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Factors Affecting the Callus Induction and GUS Transient Expression in Indica Rice Pei'ai64s

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Abstract: In the current study, the effect of different concentrations of 6-benzyladenine, the effect of light on callus induction, browning and GUS transient expression in Indica rice Pei'ai64s was investigated. The results indicated that embryos callus from seeds and GUS transient expression is favored by the addition of concentration of 6-benzyladenine to cultured medium and culturing under light condition. High 6-benzyladenine concentration(0.5, 1 mg L^{-1})reduces the frequency of callus, GUS transient expression and enhances browning ratio. Light was also observed as browning for promoting factor.

Key words: Pei'ai64s, 6-benzyladenine, GUS transient expression, light

INTRODUCTION

In recent years, gene transformation has become an important way to research gene function and to improve plant breeding program. Transformation of Indica rice has been successful in a few laboratories using protoplast transformation with electroporation[1] or PEG[2], particle bombardment[3]. Chan *et al.*[4], Hiei *et al.*[5], Rashid *et al.*[6] and Dong *et al.*[7] used Agrobacterium to reproducibly transform Japonica, Indica and Javanica rice cultivars, respectively. These recent studies on the transformation of monocot plants by Agrobacterium have provided evidence for the hypothesis that T-DNA is transferred to dicots and monocots by an identical molecular mechanism. However, Indica rice transformation is still considered difficult to be transformed, especially by Agrobacterium mediated delivery. Pei'ai64s is the most important PGMS in China, as a kind of indica rice, it's also difficult to be transformed by Agrobacterium-mediated delivery. A few laboratories has achieved transgenic plants[8,9] with low effect of transformation. In order to improve effect of indica rice transformation including Pei'ai64s via Agrobacterium mediated delivery, well established tissue culture system, particularly in improved procedure for callus induction, is a prerequisite. We have evaluated some of these factors for rice transformation. In this study, we report improved somatic embryogenesis and relatively high efficiency of GUS transient expression.

MATERIALS AND METHODS

Plant material: Mature seeds the cultivar Pei'ai64s [10] was dehulled and rinsed with alcohol (70%) for 3 min and then surface sterilized in calomel (0.1%) for 20 min, followed by three rinses with sterile distilled water. The seeds were dried on sterilize filter paper and cultured in Petri dishes which contained 25 ml of callus induction medium for ten days. Callus was excised from scutellum. Before cultured, calli were pre-cultured for four days on hormone-free MS medium.

Culture media: Callus induction medium was MS medium[11] containing MS basal medium salks, 30 g L^{-1} sucrose and 3 g L^{-1} Phytayel (Sigma), supplemented with 2 mg L^{-1} 2,4-D, either alone or in combination with 6-benzyladenine. Media were adjusted to pH 5.8 using 1N NaOH or 1N HCl, autoclaved at 121°C for 20 min.

Plasmid construct and Agrobacterium tumefaciens strain: The binary Plasmid pCAMBIA1301 was kindly supplied by genetic institution of China, which contains GUS sequence under the control of CaMv35S promoter and linked a hpt and CryIA© gene (Fig. 1). The A. tumefaciens strain EHA105 was employed to deliver the plasmid construct to plant tissue.

Agrobacterium-mediated transformation: The A. tumefaciens strain EHA105 containing pCAMBIA1301 was grown for one day at 28°C in YEP liquid medium, supplemented with 50 mg L^{-1} Kanamycin and hygromycin on a rotary shaker set at 260 rpm. The grown bacteria were sampled 100ul into 25 ml AB medium[12] and grown overnight to obtain an A600 of 0.5-0.6 (A600 1.0 corresponds to $1*10^8$) on the same condition and then also sampled 100 ul bacteria into 25 ml AB medium plus 100 mg L^{-1} acetosyringone (Fluka) cultured overnight on

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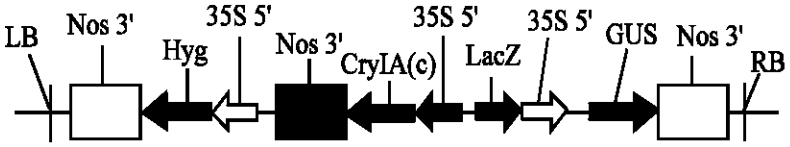

Fig. 1: The plasmid of P^{CAM} - CrylA(c)-GUS

the same condition. The bacteria cells were centrifuged at 5000 rpm in eppendorf (5417R) for 5 min and suspended in MS liquid medium containing 100 mg L^{-1} acetosyringone. Scutellum calli were immersed in MS bacteria suspension for 15 min and transferred onto filter paper to remove excess bacteria liquid. The calli were cultured on MS containing 100 mg L^{-1} acetosyringone for three days at 28°C in the dark.

