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Selection of Herbicide Tolerant Cell Lines from γ-ray-Irradiated Cell Cultures in Rice (*Oryza sativa* L. cv. Ilpumbyeo)

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Abstract

Herbicide tolerant rice (*Oryza sativa* L. cv. Ilpumbyeo) cell lines were selected from γ-ray-irradiated anther-derived cell cultures. The anther-derived cell clusters were small (300 to 400 μm in diameter) and uniform ones that were screened by miracloth filtering. The cell suspensions were very efficient to plate one layer onto agar medium and to screen target cell lines. Herbicide tolerant cell lines were selected by 5 mg/L cyhalofop butyl (CHB) treatment by using the small cell suspensions on agar N6 medium containing 1 mg/L 2,4-D and 0.2 mg/L kinetin. Of the cell lines, one line (CHB-1) showed stable tolerance at 10 mg/L concentration after 6-month culture without herbicide suspension. Growth stability of CHB-1 was similar to that of control cell line on 10 mg/L CHB containing medium. In this experiment we established herbicide tolerant cell line selection system by using anther-derived uniform-cell suspensions with γ-ray-irradiation.

Introduction

Anther culture has been quite useful for the production of homozygous double haploid lines and for accelerating the introgression of desirable traits in rice (Jahne and Lorz, 1995; Raina et al., 1987). Thus anther culture has been well integrated into the rice breeding programs especially in China and a number of high yielding, disease resistant and better quality rice varieties have been selected from microspore-derived plants (Jahne and Lorz, 1995; Yang, 1997).

Many resistant cell lines and somatic hybrids were screened by somaclonal variation in the cell cultures of plant, such as rice (Lee and Kameya, 1989; 1991; Sathish et al., 1995; 1997; Wakasa and Widholm, 1987), tobacco (Santandrea et al., 2000), and *Datura* species (Brotherton 1996). Cell suspensions are useful in screening resistant cell lines. In the case of rice, very small and uniform anther-derived cell suspensions were successfully used in the selection of target mutants (Lee and Kameya, 1989).

Gamma rays are also effective mutagenic agents that can be applied easily to any stages of anther culture (Nakamura and Hattori, 1997). In rice anther culture, some useful mutants were obtained among the progenies of doubled haploids (Kinoshita et al., 1989). Also, both dominant and recessive mutants could be detected effectively after *in vitro* selection of calli on medium containing NaCl with irradiation (Zapata and Aldemita, 1989) in rice.

In the present study we describe the effect of gamma-ray irradiation on screening efficiency of herbicide tolerant micro-callus on agar plate. We also report selection and growth of the herbicide tolerant cell lines screened after cyhalofop-butyl treatment.

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Materials and Methods

Plant materials and anther culture

Seedlings of 20 day-old rice (*Oryza sativa* L. cv. Ilpumbyeo) were transplanted and grown in field using standard agronomical practices (RDA, Korea).

The first two to three panicles were collected from the plants, when the distance between the auricles of flag leaf and penultimate leaf was 4-6 cm. Anthers obtained from the central spikelets of these panicles were in the mid-to-late uninucleate stage (Afza et al., 2000; Bishnoi et al., 2000). The panicles were sealed with Parafilm followed by surface-sterilization with 70% (v/v) ethyl alcohol, and kept in the dark for 8 days or 21 days at 7±1°C before using them for anther culture. Spikelets were removed from the sheath leaf, cut at the base with scissors so as to cut the anther filaments. Each spikelet was picked up by the uncut end using forceps and anthers were released on the medium by tapping the forceps on the rim of a test tube (1.2 cm ×10 cm). Approximately 15 to 20 anthers from one or two spikelets were cultured in the test tube. The cultures were plated on 0.8% agar-solidified N6 medium (Chu et al., 1975) containing 2 mg/L 2,4-D and 0.1 mg/L kinetin under continuous illumination (30 μmol/m²/s) at 25±°C. pH was adjusted to 5.8±0.1 before autoclaving at 121°C for 15 min. The percentage of anthers forming calli in 30 days (callus induction frequency) was recorded.

