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Regeneration and *Agrobacterium*-Mediated Transformation of *Japonica* Rice Varieties Developed for a Cold Region

Mingfang FENG[1], Jing CANG[1], Junhong WANG[1], Jian SUN[2], Jing YU[1], Qinghua XU[1], Da ZHANG[1], Ning YANG[1], Qiuwei LU[1] and Yan LV[1]*

[1]College of Life Science and [2]College of Agriculture,
Northeast Agricultural University, Harbin, P.R. China

**Corresponding author: cangjing2003@163.com*

Abstract

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So far, a large number of transformation systems have been established for *japonica* rice, but only a few have been reported for cold-region varieties. In our study, we established highly efficient tissue culture systems for two cold-region rice cultivars, Dongnong 427 and Longdao 14. Plant growth regulator (PGR) levels were optimized by an orthogonal experimental design. The culture ability, constituted by induction and differentiation rate, served as the detection index of orthogonal experiments. The optimal combinations of PGRs for callus induction and regeneration of Dongnong 427 and Longdao 14 were 1 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) + 2 mg/l 6-benzyladenine (BA) + 4 mg/l kinetin (KIN) + 0.2 mg/l α-naphthaleneacetic acid (NAA) and 1 mg/l 2,4-D + 4 mg/l 6-BA + 4 mg/l KIN + 0.5 mg/l NAA, respectively. *Agrobacterium* strain EHA 105 containing the plasmid pCAMBIA1301 was used for transformation. The frequency of transient transformation was expressed as the ratio between the number of calli showing GUS expression and the total number of calli kept for staining. The highest transformation efficiency in Dongnong 427 was obtained when calli were immersed in 0.272 OD_{600} (optical density determined at 600 nm) for 10 min. While it was best for Longdao 14 calli to be infected with 0.592 OD_{600} for 20 min. Infected calli of the two varieties were co-cultivated on two pieces of sterile filter paper moistened with 1 ml liquid co-cultivation medium for three days. The expression of the *GUS* gene was confirmed by PCR analysis of plants of both varieties.

Keywords: *Agrobacterium*; *GUS* expression; round-grained non-glutinous rice; tissue culture; transformation efficiency

Rice (*Oryza sativa* L.) is one of the most important foods and is a staple food of over half the world's population. Rice consumers are increasing at the rate of 1.8% every year but the rate of growth in rice production has slowed down (Khush & Virk 2000). The major aim of rice breeding program in future will be to obtain new high-yield varieties with superior production quality and resistance against biotic and abiotic stresses by modern genetic engineering.

Rice is also a model monocot plant for genetic modification. An efficient plant regeneration system is a prerequisite for genetic transformation of plants and crop improvement. There are variations in tissue culture responses even within specific subspecies. Therefore, research on rice regeneration technology independent of the genotype might be a key aspect for the improvement of breeding efficiency in rice biotechnology.

Many reports have demonstrated efficient protocols for genetic transformation of rice by *Agrobacterium*-mediated methods (Hiei *et al.* 1994; Fujiwara *et al.* 2016). There are a great number of factors related to *Agrobacterium*-mediated rice transformation, such as explants used, infiltration time, bacterial density, co-cultivation period, and the concentration of acetosyringone.

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Although there is some evidence of successful rice transformation (Hiei *et al.* 2014), the studies on *japonica* (*O. sativa* ssp. *japonica*) varieties, especially those developed in cold regions, are scarce. In this study, we established efficient regeneration systems of Dongnong 427 and Longdao 14, which are typical *japonica* rice varieties grown in the northeast of China (the coldest zone) by an orthogonal experimental design. In addition, the optimal conditions for *Agrobacterium*-mediated transformation using callus derived from mature seeds of the two *japonica* rice varieties were obtained through determination of transient expression of GUS. Finally, transgenic positive plants of the two varieties were obtained. Our results could be used for the development of near-isogenic lines and provide valuable reference of significance for other cold-region rice cultivars.

