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Comparison of Regeneration Efficiency of Different Genotypes of Indica Rice Cultivars

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Summary

Establishment of high efficiency *Agrobacterium*-mediated transformation techniques for indica rice (*Oryza sativa* L.), offers the potential for the introduction of specific genes from any source into existing elite lines, for the purpose of varietal improvement. In this study, we identified optimal tissue culture conditions for callus induction and regeneration, the two major steps in the transformation process. The tissue culture conditions were tested using 15 indica cultivars and one japonica cultivar. The results of the present study revealed that callus induction was enhanced when used MS as the basal in callus induction medium. On the other hand, calli which were induced on N 6 based medium, has greatly improved the regeneration ability of indica rice. In addition, cultures which were incubated under light at 30^{0}C enhanced both callus induction and regeneration ability. IR 24, K-13, K-14, K-15 and Tetep have been identified as best varieties for transformation experiments since both callus induction and regeneration ability of these varieties were significantly high.

Key words: *Agrobacterium*-mediated transformation, indica rice, tissue culture conditions.

Introduction

Genetic transformation is an essential aspect of gene manipulation in plants which can break the species barriers and make it possible to transfer any gene to the desired crop. *Agrobacterium*-mediated transformation has greatly facilitated the widespread application of transformation into rice genome for the purpose of varietal improvement[1)]. However, *Agrobacterium*-mediated transformation of rice has been limited to japonica and javanica varieties and there are only couples of reports on indica rices[2),3)]. Even, these reports in which transformation was succeeded, the results showed either low transformation efficiency or highly specific to certain genotypes[2)].

Prior to genetic transformation, regeneration techniques have to be perfected since the high regenerability is an important prerequisite for successful transformation via *Agrobacterium*-mediated transformation or any other transformation techniques[3)].

As such, an experiment was conducted with the objective of optimizing the tissue culture conditions and identification of suitable varieties for efficient transformation of different indica rice. It was observed that the basal medium, light and temperature during callus induction, have great effect on regeneration ability of callus. Hence basal medium and culture conditions at callus induction are immense important

in improving transformation efficiency of selected indica varieties.

Materials and Methods

Seed materials

Indica rice cultivars, Blue Bonnet, Dvdh Rai, Hod sodri, K-13, K-14, K-15, K-16, Ladywright, Lemont, M 202, M 302, IR 24, Simanoce, Star Bonnet and Tetep were obtained from the Laboratory of Crop Science, Saga University. Japonica cultivar, Reihou was obtained from the Laboratory of Genetics and Plant Breeding, Saga University.

Medium and culture conditions

The culture medium and conditions were described by Toki[4],with few modifications (Table 1). Modified medium compositions for callus induction (CI) were indicated within parenthesis. Cultures were incubated at two levels of illumination (light and dark) at two different temperature levels (25^{0}C and 30^{0}C).

Table 1. Media used for tissue culture and transformation of indica rice.

Medium	Composition
CI	N 6(or MS) salts and vitamins, 30 mg/l sucrose, 1 g/l casamino acids, 2800 mg/l (or 500 mg/l) proline, 2 mg/l (or 3 mg/l) 2, 4－D, 2 g/l gelrite, pH 5.7
RE	MS salts and vitamins, 30 mg/l sucrose, 30 g/l sorbitol, 2 g/l casamino acids, 0.02 mg/l NAA, 2 mg/l kinetin, 4 g/l gelrite, pH 5.7
CO	N 6 salts and vitamins, 30 mg/l sucrose, 1 g/l casamino acids, 10 g/l glucose, 200 mg/l aceto-syringone, 2 g/l gelrite, pH 5.7
SE	N 6 salts and vitamins, 30 mg/l sucrose, 2 mg/l 2, 4-D, 2800 mg/l proline, 1 g/l casamino acids, 10 g/l glucose, 2 g/l gelrite, pH 5.7, 40 mg/l hygromicin B and 500 mg/l carbenicilin

CI=callus induction, RE=regeneration, CO=co-incubation and SE=selection medium

Callus induction

Varieties; Tetep, K-13 and Ladywright were selected to represent the indica group and variety Reihou was selected to represent the japonica group for the experiment. Dehusked seeds were sterilized by sodium hypochlorite and Tween 20. After 20 minutes, the solution was removed and seeds were thoroughly washed with sterilized water. Twenty sterilized seeds were transferred into each Petri dish containing different callus induction medium as described above. Seeds were incubated for 14 days under different culture conditions and the callus induction ability was recorded according to the 1-9 standard scale where, 1=no callus induction, 3=poor callus induction, 5=moderate callus induction, 7=good callus induction and 9=very good callus induction.

