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Factors Affecting *Agrobacterium*-Mediated Transformation Efficiency in Rice

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Abstract: Several important factors affecting the efficiency of *Agrobacterium*-mediated rice transformation were studied with several predominant commercial indica and japonica rice cultivars. As far as indica rice callus was concerned, CC medium was the best and the quality of callus was improved with the addition of 1.0 to 2.0 mg/L ABA. It decreased the percentage of browning calli and improved the callus growing state by addition of a certain amount of sorbitol to the subculture medium. NB medium was the best for callus initiation of japonica rice, but the improvement in the quality of callus of japonica subspecies was not obvious by adding ABA. During the period of subculture, to a certain degree, increasing the sucrose concentration could improve the proportion of hygromycin resistant calli. Furthermore, the transformation efficiency would be higher by applying selection pressure in the selection stage, removing selection pressure during the plantlet differentiation period and applying selection pressure again during seedling hardening period. Besides, suitable combination of plant hormones was beneficial for callus differentiation. An efficient *Agrobacterium*-mediated rice transformation system had been established for several rice cultivars and a lot of transgenic rice plants had been obtained.

Key words: rice; *Agrobacterium*; callus; hormones; transformation

Rice is one of the world's major food crops. The cultivated rice in Asia mainly included indica and japonica subspecies. Since Hiei et al. [1] obtained transgenic rice plants using the method of *Agrobacterium*-mediated transformation in 1994, the method had been developed into an effective system for japonica rice. As to indica rice with relatively large cultivated area, it was more difficult to establish transformation system. The main reason was the relatively poor in vitro response for indica rice. Even so, in recent years there still were some successful examples of *Agrobacterium*-mediated transfor mation in indica rice. The in vitro response is highly genotype-dependant, so it was difficult to find a uniform *Agrobacterium*-mediated transformation system for indica rice.

We studied several key factors that affected the efficiency of *Agrobacterium*-mediated rice transformation, in order to enhance the callus growing state and quality, to increase the percentage of hygromycin-resistant calli and callus differentiation and to establish *Agrobacterium*-mediated high efficiency transformation system by improving some key links.

MATERIALS AND METHODS

Plant materials

Six indica and japonica rice cultivars were used, which included Qimiaoxiang (indica) obtained from Guangdong Academy of Agricultural Sciences, R6547 (indica), Yangdao 6 (indica), R917 (japonica) and 9538 (japonica) provided by Lixiahe Area Research Institute of Agricultural Sciences of Jiangsu Province and Hui 236 (japonica) provided by Changshu Research Institute of Agricultural Sciences of Jiangsu Province.

Bacterial strains and plasmid

The plasmid pBUSCK with improved *CpTi* gene and the binary plasmid pCUBAC with *CpTi* and *Bt* genes were obtained from Zhu Zhen's laboratory of Institute of Genetics and Developmental Biology, the Chinese Academy of Sciences. The *Agrobacterium* strain used was EHA-105.

Rice callus culture

NB [4], CC [5] and MS medium were used in this study. The basic medium for callus induction was supplemented with 2 mg/L 2,4-D and 1.0 to 2.0 mg/L ABA. The ingredient of regeneration medium was basic medium supplemented with 2 mg/L 6-benzyladenine (6-BA), 0.5 mg/L KT and 0.5 mg/L NAA. The rooting medium was 1/2 MS supplemented with 0.5 mg/L NAA and 1 mg/L chlorocholine chloride (CCC).

Mature embryo callus induction

Mature seeds were dehulled manually and soaked in 70% alcohol for 1 to 2 min then sterilized with 0.1% $HgCl_2$ followed by washing 4 to 5 times with copious amount of sterile water. Finally, they were inoculated on

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corresponding medium. After culturing in the dark for 2 to 3 weeks the proliferating calli were dissected and subcultured on the same fresh medium. The subculture was repeated once every two to three weeks.