MATERIAL AND METHODS

Explant preparation. Seeds of *japonica* rice, Dongnong 427 and Longdao 14 varieties, provided by the College of Agriculture, Northeast Agricultural University, Harbin, Heilongjiang, China, were used in this study. Mature seeds of two cultivars were dehusked and immersed in 75% ethanol for 1 min, followed by immersion in 10% sodium hypochlorite (*v*/*v*) supplemented with 2–3 drops of Tween-20 for 30 min (Tianjin Damao Chemical Reagent Factory, P.R. China). The treated seeds were washed with sterile distilled water 3–5 times and were placed on different callus induction media.

Callus induction and regeneration. Different concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) (1, 2, and 3 mg/l) were added into NB medium [containing N6 macronutrient components (Table S1 in Electronic Supplementary Material (ESM), B5 micronutrient and organic components, Tianjin Damao Chemical Reagent Factory, P.R. China] for callus induction. The NB medium was supplemented with 2.87 g/l proline, 0.3 g/l casein hydrolysate, 30 g/l sucrose, and 8 g/l agar, pH 5.8. The sterilized explants were inoculated in separate Petri dishes containing callus induction media with different concentrations of plant growth regulators (PGRs) and incubated for two to three weeks at 26 ± 2°C. Embryogenic calli produced on NB medium were transferred to MS (Murashige & Skoog 1962) basal medium for plantlet formation. Different concentrations of 6-benzylaminopurine (6-BA) (2, 4, and 6 mg/l), kinetin (KIN) (0, 2, and 4 mg/l), and α-naphthaleneacetic acid (NAA) (0.02, 0.2, and 0.5 mg/l) were added to MS medium to induce plant regeneration. The MS medium was also supplemented with 2 g/l casein hydrolysate, 30 g/l sorbitol, 30 g/l sucrose, and 8 g/l agar, pH 5.8. The calli were kept under 16/8 h light/dark cycle (RH 70–80%, 3000 lx).

***Agrobacterium* strain and plasmid vector**. The *Agrobacterium tumefaciens* strain EHA 105 harbouring the binary plasmid pCAMBIA 1301 (Figure S1 in ESM) was used.

***Agrobacterium*-mediated transformation**. *Agrobacterium*-mediated transformation was performed following the protocol described by Hiei *et al.* (1994) with a few modifications. The calli were infected in AB medium [a medium combining AAM macronutrient components (Table S2 in ESM) and B5 micronutrient and organic components) (Tianjin Damao Chemical Reagent Factory, P.R. China)] supplemented with 0.5 g/l proline, 0.5 g/l casein hydrolysate, 30 g/l sucrose, and 100 μM acetosyringone (pH 5.2) with various OD_{600} (0.2–2.0) and co-cultivated for different time periods (10–30 min). Then the calli were transferred to a solid co-cultivation medium (induction medium plus 100 μM acetosyringone) with or without sterile filter paper on the surface or to two pieces of sterile filter paper moistened with 1 ml liquid co-cultivation medium only. The infected calli were incubated at 24 ± 2°C for 3–5 days in the dark. After that, the calli were treated with carbenicillin and sterile water, and transferred to a recovery medium (induction medium supplemented with 250 mg/l carbenicillin and 250 mg/l cefotaxime) for one week. And then the calli were cultured on a selection medium (regeneration medium supplemented with 50 mg/l hygromycin, 250 mg/l carbenicillin and 250 mg/l cefotaxime). The calli were passed through two selection cycles of 15 days each. After two rounds of selection, the regeneration shoots were transferred to half-strength MS for rooting. The rooted plantlets were acclimatized and they were actively growing in greenhouse conditions and watered daily.