Regeneration

Seeds of fifteen indica varieties (Blue Bonnet, Dvdh Rai, Hod sodri, K-13, K-14, K-15, K-16, Ladywright, Lemont, M 202, M 302, IR 24, Simanoce, Star Bonnet, Tetep) and one japonica variety (Reihou) were incubated on CI medium, changing basal medium, N 6[5] or MS[6]. After 14 days intact calli were separately transferred to regeneration medium, RE (Table 1). Cultures were incubated for 14-21 days un-

der light at 30^{0}C. Regeneration ability was scored according to 1-9 standard scale at 14 day and 21 day after transferring to regeneration medium.

Transformation

Enhanced green fluorescent protein (EGFP) was used as a marker for transient expression in this experiment. *Agrobacterium tumefaciens* strain EHA 101 harboring the plasmid pBI-AT-EGFP was grown on AB medium having 50 mg/l hygromycin B and 50 mg/l kanamycin for 3 days at 22-25^{0}C. *Agrobacterium* was scrapped from the AB plate and resuspended in liquid co-incubation medium supplemented with 200 mg/l acetosyringone. Optical density of bacterial suspension was 0.05-0.1 at 600 OD.

Three days prior to co-incubation calli were sub cultured into fresh N 6 medium. Embryogenic calli were immersed in *Agrobacterum* suspension for 10 minutes. Then the calli were blotted dry and transferred into co-incubation medium, CO (Table 1). Co-incubation was done for 3-4 days under dark at 22^0 C.

Agrobacterium infected calli were first washed with sterile distilled water and followed by N 6 liquid medium supplemented with 500 mg/l carbenicilin and 1% plant preservative mixture (Plant Cell Technology, Japan). These washed calli were transferred into the selection medium, SE (Table 1). Cultures were incubated under light at 30^{0}C for 14-21 days. Only growing calli were transferred to RE medium(Table 1).

Results and Discussion

Callus induction

Callus induction ability of three indica varieties and one japonica variety were tested on different modified CI medium. The callus induction ability of three indica varieties were higher than that of japonica variety. Particularly, Tetep and K-13 showed extremely high callus induction ability. When use MS as the basic medium, indica varieties showed efficient callus induction. Increasing proline content (2800 mg /l) also helped to enhance callus production. Irrespective of variety groups (indica or japonica) temperature at 30^{0}C showed significant increase in callus induction. Four varieties used for the experiment showed no response to different levels of 2, 4-D (Table 2).

Table 2. Callus induction ability of different rice varieties under different tissue culture conditions.

Variety	Basic Medium		Proline (mg/l)		2, 4-D (mg/l)		Illumination		Temp. (^{0}C)	
	MS	N 6	500	2800	2	3	Light	Dark	25	30
Tetep	7.87*	7.16	7.22	7.81*	7.62	7.40	7.66	7.37	7.00	8.03*
K-13	7.54*	7.00	6.69	7.84*	7.16	7.37	7.41	7.14	6.91	7.62*
Ladywright	5.87*	5.47	5.62	5.72	5.75	5.59	6.00*	5.34	4.97	6.37*
Reihou	4.59	4.25	4.41	4.44	4.47	4.37	4.62	4.22	3.81	5.03*

* Significant at 0.05 level probability.

Callus induction of indica rice showed significantly increase when use MS salts and vitamins instead of N 6 salts and vitamins in CI medium. According to Tahiliani and Kothari[7] callus induction could be improved by $CuSO_4$ in the MS basal medium. It has been suggested that copper ions promote callus in-

duction, since they are components or activators of many enzymes involved in electron transport, protein and carbohydrate biosynthesis. Khatun et al.[8] have demonstrated that amount of nitrogen given by proline may improve *in vitro* culture of the rice genotype. Different genotypes require very specific nutrient elements at specific stages. A lack or deficiency in any of these nutrients may cause severe difficulty to the growth and development of callus.

