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Short Communication

Doubled haploid plant production of transgenic rice (*Oryza sativa* L.) using anther culture

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Abstract Anthers of transgenic rice plants with two foreign genes were cultured to obtain doubled haploid plants that possessed the introduced foreign genes homozygously. Anther culture response varied with the transgenic line. Green plantlets were regenerated from the microspore-derived calli, and 5.9–88.9% of the regenerated plants set numerous seeds in a greenhouse. The haploid or doubled haploid green plants were confirmed to have the foreign genes by Southern hybridization analysis. They had both or no foreign genes, and no plants with heterozygosity in transgenes were observed. The ratio of the plants with the transgenes homozygously to those without the transgenes varied with the donor transgenic lines.

Key words: Anther culture, doubled haploid, rice.

We had established an efficient transformation system using rice mature embryo through particle bombardment and analyzed the transgenic rice plants with some foreign genes (Shimada et al. 1995; Makino et al. 1997). In many cases, the foreign genes were integrated in the genome heterologously, and they were transmitted to their progenies in a MendelIian manner (Wakita et al. 1998). In practical breeding, plants homozygous for the introduced gene might be necessary. In general, the regenerated transgenic plants self-fertilized to obtain homozygous plants. However, it needs much time to select homozygous plants from the progenies of self fertilization. In the present study, we demonstrated the quick production of homozygous transgenic plants by anther culture of the transgenic lines with two foreign genes.

Transgenic rice plants that possessed *bar* and *NtFAD3* (tobacco microsomal ω-3 fatty acid desaturase) genes and non-transformed 'Notohikari' were used in this study. Two plasmids were used in this study. Plasmid pDM302 contains a *bar* gene fused to rice *Actin 1* promoter region and NOS terminator (Cao et al. 1992). Plasmid pTF1SN is a derivative of pBI121 and was constructed by replacement of *gusA* with *NtFAD3* cDNA fragment from 52 to 1381 (Accession no. D26509). The *NtFAD3* gene is a tobacco microsomal ω-3 fatty acid desaturase gene which catalyzes the conversion of linoleic acid (18 : 2) to linolenic acid (18 : 3) in lipids (Hamada et al. 1994). These transgenic plants were obtained by the particle bombardment system (Wakita et al. 1998).

Eleven independent transgenic plant lines and untransformed 'Notohikari' were grown in a greenhouse. The tillers of these transgenic plants were collected when the microspores were in the middle- to late-uninucleate stages of development, and kept at 8°C for 7 days. After cold treatment, donor spikes were sterilized with 70% (v/v) ethanol for 30 s, and anthers were placed onto 18×108 mm glass tubes with 8 ml of callus induction medium, which was N6 medium (Chu et al. 1975) supplemented with 5% (w/v) sucrose, 4 mg l^{-1} 2,4-D and 0.25% (w/v) gellan gum. Cultures were kept at 26°C in the dark. After 4 weeks of incubation, pollen-derived calli were transferred to regeneration medium composed of LS basal medium (Linsmaier and Skoog 1965) containing 2 mg l^{-1} of NAA, 1 mg l^{-1} of kinetin, 1 g l^{-1} of MES, 2 g l^{-1} of casein hydrolysate, 3% (w/v) D-sorbitol and 0.32% (w/v) gellan gum. Then regenerated shoots were transferred to the rooting medium composed of LS hormone-free medium.

Southern hybridization analysis of regenerated plants and their progenies were performed according to the method of Wakita et al. (1998).

Nine of the eleven transformed plant lines formed microspore-derived calli in the anther culture. Frequency of callus formation in each transgenic plant ranged from 0% to 110.9%, while the frequency in non-transgenic 'Notohikari' was 188.4% (Table 1). On the other hand,

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Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; NAA, 1-naphthaleneacetic acid; MES, 2-(N-morpholino) ethanesulfonic acid.