Histochemical assay of GUS activity. After these 3–5 days of co-cultivation with *Agrobacterium*, the infected calli were subjected to a GUS assay. A GUS assay buffer containing 50 mM phosphate buffer, TritonX-100, 20% methanol (Tianjin Damao Chemical Reagent Factory, P.R. China), and 1 mM substrate 5-bromo, 4-chloro, 3-indolyl β-D-glucuronide (Beijing Coolaber Technology Co., Ltd., P.R. China) was used for histochemical detection. The samples were then incubated at 37°C for 24–48 h in the dark. The

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frequency of transient transformation was expressed as the ratio between the number of calli showing GUS expression and the total number of calli kept for staining (more than 20 explants per experiment, in triplicates).

Genomic DNA extraction and PCR analysis. Genomic DNA was extracted from the transformed and non-transformed rice leaf blades by the CTAB method (Murray & Thompson 1980). The GUS expression of the regenerated plant level was confirmed by PCR analysis. The primer sequences were: GUS-F-5'-CTGTGGGCATTCAGTCTGGA-3' and GUS-R-5'-ATTGTTTGCCTCCCTGCTGC-3'. PCR was performed for GUS in a 20-µl reaction volume containing 10 µl 2× Taq MasterMix, 0.8 µl of each primer, 0.5 µl of template DNA, and 7.9 µl RNase-free water (Beijing Kangweishiji Biotechnology Co. Ltd., P.R. China). Amplification was performed in a programmable thermal cycler (MYCYCLER, Bio-Rad, USA) under following conditions: 1× (94°C for 1 min), 30× (94°C for 30 s, 55°C for 30 s, and 72°C for 1 min), 1× (72°C for 10 min).

Data recorded. The rate of callus induction, plant regeneration, and culture ability were calculated using the following formulas:

$$\text{Induction rate} = \frac{\text{number of calli}}{\text{number of incubated seeds}} \times 100\%$$

$$\text{Regeneration rate} = \frac{\text{number of regenerated calli}}{\text{number of incubated calli}} \times 100\%$$

$$\text{Culture ability} = \text{induction rate} \times \text{regeneration rate}$$

$$\text{Transformation rate} = \frac{\text{number of GUS expressing calli}}{\text{number of stained calli}} \times 100\%$$

Experimental design and statistical analysis. The systems for regeneration of Dongnong 427 and Longdao 14 varieties were arranged by the orthogonal experimental design with three replications. Each replication contained 20 seeds per treatment for callus induction and 10–20 embryogenic calli for plant regeneration. More than 20 calli per experiment with three replications were used for transformation studies. A four-factor three-level orthogonal design L_9 (3^4) (Table S3 in ESM) was employed for investigating the effects of PGRs on culture ability. The PGRs were 2,4-D, 6-BA, KIN, and NAA. Levels and factors of orthogonal design are reported in Table S4 in ESM. Analysis of variance (ANOVA) was performed using SPSS (Ver. 19.0, 2010) software. The experimental data obtained from the orthogonal design were reported in tables, in which K being the sum of culture ability for certain factors at one level, k being the average total culture ability for certain factors at one level, the range value (*R*) being k_{max}–k_{min}, and k_{max} and k_{min} being the maximum and minimum values of k, respectively.

RESULTS

Effect of PGRs on rice callus induction and regeneration. In this study, 2,4-D was used for callus induction (Figure 1a, b), whereas 6-BA, KIN, and NAA were utilized for regeneration (Figure 1c, d). The optimal combinations of four PGRs for Dongnong 427 and Longdao 14 were obtained through orthogonal experiments. The highest culture ability values obtained were 40% for Dongnong 427 and 76% for Longdao 14 (Table 1). A significant influence of 2,4-D and KIN was established on the culture ability of Dongnong 427 (Table S5 in ESM). However, all PGRs, except for KIN, had a significant effect on the culture ability of Longdao 14 (Table S6 in ESM). The factor with the largest range value demonstrated the greatest effect on the culture ability. Table 1 reported that the rank order of PGRs was 2,4-D, KIN, NAA, 6-BA for Dongnong 427 and 2,4-D, NAA, 6-BA, KIN for Longdao 14, respectively. The optimal level of PGRs was the corresponding level of k_{max}. As a result, the optimal culture conditions for