Regeneration

The regeneration ability of 15 indica varieties and one japonica variety was tested. To identify the regeneration ability of these varieties, calli which, were induced separately on N 6 and MS basal in CI medium were transferred into RE medium (Figure 1).

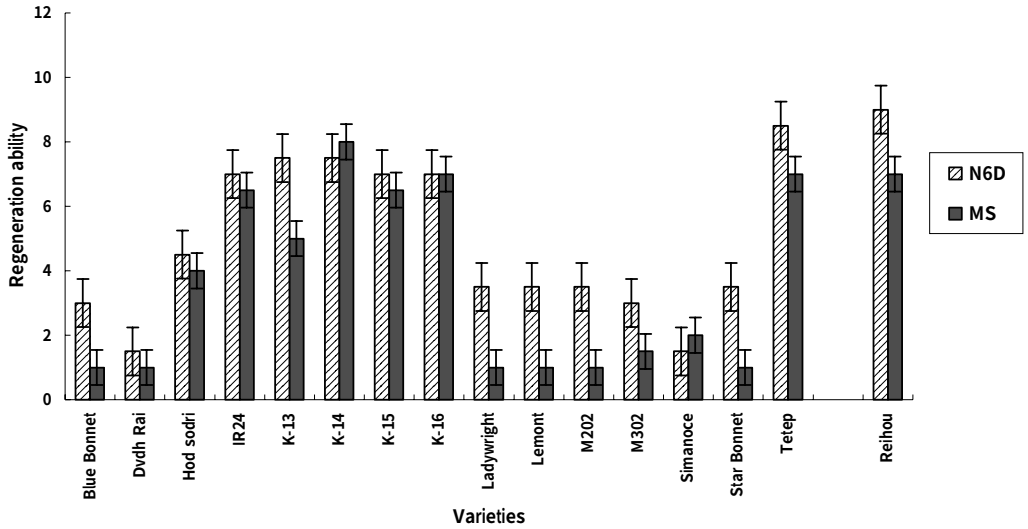

Figure 1. Regeneration ability of different indica rice varieties. Calli were induced on MS and N6 basic medium separately. Bars represent standard error of n=34.

In this experiment both callus induction and regeneration ability were estimated using 1-9 standard scale. As the size and the quality of the callus may vary even the number of calli were similar in different genotypes, the use of callus number as a quantification unit may deviate from the real situation. As such, use of 1-9 standard scale is more meaningful when estimate the callus induction ability by comparing the size and the quality of calli in different varieties.

Japonica variety; Reihou was used as a control in this experiment since it was identified as one of the best performing varieties among japonica group[9].

Table 3 summarized both callus induction and regeneration ability of different indica varieties. Tetep, K-15, IR 24, K-13 and K-14 were showed efficient callus induction as well as regeneration. Though, Hod sodri and Dvdh Rai showed high callus induction their regeneration ability was poor. On the contrast varieties, K-16 and Reihou showed poor callus induction while their regeneration ability was very high.

Regeneration ability of most varieties showed significantly higher when the calli were induced on CI medium with N 6 salts and vitamins. Optimized mineral nutrients in the N 6 medium can have a positive influence on explant development.

Various factors like basal medium, plant growth regulators, age of the explant and genotype have

Table 3. Comparison between callus induction and regeneration ability of different indica rice varieties.