Callus induction from immature embryos

Twelve to fifteen days after flowering, immature rice seeds were collected. They were soaked in 70% alcohol for 1 to 2 min then soaked in 25% $NaClO_3$ for 90 min followed by washing 4 to 5 times with copious amount of sterile water. Immature embryos were dissected with scalpel then inoculated on corresponding medium and cultured at 25℃ in the dark for 2 to 3 weeks. After 2 to 3 weeks the proliferating calli were dissected and subcultured on fresh medium. The subculture was repeated once every two to three weeks.

***Agrobacterium*-mediated transformation of calli**

Single *Agrobacterium* colony was chosen and sorted out from YEB medium to YM medium (containing Km 50 mg/L) with sterile transferring loop and cultured at 28 ℃ in the dark for 2 to 3 days. After that, the bacterial cells were collected and suspended in liquid MS medium (containing AS 100 μmol/L). When the OD_{600} of the cell density was between 0.6 and 0.8, embryogenic calli were immersed in bacterial suspension for 20 min with occasional shaking. The excess of bacteria was removed by decanting the liquid. The calli were blotted up and transferred to the layer of sterile filter paper on the co-cultivation medium at 25℃ in the dark for 3 days.

Selection and regeneration

The co-cultivated calli were then transferred onto the selection medium (NB or MS + 50 mg/L hygromycin + 400 mg/L cephaloridine) and cultured at 26℃ in the dark for three weeks. The hygromycin-resistant calli survived after second cycle of selection were transferred to regeneration medium and cultured at 25℃ under photoperiod of 16 h illumination. Plantlets with 3 to 4 cm in height were transferred to rooting medium for root establishment.

RESULTS

Effect of different basic media on the callus initiation rate of rice mature embryo culture

Table 1 indicated that various basic media brought about different callus initiation rate. Average callus initiation rate of japonica rice was higher on the NB medium (88.2%) than on N_6 medium (75.9%). However, on the whole, average callus initiation rate of indica rice cultivars was higher on CC medium than on MS medium, though callus initiation rate of different indica rice cultivars was remarkably different with a range from 92.3% to 12.9%.

Effect of ABA concentration on callus induction rate and growth state

To a certain degree, addition of ABA could promote the callus induction of indica rice. Fig. 1 and Table 2 showed that the rate of R6547 and Yangdao 6 increased remarkably with supplementation of ABA, the optimum concentration range of which was 1.0 to 2.0 mg/L. Besides, ABA could improve the callus growing state and regeneration efficiency and make the calli stronger and brighter. As to japonica rice R917 and Hui 236, ABA had not any remarkable effect on the callus induction rate.

Effect of different carbohydrate source combinations on callus quality

Mixture of different carbohydrates with certain proportion not only provided carbon source for plant cell growth, but also played a vital role while keeping osmotic pressure in plant cells. In 2000, Wang [6] found that sorbitol and mannitol influenced the induction rate

Table 1. Effects of different basic media on callus initiation rate in rice mature embryo culture.

Cultivar	Medium	No. of plants	No. of calli	Callus induction rate (%)	*F* value
R917	NB	99	88	88.9	49.82**
	N_6	102	79	77.4	
9538	NB	96	82	85.4	356.05**
	N_6	98	73	74.4	
Hui 236	NB	100	88	88.0	17.20**
	N_6	102	81	79.4	
R6547	CC	105	97	92.3	8.67*
	MS	103	94	91.3	
Qimiaoxiang	CC	101	34	33.7	126.27**
	MS	99	15	15.2	
Yangdao 6	CC	96	59	61.2	352.70**
	MS	93	12	12.9	

*,**Significant at 0.05 and 0.01 levels, respectively.