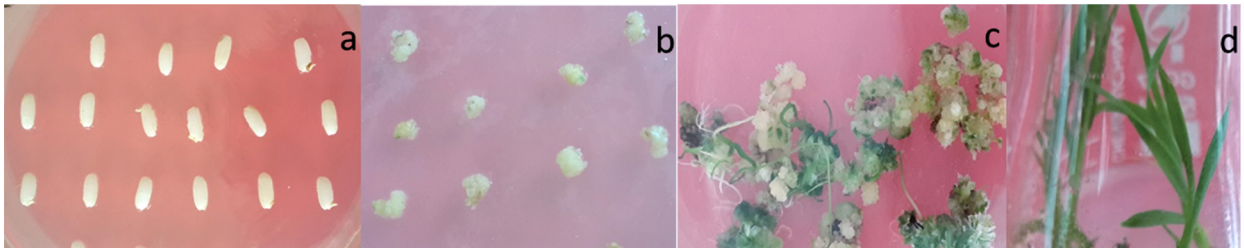

Figure 1. Induction and regeneration of rice plantlets from mature seed derived callus: inoculation of seeds onto NB medium (a), subculture of calli (b), regeneration of calli on MS medium (c), root formation of rice in half strength MS medium (d)

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callus induction and regeneration of Dongnong 427 and Longdao 14 were: 1 mg/l 2,4-D + 2 mg/l 6-BA + 4 mg/l KIN + 0.2 mg/l NAA; and 1 mg/l 2,4-D + 4 mg/l 6-BA + 4 mg/l KIN + 0.5 mg/l NAA, respectively (Table 1).

Effect of bacterial density on transformation. For Dongdong 427, the highest transformation rate revealed by transient GUS expression was 91% at 0.272 OD_{600}, while the lowest transformation rate was 61% at 1.814 OD_{600} (Figure 2a). On the contrary, the highest transformation frequency for Longdao 14 was 72%, which was observed at OD_{600} 0.592 (Figure 2b).

Effect of infiltration time. The exposure of Dongnong 427 explants to *Agrobacterium* culture with OD_{600} value of 0.870 for 10 min was found to be optimum as significant GUS staining was observed (Figure 3a). However, the infection of Longdao 14

Table 1. Orthogonal experimental results of Dongnong 427 and Longdao 14 rice cultivars

Treatment	2,4-D**	6-BA	KIN*	NAA	Culture ability
	(mg/l)				
Dongnong 427					
1	1	2	2	0.02	0.29
2	1	4	0	0.20	0.35
3	1	6	4	0.50	0.40
4	2	2	0	0.50	0.20
5	2	4	4	0.02	0.20
6	2	6	2	0.20	0.16
7	3	2	4	0.20	0.37
8	3	4	2	0.50	0.23
9	3	6	0	0.02	0.23
K1	1.04	0.86	0.68	0.72	
K2	0.56	0.78	0.78	0.88	
K3	0.83	0.79	0.97	0.83	
k_1	0.35	0.29	0.23	0.24	
k_2	0.19	0.26	0.26	0.29	
k_3	0.28	0.26	0.32	0.28	
Range	0.16	0.03	0.09	0.05	
Longdao 14					
1	1	2	2	0.02	0.67
2	1	4	0	0.20	0.76
3	1	6	4	0.50	0.74
4	2	2	0	0.50	0.20
5	2	4	4	0.02	0.17
6	2	6	2	0.20	0.08
7	3	2	4	0.20	0.14
8	3	4	2	0.50	0.28
9	3	6	0	0.02	0.07
K1	2.17	1.01	1.03	0.91	
K2	0.45	1.21	1.03	0.98	
K3	0.49	0.89	1.05	1.22	
k_1	0.72	0.34	0.34	0.30	
k_2	0.15	0.40	0.34	0.33	
k_3	0.16	0.30	0.35	0.41	
Range	0.57	0.10	0.01	0.11	