Variety	Callus induction	Regeneration
Tetep	9.00 a	8.75 a
K-15	9.00 a	7.75 b
IR 24	9.00 a	7.75 b
K-13	9.00 a	7.25 b
Hod sodri	9.00 a	5.25 c
K-14	8.57 b	8.75 a
Dvdh Rai	8.25 b	2.25 d
Blue Bonnet	7.75 c	3.00 d
Ladywright	7.50 c	3.25 d
Star Bonnet	7.25 c	3.25 d
M 302	6.50 d	3.25 d
Simanoce	6.25 d	2.75 d
Reihou (japonica)	6.25 d	9.00 a
M 202	5.25 e	3.25 d
K-16	4.75 e	8.00 b
Lemont	4.50 e	3.25 d

Means followed by common letters are not significantly different at 0.05 level probability by DMRT.

been reported to increase regeneration frequency of indica rice[7]. In addition, Saharan[3] have showed that the regeneration ability of indica varieties (HKR-46 and HKR-126) could be increased when calli were subjected partial desiccation treatment. This finding let to suggest that some physiological changes during dehydrating of callus would enhance the regeneration ability. It can be speculated that physiological state of callus induced by N 6 based medium would also have influenced on the regeneration ability.

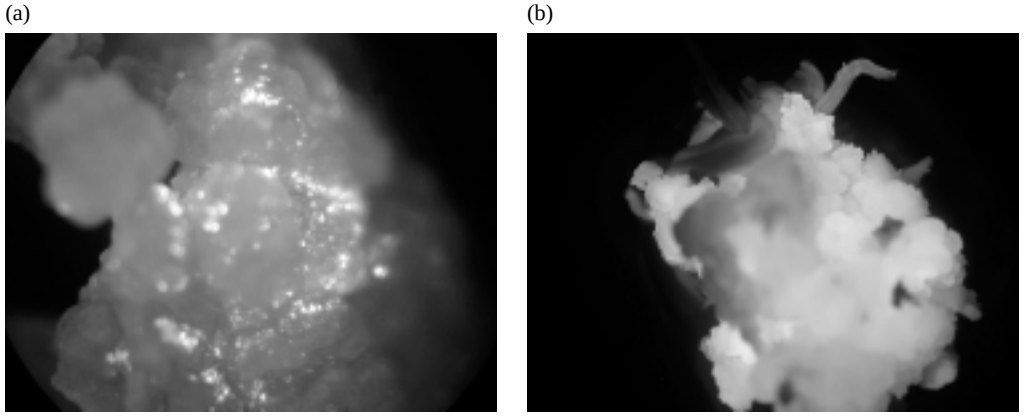

Figure 2. Transgenic calli of indica rice (c.v. Tetep). (a) Transformed callus with EGFP gene on SE medium and (b) regenerating callus on RE medium under fluorescence stereomicroscope.

Transformation

Varieties; IR 24, K-13, K-14, K-15 and Tetep were used for transformation as their callus induction and regeneration ability were significantly higher than other indica varieties that were tested. Tissue culture conditions as described by Toki[4], except for the co-cultivation period were carried out at 22^{0}C under dark conditions.

Under this experimental condition, transformation efficiency of these indica varieties was slightly lower than that of Reihou (data not shown). However, this level of transformation efficiency is sufficient to obtain transgenic indica rice.

The results of the present study revealed that calli induced on CI medium supplemented with N 6 salts and vitamins, has greatly improved the regeneration ability of indica rice. In addition, cultures which were incubate under light at 30^{0}C enhanced the regeneration ability of indica rice. Both callus induction and regeneration ability were showed significantly high in IR 24, K-13, K-14, K-15 and Tetep. Thus, these varieties are identified as suitable for further transformation experiments.

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異なる遺伝子型を持つインディカ種のイネにおける植物体再生効率の比較

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摘　　要

インディカ種イネの品種改良を進める上で，既に存在するエリートラインへのアグロバクテリウムを用いた効率転換技術の確立は，極めて重要な課題である．本研究において，我々は形質転換の過程で重要なカルス誘導および再分化の二つのステップについての最適化を図った．この組織培養実験には，15種類のインディカ種および１種類のジャポニカ種を用いた．その結果，インディカ種のイネではMS培地をベースとしたCI培地で培養した際に，著しいカルス誘導能力の改善が認められた．一方で，N６培地をベースとした培地で誘導されたカルスは，非常に高い再分化能力を持つことが明らかになった．さらに，光照明および30℃の温度条件もカルス誘導能力を上昇させることが明らかとなった．また，今回供試したうちIR24，K-13，K-14，K-15，Tetepの５品種については，高いカルス誘導能力と再分化能力を併せ持つことから，形質転換実験に用いるのに適した品種であると考えられた．