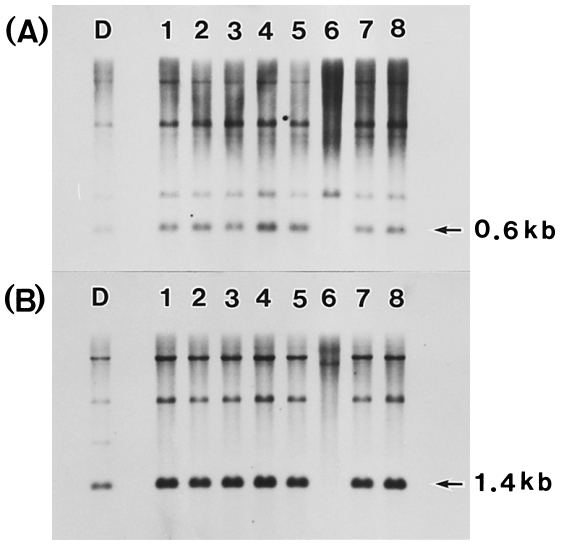
Table 1. Summary of anther culture of transgenic rice plants

T_0 Lines	Characters of donor transgenic rice plant line			No. anthers cultured	No. calli formed (%)[1]	No calli transferred	No.calli regenerating green plants (%)[2]	No. green plants grown in a green house	No. green plants with seeds (%)[3]
	Estimated copy No. of transgenes		Pollen fertility (%)						
	bar	*NtFAD3*							
Notohikari	0	0	97.8	1500	2826 (188.4)	200	100 (50.0)	17	7 (41.2)
F31-2	10	10	95.9	312	125 (40.1)	120	33 (28.0)	24	18 (75.0)
F31-3	3	2	96.8	867	89 (10.3)	89	56 (63.0)	17	1 (5.9)
F31-5	10	10	0	339	0 (0)	0	-	-	-
F31-7	5	3	95.3	576	160 (27.8)	159	20 (12.6)	9	4 (44.4)
F31-8	10	10	88.8	147	163 (110.9)	158	49 (31.0)	29	21 (72.4)
F31-9	2	1	0	383	3 (0.8)	3	0 (0)	-	-
F31-14	1	1	15.3	621	106 (17.1)	105	70 (66.7)	38	17 (43.6)
F31-15	3	1	42.7	126	0 (0)	0	-	-	-
F31-17	3	1	99.2	1036	370 (35.7)	370	274 (74.1)	73	41 (56.2)
F31-22	10	10	15.9	476	43 (9.0)	40	0 (0)	-	-
F31-31	10	10	51.4	786	429 (54.6)	428	145 (33.9)	54	48 (88.9)

[1] (Number of calli formed/ Number of anthers cultured)×100
[2] (Number of calli forming green plants/ Number of calli transferred onto regeneration media) ×100
[3] (Number of green plants with seed / Number of green plants grown in a green house) ×100

five lines (F31-5, F31-9, F31-14, F31-15 and F31-22) which had low fertility showed a low frequency of callus formation, ranging from 0% to 9.0% (Table 1). Green and albino plants regenerated from the microspore-derived calli of seven transformed plant lines. Frequency of green plant regeneration ranged from 12.6% to 74.1% in the seven lines, while the frequency of non-transgenic 'Notohikari' was 50.0%. In these 7 lines, frequency of the regenerated plants, which set numerous seeds varied from 5.9 to 88.9% in a greenhouse (Table 1). Regenerated plants setting normal seeds were considered to be spontaneous doubled haploids as confirmed previously (Niizeki et al. 1989).

Microspore-derived plants (A_0) of seven independent transgenic plant lines (T_0) were investigated for foreign gene integration by Southern hybridization analysis. Figure 1 shows the Southern hybridization patterns of eight microspore-derived plants from a line, F31-3. Seven plants had the same band pattern as its donor transgenic line, F31-3, and one did not. Table 2 summarizes the results of Southern hybridization analysis in the microspore-derived diploid plants from each donor transgenic plant line. The ratios of anther culture-derived plants with foreign genes to those without foreign genes varied with the line despite the theoretical one-to-one segregation (Table 2). In seven T_0 lines, the ratio of three lines, F31-2, 7 and 17, was one to one, and that of one line, F31-3, is 15 : 1 indicating the much higher number of regenerated plants with foreign genes than that of the plants without the genes. On the other hand, the number of regenerated plants without foreign genes was higher than that of the plants with the genes in the remaining three lines (F31-8, 14 and 31). Microspores that contained foreign genes had a tendency to be omitted during anther culture in the present study.