2,4-D – 2,4-dichlorophenoxyacetic acid; 6-BA – 6-benzyladenine; KIN – kinetin; NAA – α-naphthaleneacetic acid; *, **significant at $P < 0.05$, 0.01, respectively; K – sum of culture abilities for the factors at each level; k – the mean values of culture ability for the factors at each level; range – k_{max}–k_{min}

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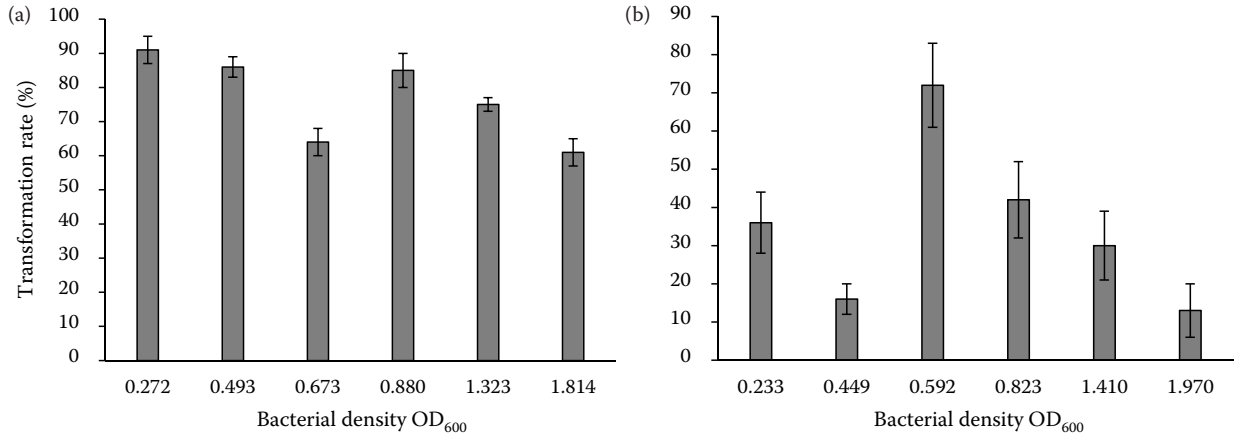

Figure 2. Effect of bacterial density on rice transformation: Dongnong 427 (a) and Longdao 14 (b) cultivars

calli in *Agrobacterium* culture with OD_{600} value of 0.684 for 20 min was the most favourable (Figure 3a).

Effect of co-cultivation period. The highest GUS activity was observed after three days of co-cultivation for both Dongnong 427 and Longdao 14 (Figure 3b). Although calluses co-cultivated for four and five days showed GUS activity, the tissues were adversely affected by the prolonged cultivation with *Agrobacterium*.

Effect of co-culture methods. After infection, calli of Dongnong 427 and Longdao 14 were divided into three parts. One part of them (about 60 calli) was transferred onto two pieces of sterile filter paper moistened with 1 ml liquid co-cultivation medium, the second part (about 60 calli) was transferred onto a solid co-cultivation medium with one piece of sterile filter paper on the surface, and the rest (about 60 calli) was transferred directly onto a solid co-cultivation medium. After three days of co-cultivation, the highest transformation efficiency for both Dongnong 427 and Longdao 14 was established in the treatment in which calli were co-cultivated on two pieces of sterile filter paper (Figure 3c).

PCR analysis. The results of GUS assay are shown in Figure 4. The presence of the transgene in the putative transformed plants was further confirmed by PCR analysis (Figure 5). Using GUS gene-specific primers, a 1800-bp fragment from calli of Dongnong 427 and Longdao 14 was amplified by PCR, as well as from the plasmid pCAMBIA1301 as a positive control. In addition, leaves of wild type rice were also tested as a negative control, showing no fragment in lane 1 (Figure 5).

DISCUSSION

The orthogonal experimental design is a design methodology that operates with multiple factors and levels.