GUS assay: Histochemical assay of GUS gene expression was carried out according to Jefferson[13]. After co-cultivation, calli were washed with sterile water and covered with filter-sterilized GUS substrate buffer[14] containing 20% methanol (v:v) and incubated at 37°C for 16–24 h. The GUS substrate buffer consisted of 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 0.3% (w:v) 5-bromo-4-chloro-3-indolyl-â-D-glucuronide acid, 0.1 M sodium phosphate (pH 7.0) and 0.3% (v:v) Triton X-100. Every blue spot, irrespective of its size, was counted.

RESULTS

***In vitro* establishment and callus culture:** Pei'ai64s was tested for calli induction response on various media. An intensive investigation was carried out aiming at the achievement of the best medium for good quality calli induction with various concentrations of 6-benzyladenine and other elements. The rate of calli induction was compared with different periods of culture (Table 1). High rate of calli (more than 70%) induction was obtained from MBA medium including MBA 0.2, MBA 0.5 and MBA 1. When the N6[15], MS, MB (B5 vitamins[16] substituted for MS vitamins and supplemented proline and 0.5 mg L^{-1}casamino acids) medium were used, the rate of induction was drastically decreased with less than 60%. This confirms the importance of 6-benzyladenine in the callus induction period in Pei'ai64s. In this experiment, the calli were also subcultured on the same primary medium for two weeks to compare brown frequencies. The results proved 6-benzyladenine can decrease callus browning ratio, proline and casamino acids in MB medium has no significant effect on reducing browning, which is different from previous report that proline and casamino can inhibit callus browning, maybe it related to different testing varieties [17]. Based on the rate of induction and callus browning, it was confirmed that MBA 0.2 is better than other medium.

Light influence: Light also influences the rate of callus induction and browning. Several investigators have reported that incubation of cell cultures under a low PFD or in darkness may be preferable for shoot induction and somatic embryogenesis from some species, such as cucumber [18,19], melon[20] and rose[21]. Pei'ai64s seeds were induced on MBA 0.2 medium under a day-length of 16 h(PFD=40-45 umolm-2s-1,PFD: photon flux density) and darkness, the light source being used was white fluorescent tubes. After ten days of culture, the calli were cut and cultured again in the same medium and observed the browning frequency. Light increased induction frequency (80.3%), but it had insignificant difference when compared with cultured in the darkness (70.8%); light makes callus growth faster, but at the same time, it also increases browning rate. After two-week of stepculture, the frequency of browning in light is 18.7%, the least significant difference compared with cultured in darkness (9.0%). After 3 days subculture on callus induction medium, the calli were inoculated by Agrobacterial suspension, GUS transient expression was detected.

Transient GUS expression in scutellar calli: Transient GUS expression was detected three days after co-cultured, calli variation were shown with a range of 9.6 to 71.8%, callus induced was highly susceptible to Agrobacterium and callus on MBA 0.2 gave the highest rate of GUS expression. However, calli induced on N6, MS, MB medium were poorly susceptible to the Agrobacterium with only about 10% GUS transient expression. Callus induced on MBA 0.2 under a day-length of 16 h resulted in a more enhancement of percent GUS expression (71.8%) than under the dark (64.9%), but there was not significant different between the class of condition.

The time of co-cultivation: After bacterial resuspension, the cultures were always incubated at 25°C for 3 days in the dark. During this process, genes in Agrobacterium T-DNA region were replicated, transferred into host cells and finally integrated into host genomes. But

Table 1: The ratio of callus production¡¢brown and GUS+

	N6	MS	MB	MBA 0.2[a]	MBA 0.2[b]	MBA 0.5	MBA 1
Percent of callus induction(%)	35.5D	45.8CD	58.4BC	80.3A	70.8AB	73.9AB	72.2AB
Percent of callus browning (%)	46.9A	41.8A	46.9A	18.7B	9.0C	15.6BC	12.7BC
Percent of GUS+(%)	9.6B	10.0B	12.6B	71.8A	64.9A	59.4A	25B

Note: MBA 0.2[a] means culture under light, MBA 0.2[b] means culture in dark;MBA 0.2, MBA 0.5, MBA 1 mean the concentration of 6-BA is 0.2, ¢0.5, 1 mg L^{-1}; Means with the same letter are not significantly different at P=0.01

Table 2: Percent of resistant calli in different selection condition

	Resistant calli(%)	Resistant calli(%)	Resistant calli(%)	Resistant calli(%)	average
HYG50(%)	26.7	26.2	29.7	25.0	26.9A
HYG100(%)	4.2	2.4	4.5	2.6	3.0B

Means with the same letter are not significantly different at P=0.01.