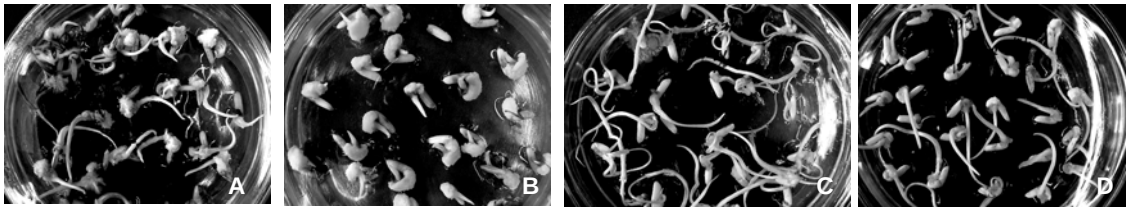

Fig. 1. Induction of callus on different media.

A, R6547 in MS medium; B, R6547 in MS medium with 1.0 mg/L ABA;
C, Yangdao 6 in MS medium; D, Yangdao 6 in MS medium with 1.0 mg/L ABA.

of callus remarkably. In this study, we observed that it was favorable for subculture of indica rice callus to replace the 30 g/L maltose with 15 g/L maltose and 15 g/L sorbitol or mannitol. The percentage of browning calli declined sharply (Table 3).

Effect of sucrose concentration on the callus quality and the percentage of hygromycin-resistant calli

During the long time (more than four months) of subculture, we found that some calli of japonica rice became wet and soft. In order to improve the calli quality and to increase the percentage of hygromycin-resistant calli, we have tried different concentrations of sucrose. Results suggested that callus growing state in long time of subculture as well as the percentage of hygromycin-resistant calli would be better improved with 40 g/L surcrose than with 30 g/L. The reason might be that sugar regulated osmotic pressure of plant cells.

Effect of different hormone combinations on callus differentiation rate of the cultivar 9538

Hormones played a key role in callus differentiation. In this study, the effect of different combinations of hormones on callus differentiation rate was estimated. Results suggested that the hormone combination of 2 mg/L 6-BA, 0.5 mg/L NAA and 0.5 mg/L KT was the best (Table 5).

Selection pressure and selection time

It was observed that callus became faint after co-cultivation and was extremely sensitive to hygromycin during differentiation, so in this research, selection pressure was increased gradually during induction stage and paused during differentiation stage (Table 6). When plantlets emerged, selection pressure was renewed. The rate of hygromycin-resistant calli increased obviously by utilizing this method. Of course, the percentage of false positive plants increased simultaneously, which in certain degree aggravated the load of molecular detection and resistance identification in the future. However, our goal was to obtain more transgenic rice plants while more differentiation was the key to reach the goal, so that the procedure adopted by

Table 2. Effects of ABA concentration on callus induction rate (%) from the mature embryo culture of rice.

ABA concentration (mg/L)	R6547	Yangdao 6	R917	Hui 236
0.0	91.3 a	75.1 c	86.8 a	87.9 a
1.0	95.3 a	92.9 a	82.4 ab	86.5 a
2.0	92.7 ab	87.2 b	81.7 b	85.7 a
3.0	69.9 c	71.9 c	70.3 c	71.5 b
F value	83.83*	37.02*	22.15*	45.07*

Within a column, data followed by the same letter indicate no significant difference at 0.05 level; *Significant at 0.05 level.

Table 3. Effect of different combinations of carbohydrates on callus quality (cultivar: R6547).

Combination of carbohydrate source	State of callus
Maltose 30 g/L	Big and rigid, yellowish or white, becoming browning after several subcultures, and few embryogenic ones.
Maltose 15 g/L+Sorbitol 15 g/L	Fabric and tight, bright yellowish, more embryogenic calli after two or three subcultures, and no browning calli, the calli being able to subculture more times.
Maltose 15 g/L+Mannitol 15 g/L	Fabric and tight, more bright, some embryogenic calli after two or three subcultures, calli becoming dry and some browning after more subcultures.

Table 4. Effect of sucrose concentration on callus quality and rate of hygromycin-resistant calli.