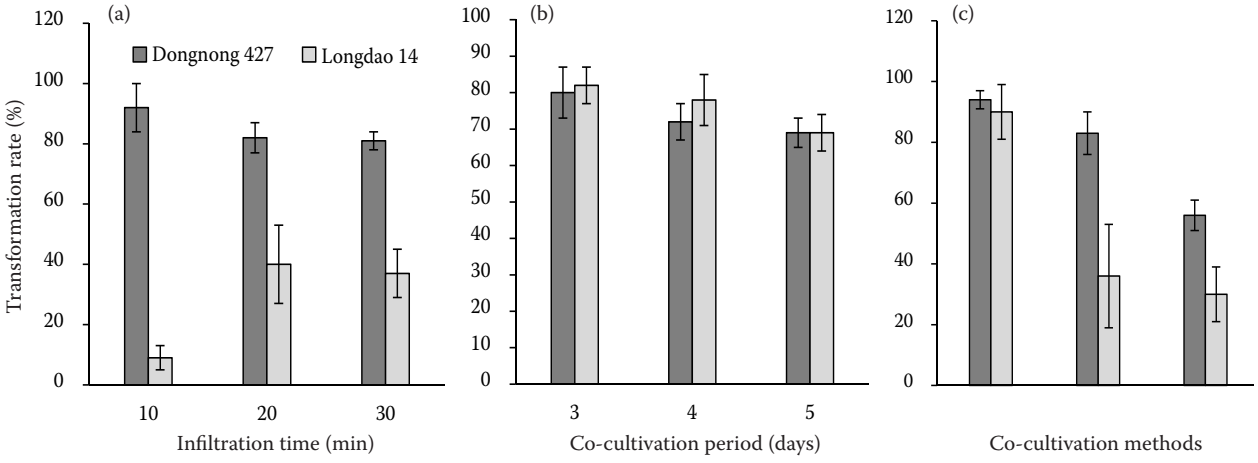

Figure 3. Effect of infiltration time (a), co-cultivation period (b) and co-culture methods (c) on Dongnong 427 and Longdao 14 rice cultivars

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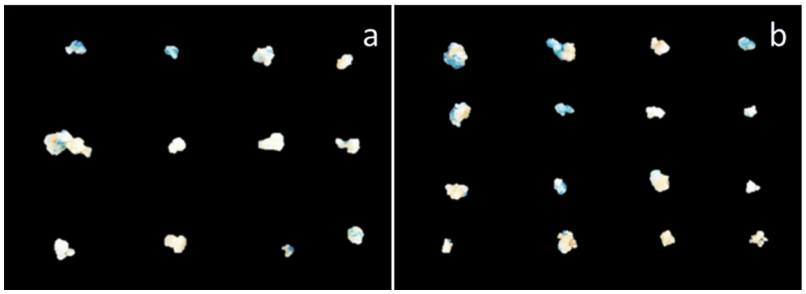

Figure 4. Histochemical detection of GUS activity in rice calli: Dongnong 427 (a) and Longdao 14 (b) cultivars

It is efficient, fast, and economic, because some representative points have been selected from the full-scale testing, and experimental numbers are considerably reduced by orthogonality. The method has been widely used earlier by a large number of scientists (Zhang *et al.* 2011; Liu & Xu 2013). Many studies have been conducted on the effect of PGRs by one-factor experiments, but the interactions between the various PGRs have been ignored. In this study, the optimum culture conditions were studied with an orthogonal design L_9 (3^4). The factors included the addition of four PGRs to the culture medium: 2,4-D, 6-BA, KIN, and NAA. The levels of each factor in the orthogonal design and the visual analysis results are listed in Table 1. Nine trials with three replications were performed according to the orthogonal design, and the relationships between the factors were determined by analysis of variance (ANOVA). Eventually, the best combinations of four PGRs for each rice cultivar were obtained by only nine experiments.