Screening of small and regular cell suspensions from anther-derived calli

Calli from each anthers were maintained separately in conjunction with our small and uniform cell suspensions screening strategy (Figure 1). The anther-derived calli (1 g FW) were transferred to 40 mL of AA (Toriyama and Hinata, 1985) liquid medium containing 1 mg/L 2,4-D, 0.2 mg/L kinetin, and cultured in 100 mL flasks with reciprocal shaking at 140 rev/min to establish suspension cultures. Three month-old subcultured cells were passed through two-fold miracloth and about 200 mg/L fresh weight of them was transferred to 40 mL of AA liquid medium containing 1 mg/L 2,4-D, 0.2 mg/L kinetin, 20 g/L sucrose. The cultures were grown in the deem light at 25±1°C and subcultured every 2 weeks at a 1 (suspension inoculum) : 4 (fresh medium) dilution rate (Lee and Kameya, 1989). The filtering was carried out more than six times by each two-week interval to make small and uniform cell suspensions.

Callus induction and proliferation from rice anthers (N6)
| (over 2 months)
Screening of micro-calli through twofold miracloth (AA)
| (over 3 months)
Determination of selection concentration of the herbicide (N6)
| (over 1 month)
Selection of herbicide tolerant cell lines on agar plate (N6)
| (1 month)
Free culture of selected cell lones (AA)
| (over 6 months)
Investigation of growth stability of the selected cell lines (AA)

Figure 1. A diagrammatic summary of the anther-culture herbicide-selection strategy.

γ-ray-irradiation and selection of herbicide tolerant cell lines

To determine the selection concentration of chyhalofop butyl tolerelant cell cultures, both solid media with calli or cell suspensions and suspended media with cell suspensions were tested on different concentration of chyhalofop butyl (0, 0.01, 0.1, 0.5, 1.0, 5.0 and 10.0 mg/L).

The suspended cultures were exposed to different strength of γ-rays (0, 30, 50, 50, 90, 120, 150 and 170 Gray) from a 60Co source in a gamma room of the KAERI (Daejeon, Korea). And then the cultures were subcultured three times by 10 day-interval. Then the cultures were plated onto N6 agar media containing various concentration of cyhalofop butyl (2.5 mg/L or 5 mg/L). After 2 to 3 weeks, rapidly growing cells that were cultured on agar media containing 5 mg/L of cyhalofop butyl were selected and considered as a putative herbicide tolerant cell line.

Growth of the selected cell lines

The putatively tolerant cells were harvested by subculturing for 1 month on AA liquid media containing 5 mg/L cyhalofop butyl, 1 mg/L 2,4-D and 0.2 mg/L kinetin and transferred to the herbicide free liquid media for six months to determine cell growth. The growth was measured by settled-cell volume 4, 8 and 12 days after culture.

Results and Discussion

Anther culture and screening of anther-derived micro-calli

The response of anthers to callus formation from the different media is shown in Table 1. Within 30 days from inoculation, callus formation was much higher on N6 medium (Figure 1A) than on AA medium in Ilpumbyeo as well as Donjinbyeo and Whayoungbyeo. Callus induction frequency of Dongjinbyeo was significantly lower than

that of other cultivars in this research. However, the callus induction frequency was surprisingly increased in Donjinbyeo as high as 48% (283/592) by increasing of pre-treatment period from 7 days to 21 days on N6 media, whereas the frequency was not increased in Ilpumbyeo (25%, 230/935). This result is compatible with the reports that cold pre-treatment (or period) of panicles plays important role in anther-derived callus induction (Afza et al., 2000; Jahne and Lorz, 1995).

To screen small and uniform cell suspensions (this cell suspensions were designated as micro-calli), 1 g FW of late log phase cells of rice cultivars were inoculated into 40 mL of liquid AA medium with reciprocal shaking at 140 rev/min. The small and uniform cell suspensions were screened by subculturing every 2-week interval for 3 months in Ilpumbyeo and Whayoungbyeo. But the cell size was somewhat large and irregular in Donjinbyeo. Of the three cultivars, small and uniform cell clusters were easily produced in Ilpumbyeo. The size of cell clusters were approximately 300 to 400 μm (Figure 1B). The cell suspensions of Ilpumbeyo were used for the selection of herbicide tolerant cell lines, because its uniformity and small size was very efficient for plating one-layer cells onto agar media and its cell character plays an important role for correct selection of the target cell lines.

Table 1. Effects of media of plated anthers of *Oryza sativa* L. on callus induction (%) cultured for 30 days.