Sucrose concentration (g/L)	Callus quality	No. of test calli	No. of hygromycin-resistant calli	Rate of hygromycin-resistant calli (%)
30	Soft, loose, wet	134	35	26.1
35	More tight, more wet	126	53	42.1
40	Tight, embryogenic callus	137	67	48.9
45	Tight, more rigid	119	45	37.8

Table 5. Effect of different hormone combination on callus differentiation rate of the cultivar 9538.

Combination of plant hormones	No. of calli	No. of regeneration plantlets	Regeneration rate of plantlets (%)
6-BA 3.0 mg/L+NAA 1.0 mg/L+KT 1.0 mg/L	64	24	37.5
6-BA 3.0 mg/L+NAA 0.5 mg/L+KT 0.5 mg/L	60	18	31.5
6-BA 2.0 mg/L+NAA 1.0 mg/L+KT 1.0 mg/L	61	24	39.3
6-BA 2.0 mg/L+NAA 0.5 mg/L+KT 0.5 mg/L	63	33	55.9

Table 6. Effect of different selection ways for hygromycin-resistant calli on transformation rate.

Cultivar	Way of selection	No. of test calli	Regeneration explants	Positive	Negative	Transformation efficiency (%)
9538	Normal selection	89	0	0	0	0
	Without selection pressure during plantlet differentiation stage	79	51	18	33	22.8
R917	Normal selection	85	2	1	1	1.12
	Without selection pressure during plantlet differentiation stage	80	57	27	30	33.8

us might have important role for obtaining more transgenic plants.

The transgenic rice plants obtained by improved procedure

Several predominant commercial indica and japonica rice cultivars were transformed and a mass of transgenic plants were obtained by utilizing our improved *Agrobacterium*-mediated transformation system (Table 7).

DISCUSSION

The aim of the present study was to increase the efficiency of *Agrobacterium*-mediated rice transformation. *Agrobacterium*-mediated high efficiency transformation mainly depended on the improvement and the optimization of the transformation conditions. In the present study, we optimized several key links of *Agrobacterium*-mediated rice transformation such as induction of callus, improvements in the callus quality and adjustment of the callus growth state during subculture stage, increase of the calli regeneration and differentiation rate. The conclusion was that addition of a certain concentration of ABA during the induction stage of indica rice would inhibit the growth of plumule (embryo bud) and increase the frequency of callus induction. The optimal concentration of ABA was 1.0 to 2.0 mg/L. If concentration of ABA were higher, the callus would become dry and its growth would draw up. This finding was different from the observation of Mi et al. [7] and Yi et al. [8], in which the optimal concentration of ABA was 5 mg/L. The main reason involved might be the genotypic factor. During subculture stage of indica rice callus, it rapidly improved the quality of callus to replace maltose partially with sorbitol and mannitol. It might relate to the difference in callus growth state and osmotic adjustment. For long time subculture of japonica rice, a proper increase of sucrose concentration

Table 7. Number and frequency of transgenic plants obtained in the present test.

Cultivar	Plasmid	No. of calli	No. of resistant calli	No. of positive plants	Transformation efficiency (%)
9538	pBUSCK	75	58	20	26.6
Hui 236	pBUSCK	66	43	15	22.7
RR917	pCUBAC	78	53	29	37.1
R6547	pCUBAC	71	27	7	9.9

remarkably improved the callus growth state as well as the percentage of hygromycin-resistant callus. This improvement might be stemmed from the reason that higher concentration of sucrose increased callus osmotic pressure and improved callus growth state with bright color, tight granule. During the stage of the callus differentiation, plant hormones played a vital role on the decision of differentiation direction and level. Suitable combination of plant hormones would make the callus differentiate along the direction we want. Our results suggested that the better combination of plant hormones was 2 mg/L ABA, 0.5 mg/L KT and 0.5 mg/L NAA. Besides, it also improved the transformation efficiency of callus to inflict selection pressure during callus induction stage, to remove the pressure during differentiation stage and re-apply the pressure afterwards.

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