Agrobacterium-mediated transformation is influenced by many factors. The analysis of variance confirmed that bacterial density exerted a significant effect on the transformation rate of Dongnong 427 and Longdao 14 ($P < 0.01$). Although the trends of changes of the two varieties were different, their optimal OD_{600} values were lower than 1.0, which was similar to the results of previous studies indicating that the optimal OD_{600} for two *indica* (*O. sativa* ssp. *indica*) rice species, IR36 and ADT43, were 0.4 and 0.8, respectively (Karthikeyan *et al.* 2012; Krishnan *et al.* 2013). Necrosis was found when calli were infected with a higher bacterial density ($OD_{600} > 1.0$).

The infiltration time for Longdao 14 was statistically significant ($P < 0.05$), while it was not for Dongnong 427 ($P > 0.05$). However, the transformation frequencies of two varieties were reduced when the infection time reached 30 min. Karthikeyan *et al.* (2012) discovered that an interval of 10 min was the optimal duration for infiltration of mature seed-derived calli supported by the intensity of the blue colour obtained. Nevertheless, Priya *et al.* (2012) found that the infection of IR 64 with 1 OD_{600} for 1 min was the most favourable, possibly because the OD_{600} was higher, resulting in a reduced infection time.

The co-cultivation period had no significant effect on the transformation rate of Dongnong 427 and Longdao 14 by the analysis of variance. With the increase of the co-cultivation duration, all transformation rates of the two varieties were reduced. In general, the co-cultivation periods used until now have varied from three to five days (Hiei *et al.* 1997). However, in the present study it was found that three days were optimal for rice transformation (Figure 3b). Identical results have been reported for *indica* rice transformation (Hoque *et al.* 2005). In an examination, there was

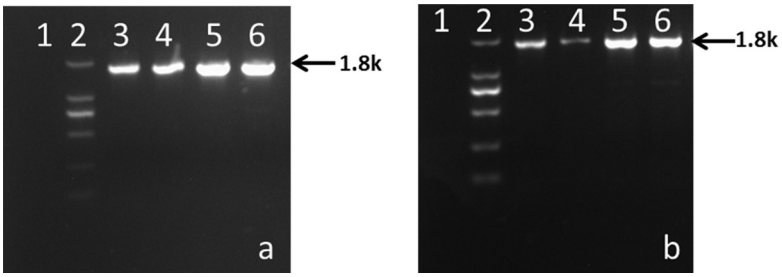

Figure 5. PCR analysis of transgenic rice plants of Dongnong 427 (a) and Longdao 14 (b); 1 – untransformed control; 2 – 2kb DNA ladder marker; 3–5 – putative transgenic plants; 6 – positive control (plasmid pCAMBIA1301 DNA amplified with GUS specific primers)

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evidence that although calli, which were co-cultivated more than three days, showed some GUS activity, they were adversely affected by *Agrobacterium* overgrowth and subsequently died (Ozawa 2009).

Our findings revealed that the effect of co-culture modes on transformation frequency was highly significant in Dongnong 427 and Longdao 14 ($P < 0.01$). Although a large number of researchers used solid media in their experiments (Ozawa 2009), in this study, calli co-cultivated on two pieces of sterile filter paper moistened with 1 ml liquid co-cultivation medium were the optimal choice.

After co-cultivation for 3–5 days with *Agrobacterium*, the transient expression of GUS was determined (Figure 4). Hence, the transformation rate discussed in the present paper was equal to the GUS staining rate. As the results indicated, the transformation efficiencies of Dongnong 427 and Longdao 14 varied. The reasons were complicated, and one reason was that the experimental conditions of the single-factor analysis were different. For example, after infection with various concentrations of *Agrobacterium* (OD_{600} 0.2–2.0), calli of Dongnong 427 were co-cultivated on two pieces of sterile filter paper moistened with 1 ml liquid co-cultivation medium, while calli of Longdao 14 were transferred onto a solid medium. Another reason for a difference in the transformation rate of Dongnong 427 and Longdao 14 was that the transformation rate is genotype-dependent (Hiei *et al.* 1997).

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