Table 3: PCR reaction system for identification of transgenic plantlet

Ingredient	ddH_2O	10¡ÁBuffer (including Mg^{++})	dNTPs(2.5 mM)	Primer 1 (10 μM)	Primer 2 (10 μM)	Taq (5 U μL^{-1})	Template (20ng μL^{-1})
Volume(μL)	9.5	1.5	1	1	1	0.2	1

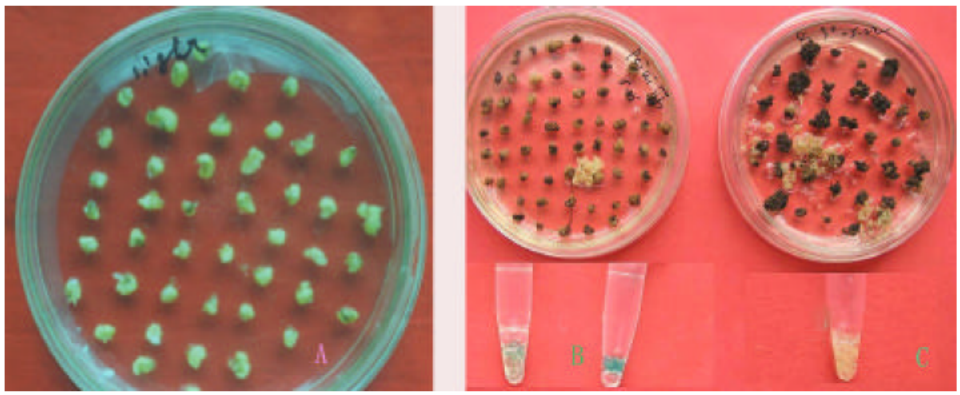

Fig. 2: The callus of peiai64S and selection in Hygromycin
A is the callus of peiai64S in MBA 0.2; B is callus selected in 100 mg L^{-1} Hyg and GUS array; C is callus selected in 50 mg L^{-1} Hyg and GUS array

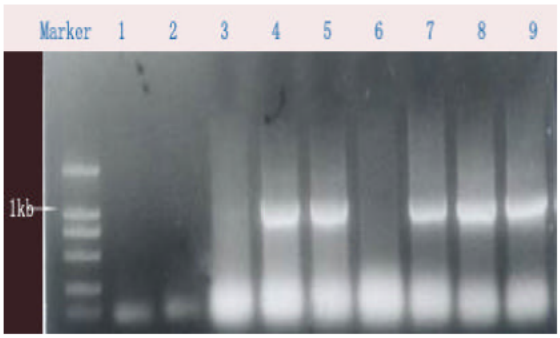

Fig. 3: PCR analysis of CryIA© in putative transgenic Peiai64S plantlets
1,2 are water of PCR 3,6 are pseudo-positive transgenic plants, 4,5,7,8,9 are transgenic peiai64S plants

Agrobacterium is also harmful to explant, it can cause browning, rotting and death of tissue at last. During the 3 days co-cultivation, the frequency of GUS transient expression is different, they are 33.3, 56.7 and 64.9% (callus culture in darkness). In our research, there is no significant difference between 2 days and 3 days concultivation period, so we adopt two days concultivation, while only two days concultivation can lessen Agrobacterium's harm, it is of beneficial for callus to long-time selection and important to easy-browning indica rice.

