table 3. Transformation response of rice varieties divided by group (aromatic/traditional vs. high yielding)

Rice group	No. of tested varieties	No. of GUS^+ varieties	GUS^+(%)
Aroma/Traditional	20	15	75.0
High yielding	31	20	64.5
Total	51	35	68.6

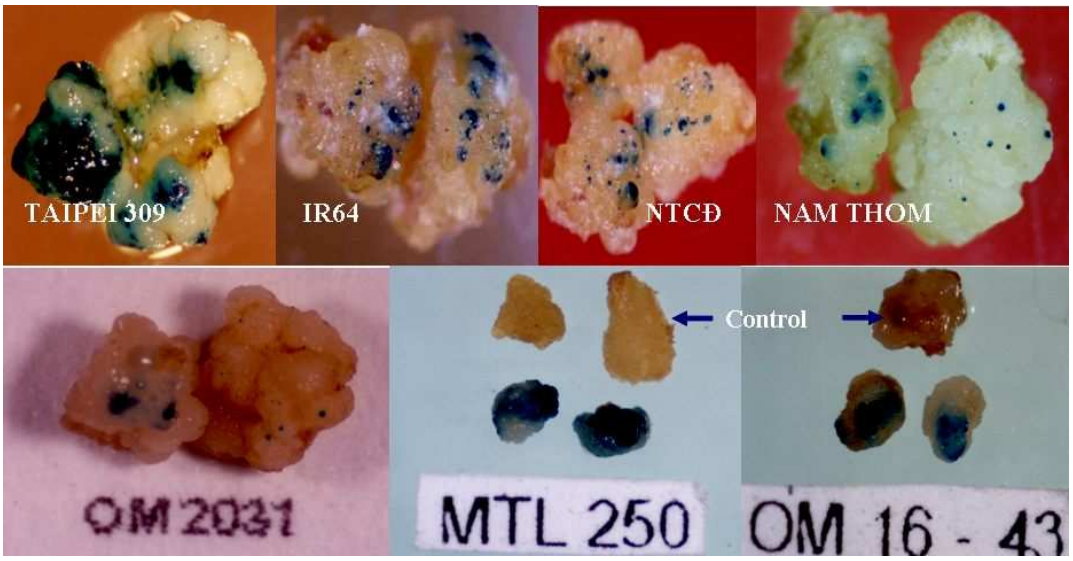
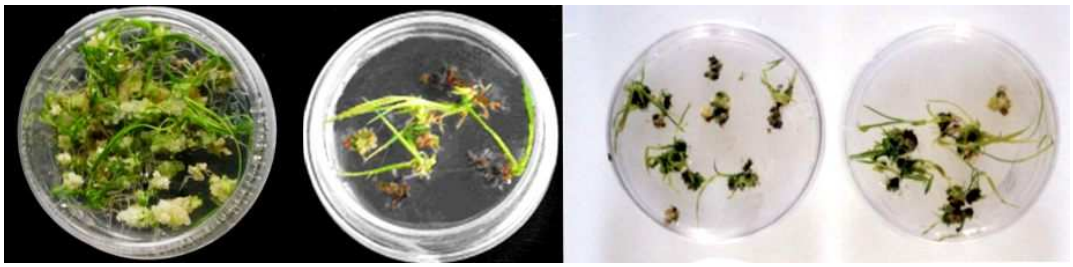


Fig. 3. GUS staining of tested rice varieties showing various levels of transient gene expression



Mot bui do/MSReg3 IR64/MSReg3 Nang Thom Cho Dao/MSReg3

Fig. 4. Regeneration of tested rice varieties on MSReg3 medium

Table 4. Regeneration capability of calli (% of calli producing green plants) induced from mature emryos of different rice varieties under four media.

No.	Designation	Regeneration media				Variety mean*
		MSReg1	MSReg2	MSReg3	MSReg4	
1	Mot bui do	63.3	79.3	87.3	67.3	74.3 a
2	Nang thom Cho Dao	56.3	72.0	81.0	67.3	69.2 b
3	IR64	61.3	61.7	78.7	63.3	66.3 b
4	Nam Thom	55.3	58.3	83.0	58.0	63.7 b
5	MTL250	53.7	53.7	70.7	58.3	59.1 b
6	IR68144	54.7	55.3	60.7	53.3	56.0 b
7	OM3536	18.0	23.7	44.0	28.0	28.4 c
	Medium mean*	51.8 b	57.7 b	72.2 a	56.5 b	

*: The means followed by the same letter were not significantly different in Duncan's Multi-Range Test (α=0.05)

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Nghiên cứu khả năng chuyển nạp gen bằng *Agrobacterium* của các giống lúa indica Việt Nam bằng thử nghiệm sự biểu hiện tạm thời của gen và hiệu quả tái sinh cây của từ mô sẹo sinh phôi của các giống lúa

Sự đáp ứng của lúa đối với chuyển nạp gen bằng vi khuẩn *Agrobacterium tumefaciens* tùy thuộc vào giống lúa, vì vậy rất cần thiết để xác định các giống có tiềm năng để sử dụng chuyển nạp gen bằng *A. tumefaciens* . Trong nghiên cứu này, chúng tôi đánh giá khả năng đáp ứng của 51 giống lúa indica Việt Nam đối với chuyển nạp gen bằng *A. tumefaciens* qua thử nghiệm sự biểu hiện tạm thời của gen *gusA* được chuyển nạp vào các giống lúa này. Sự biểu hiện gen được nhận biết với sự xuất hiện của các đốm màu xanh trên mô sẹo được chuyển nạp với plasmid pTOK233 bằng *A. tumefaciens.* Kết quả ghi nhận mức độ biểu hiện của gen khác nhau giữa các giống lúa. Dựa vào mức độ biểu hiện định lượng bằng tỷ lệ số mô sẹo có biểu hiện GUS (đốm màu xanh), có thể chia các giống lúa được nghiên cứu vào năm nhóm phản ứng đối với chuyển nạp gen bằng *A tumefaciens*: rất cao, cao, trung bình, kém và không phản ứng. Các giống lúa có khả năng chuyển nạp gen cao đã được xác định và khuyến cáo sử dụng để chuyển nạp gen bằng *Agrobacteium.* Kết quả ghi nhận có đến 28.07% số giống lúa không biểu hiện gen khi chuyển nạp. Trong nghiên cứu này chúng tôi cũng đã tiến hành đánh giá hiệu quả tái sinh cây của các mô sẹo từ 7 giống lúa india đã biểu hiện GUS^+, sử dụng 4 môi trường nuôi cấy khác nhau, các môi trường khác ở thành phần chất điều hoà sinh trưởng và một vài thành phần khác như đường. Kết quả cho thấy môi trường MSReg3 với thành phần MS cơ bản cho thêm 20 gl^{-1} sucrose, 30 gl^{-1} sorbitol, 0.5 mgl^{-1} NAA and 2 mgl^{-1} BAP cho hiệu quả tái sinh cây cao nhất qua tất cả các giống. Việc thêm đường sorbitol có thể làm tăng hiệu quả tái sinh cây ở lúa indica.