Cultivar	N6 medium		AA medium	
	No. of anthers plated	No. of callus induced (%)	No. of anthers plated	No. of callus induced (%)
Ilpumbyeo	850	76 (9)	167	0 (0)
Dongjinbyeo	576	12 (2)	295	6 (2)
Whayoungbyeo	291	79 (27)	367	42 (11)

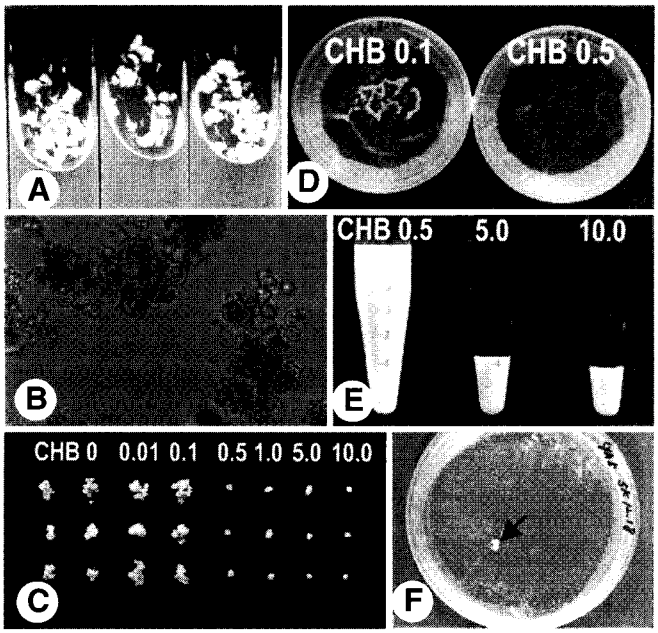

Figure 2. Selection of herbicide tolerant cell lines. A: Anther-derived callus formation after 35 days culture, B: Small cell clusters after miracloth filtering for 3 months. Bar indicates 100 μm. C, D, E: Selection of callus (C) and cell suspensions (D) on agar medium and cell suspensions (E) on suspended media with different concentrations of cyhalofop butyl (CHB), F: A independent cell line selected by 5 mg/L cyhalofop butyl treatment for 2 weeks.

Selection of herbicide tolerant cell lines

To determine the selection concentration of chyhalofop butyl tolerelant cell cultures, both solid media with

Table 2. Callus growth from anthers of rice (*Oryza sativa* L.) on various concentration of cyhalofop butyl after 35 days culture on agar plated media[a].

Concentration (mg/L)	Fresh weight (mg)[b]	Relative growth (%)
0	20.0±0.4	100
0.001	16.4±0.4	82
0.01	14.8±0.3	74
0.1	13.8±0.3	69
0.5	6.0±0.4	30
1.0	5.0±0.4	25
5.0	3.0±0.3	15
10.0	2.0±0.3	10

[a]Callus was cultured on N6 medium containing 1 mg/L 2,4-D, 0.2 mg/L kinetin.
[b]Callus growth appeared average fresh weight (mg) of ten calli.

Table 3. Screening of cyhalofop butyl (2.5 mg/L) tolerant cell colonies on solid agar medium at various intensities of γ-radiation from anther-derived cell suspensions of rice for 2 to 3 weeks culture.

Irradiation intensity (Gy)	A/C[a]	B/C[b]
0	0/60	0/60
30	0/60	0/60
50	0/60	0/60
70	0/60	0/60
90	1/60	6/60
120	2/60	6/60
150	0/60	0/60
170	0/60	0/60

[a]No. of petri-dish formed cell colony/Total no. of plated petri-dish.
[b]No. of cell colony formed/Total no. of plated petri-dish.

calli or cell suspensions (Figure 1C, D) and suspended media with cell suspensions (Figure 1E) were used. Callus and cell suspensions plated on agar media significantly inhibited cell growth at 0.5 mg/L chyhalofop butyl treatment (Table 2, Figure 1C, D). Especially, suspensions plated on agar medium inhibited cell growth perfectly at the chyhalofop butyl treatment (Figure 1D). As with all of the treatments, all growth was inhibited in sensitive cultures by 2.5 mg/L chyhalofop butyl. Thus we used the herbicide selection concentration of 5 mg/L in the next experiment.

Effect of gamma-ray irradiation on screening efficiency of herbicide tolerant micro-callus on agar plate was tested. As shown in Table 3, both 90 Gy and 120 Gy yielded highly growing cell lines at the selection concentration. However, no large difference of the selection frequency was observed at different irradiation intensities.