Figire 1. Southern hybridization of *Bam*HI-digested DNAs from the microspore-derived plants of a transgenic rice, F31-3. (A) Probed with the 402-bp product of *bar* gene and (B) 490-bp product of *NtFAD3* gene after PCR amplification. D, donor transgenic plant line F31-3; 1-8: microspore-derived plant lines. Arrows indicate *bar* gene (A) and *NtFAD3* gene (B).

The integrated genes were stably transmitted into all the progenies (T_0A_1) of anther culture-derived plants with transgenes by self-fertilization confirmed by the Southern hybridization (Figure 2). The bialaphos resistance test on microspore-derived plants supported these results (data not shown). These findings suggested that anther culture of transgenic rice plants could offer doubled haploids plants which possess the introduced foreign genes homozygously.

The results of the present study showed that doubled

Table 2. Segregation of transgenes in A_0 plants

T_0 lines	No. of A_0 plants examined	No. of A_0 plants with both *bar* and *NtFAD3* genes	No.of A_0 plants without both *bar* and *NtFAD3* genes	Expected ratio (*bar*-/*NtFAD3*+ : *bar*-/*NtFAD3*−)	χ^2
F31-2	24	12	12	1 : 1	0.00ns
F31-3	16	15	1	1 : 1	12.25**
F31-7	10	7	3	1 : 1	1.60ns
F31-8	24	5	19	1 : 1	8.17**
F31-14	24	7	17	1 : 1	4.17*
F31-17	24	16	8	1 : 1	2.67ns
F31-31	24	1	23	1 : 1	20.17**

* and **: Significantly different from the expected 1 : 1 ratio at 5 and 1 % levels, respectively.

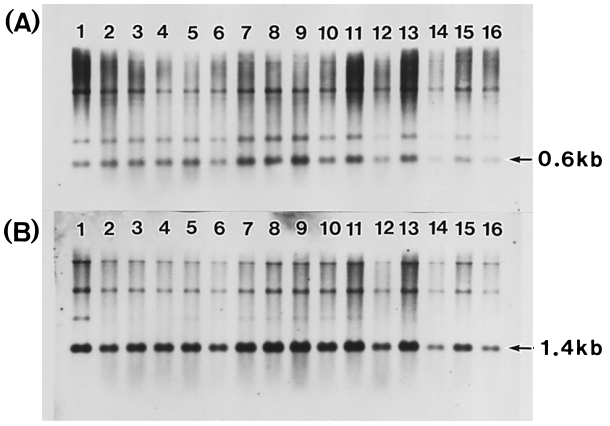


Figure 2. Southern hybridization of *Bam*HI-digested DNAs from the progenies of microspore-derived transgenic rice plant, F31-3, by self-fertilization. (A) Probed with the 402-bp product of *bar* gene and (B) 490-bp product of *NtFAD3* gene after PCR amplification. 1-16: Progenies of microspore-derived plant line, F31-3-1, by self-fertilization. Arrows indicate *bar* gene (A) and *NtFAD3* gene (B).

haploid rice plants possessing homozygous foreign genes could be produced by an anther culture method. Homozygous diploid plants with the introduced foreign gene can be obtained in one generation and homozygous seeds (A_1) can be obtained in a half year. Recently, Massiah et al. (2001) performed anther culture of transgenic wheat plants and succeeded in obtaining fertile homozygous transgenic lines.

In some transgenic plant lines, F31-8 and F31-14, however, microspores that contained foreign genes had a tendency to be omitted during anther culture in the present study. Selection with antibiotics or chemical matter during anther culture might be needed for efficient production of regenerated plants possessing the introduced foreign genes.

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