Selection pressure: Selection pressure has great influence on resistant calli production. As we known, hygromycin is a kind of most common used selection agent in rice transformation. The concentration used is 50 mg L^{-1}, same as Zhao *et al.*[8] and Zai *et al.*[9] in their research on pei'ai64s transformation. But in this experiment, we found many pseudo-resistant calli under 50 mg L^{-1} hygromycin selection pressure. This kind of calli grow up more quickly than true resistant calli, so they overshadow the true resistant calli eventually and cause them to die. For this reason, the calli were selected in

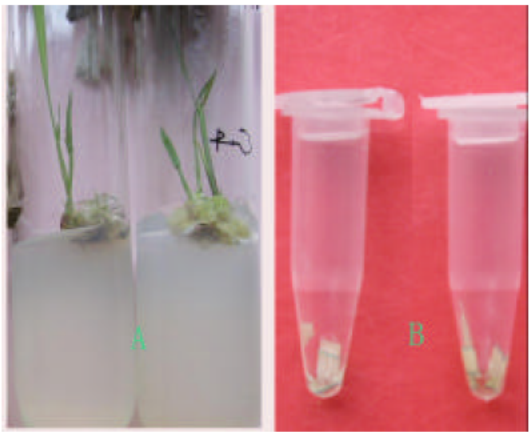

Fig. 4: Transgenic Peiai64S plantlets and GUS assay
A is transgenic Peiai64S plantlets, B is leaf GUS assay

30 mg L^{-1} pressure one week for natural growth first, then transferred into 100 mg L^{-1} selection pressure. According to this process, all produced resistant calli can be stained blue by X-Gluc (Fig 2), of course the percent of resistant calli (3%) is lower than in 50 mg L^{-1} selection pressure (26.9%) and significantly different, maybe the reason why true resistant calli became brown and then dead.

Regeneration of transgenic lines: Bright yellow hygromycin resistant calli were transferred to fresh MS regeneration medium(MS with 3% sucrose, 4 mg L^{-1} KT, 0.5 mg L^{-1} NAA, 0.3% Phytagel). In approximately 3 weeks, shoots of plantlets were recovered and transferred into new regeneration medium. Independent transgenic Plantlets 7 to 10 cm high with vigorous root development were transferred to potting soil in the greenhouse.

PCR analysis and GUS staining: DNA samples from putative transgenic rice plants were extracted from leaf tissue. PCR analysis was carried out using the following primers (CryIA© primer°5' GACCACTGCTATCCCATTGT 3', 5' TCTAACTA AGTCCCCACCAG 3'). The PCR conditions were as follows: 94°C for 5 min; 94°C for 1 min, 58°C for 1 min, 72°C for 1 min, 30 cycles: the final cycle was carried out at 72°C for 5 min. The expected amplified products for these reactions were 1078 bp fragment from CryIA© genes. The result of GUS staining demonstrated that GUS gene has expressed in transgenic plants.

DISCUSSION

The heterosis of inter-subspecific hybrids is much stronger than that of inter-varietal hybrids. Therefore, utilization of inter-subspecific hybrids is the most feasible approach for realizing super high yield. Pei'ai64s is an intermediate type between indica and japonica with good wide compatibility, several pioneer hybrids of Pei'ai64s such as Pei'ai64s/E32[22] have been certificated for commercial use. Although Pei'ai64s is an intermediate type, it leans to indica much more and like other indica rice, is also recalcitrant in tissue culture. The results revealed that inclusion of a low concentration of 6-benzyladenine in the callus induction medium for mature seeds resulted in the formation of a very compact, white yellowish, embryonic structure on callus at a very high percentage. Other media without 6-benzyladenine yielded non-embryonic calli which were unorganized and had loosely tentive, washy and white appearance. 6-Benzyladenine evoked higher embryogenesis and GUS transient expression than any other medium. 6-Benzyladenine can enhance cell division and Agrobacterium infection, 6-Benzyladenine at 0.5,1 mg L^{-1} reduced GUS expression significantly, although the rate of embryogenesis didn't change much. Too high 6-Benzyladenine had a negative effect on the number of embryos produced and Agrobacterium infection. Light treatment is likely to limit water uptake by tissues and make them drier and harder than in the dark, just as water stress treatment using mannitol and dehydration treatment, significantly improve the frequency of somatic embryogenesis. At the same time, soluble proteins of molecular masses of 21,22 and 26 kDa in rice calli were produced and improved the quality of calli after light culture, it is of beneficial for Agrobacterium transformation[23]. Before transformation, calli were transferred on a hormone-free MS medium to fully express their embryonic potential, allowing embryo development and subsequent conversion.

As we known, indica rice is not only difficult to induce embryonic callus but also easy to brown. Among the different 6-benzyladenine concentration, the optimal level was 0.2 mg L^{-1} combined with 2 mg L^{-1} 2,4-D, so the rate of auxin and cytokinin is also important. In rice tissue culture, there is few report that cytokinin including 6-benzyladenine was used to induce callus. However, in turf-type bermudagrass and maize were combined with auxin to induce callus and the results confirmed the function of the 6-benzyladenine.

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