To select the herbicide tolerant cell lines, suspensions of the micro-calli were plated onto N6 agar medium containing 5 mg/L chyhalofop butyl, 1 mg/L 2,4-D and 0.2 mg/L kinetin. Two weeks after plating, three independent chyhalofop butyl tolerant colonies were selected, which were growing at high speed. A line is shown in Figure 1F. These three tolerant cell lines were designated as CHB-1, CHB-2 and CHB-3, and used to investigate whether the herbicide tolerance is stable or not.

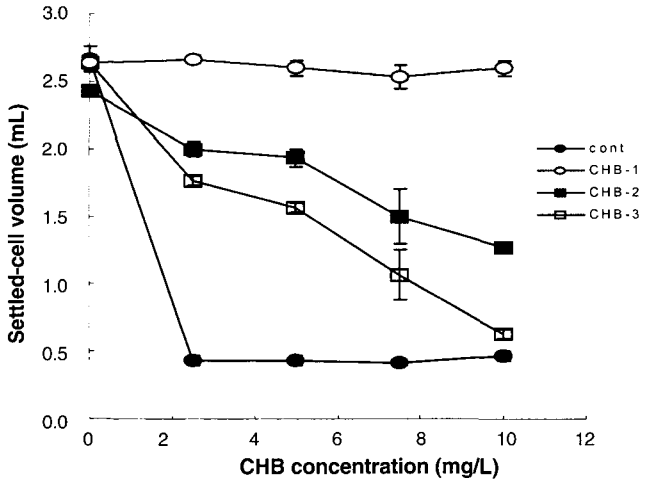

Figure 3. Stability of selected cell lines tolerant to cyhalofop butyl (CHB). CHB tolerance was determined 12 days after culture with or without 10 mg/L CHB.

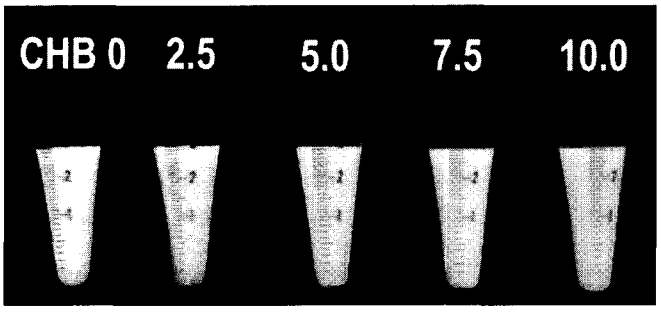

Figure 4. Effect of herbicide (cyhalofop butyl, CHB) on cell growth expressed as packed cell volume as compared to that in AA media containing 1 mg/L 2,4-D, 0.2 mg/L kinetin. The packed cell volume was determined after 14 days culture.

Growth stability of the herbicide tolerant cell lines

After 6 months of culture in AA medium containing 1 mg/L 2,4-D and 0.2 mg/L kinetin without the herbicide, CHB-1 was still tolerant to the herbicide but CHB-2 and CHB-3 lost its tolerance by the increased concentration of cyhalofop butyl treatment (Figure 3). CHB-1 could grow well in 10 mg/L cyhalofop butyl (fresh weights near that of control), whereas the unselected cell was completely inhibited. CHB-1 has been showing tolerance even when grown without the herbicide for more than six months (Figure 4). It means that the tolerance does not result in physiological adaptation of the cell line. Therefore, it is expected that the cell line was stable to cyhalofop-butyl treatment.

Attempt to regenerate plants from CHB-1 using plant regeneration medium is under progress. However, anther-derived rice cultures subcultured for 6 months were not able to produce plants on regeneration medium (Lee and Kameya, 1989; Lee et al., 1989). Thus, the tolerant cell line may be needed to protoplast fusion with a new cell line for plant regeneration and the cell line will be used effectively as a dominant marker for selection of somatic hybrid (Lee and Kameya, 1989; Lee et al., 1989).

From the above results, the selected cell lines did not have physiological adaptation but have stable herbicide tolerance. This selected cell lines also would be able to be used in the study of fatty acid biosynthesis, as cyhalofop butyl is a kind of herbicide that acts on inhibitors of fatty acid biosynthesis (Kondo et al., 1996; 1998; Moreland, 1999).

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