Effects of a Gamma Irradiation and 5-methyltryptophan on the Selection of High Tryptophan Accumulating Rice Mutants by an Embryo Culture System

Dong Sub Kim*, Jin-Baek Kim, Jae Young Song, Jae Beom Jeon, Young Mi Lee, Geung-Joo Lee, Kwon-Kyoo Kang[1], Yong Gu Cho[2], Bo Kyeong Kim[3] and Si-Yong Kang*

Radiation Research Center for Bio-technology, Advanced Radiation Research Institute, Korea Atomic Energy Research Institute, Jeongeup, 580-185, Korea
[1]Division of Life Science, Hankyong National University, Anseong, Gyeonggi 456-749, Korea
[2]College of Agriculture, Life & Environment Sciences, Chungbuk National University, Cheongju 361-763, Korea
[3]Division of Rice Breeding & Cultivation, Honam Agricultural Research Institute, NICS, Iksan 570-080, Korea

Abstract − For an increase of specific free amino acids through embryo cultures, the rice (*Oryza sativa* L.) mutant lines resistant to a growth inhibition by 5-methyltryptophan (5MT) were selected from calli irradiated with 0～90 Gy gamma rays in 3 rice cultivars, Dongjinbyeo, Donganbyeo and Jakwangdo. The optimum 5MT concentrations for a resistance selection were 0.25 mM at the callus level. In the test of the radiation sensitivity for the callus level, radiation doses of RD_{50} (50% reduction on fresh weight) were 71.2, 64.0, and 68.2 Gy in the cvs. Dongjinbyeo, Donganbyeo and Jakwangdo by each exponential function. The appearance of 5MT resistant calli seemed to be dependent on a combination between the radiation dose and the 5MT selection pressure. The treatment of mutagens for the selection of amino acid analog resistant mutants has a different influence among cultivars on the resistance frequency. In the effect of growth regulators on a regeneration, a combination of 0.1 mg/l IAA and 5 mg/l kinetin, was the optimum concentration for a regeneration of calli induced from rice embryo. The regeneration rate of cv. Donganbyeo was 14.8%, which was 3.5 times and 2.6 times higher than cv. Dongjinbyeo (4.2%) and cv. Jakwangdo (5.6%), respectively. In the progeny test, the 5MT resistance character is inherited to the next generation and is expressed in the germinating M_2 seeds and would appear to be a dominant trait. The 5MT resistant mutants will be useful in molecular and biochemical studies for the regulation of the nutritional quality in rice.

Key words : Gamma rays, rice (*Oryza sativa* L.), Embryo culture, 5-methyltryptophan (5MT)

INTRODUCTION

A matter of concern for the world's nutrition problem is the intake of sufficient quantities of essential amino acids, which are not synthesized in human and monogastric ani-

* Corresponding authors: Dong Sub Kim, Tel. +82-63-570-3311, Fax. +82-63-570-3319, E-mail. bioplant@kaeri.re.kr
Si-Yong Kang, Tel. +82-63-570-3310, Fax. +82-63-570-3319, E-mail. sykang@kaeri.re.kr

mals (Nelson 1969). Therefore, these essential amino acids must be supplied from food sources in sufficient quantities and at regular time intervals to optimize a growth, health, and normal bodily functions (Steinke and Hopkins 1983). Wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.) are also low in threonine. Maize, which is one of the major carbohydrate providers for animal feed, is low in tryptophan. The legume soybean (*Glycine max* Merr.), while high in protein, has a complement in methionine (Widholm 1977; Lee *et al*. 1992). Rice (*Oryza sativa* L.) which forms a staple food for a majority of people in many East Asian countries is very low in tryptophan and lysine in endosperm storage proteins (Dutta and Barua 1978; Lim *et al*. 1996).

Since Carlson (1970) demonstrated that it is possible to utilize selective techniques to recover mutant individuals from a population of single haploid cells cultured *in vitro*, rapid progress in culture methods is attributed to the availability of many mutants that could be generated for specific biochemical events which are difficult or impossible to use with intact plants (Larkin and Scowcroft 1981).

In many cases, plant cell cultures resistant to various amino acid analogs have produced elevated levels of the corresponding natural free amino acids (Widholm 1972; Ranch *et al*. 1983). Selection for a resistance to 5-methyltryptophan (5MT), tryptophan (Trp) analog, usually results in cell lines with greatly elevated free Trp levels (Ranch *et al*. 1983; Wakasa and Widholm 1987; Lee and Kameya 1991; Lim *et al*. 1996). Trp biosynthesis is controlled by a feedback inhibition of anthranilate synthase, the key feedback control enzyme in the Trp biosynthetic branch of the shikimate pathway, by tryptophan or its analogs (Widholm 1973). However, mutants with an altered anthranilate synthase showed more resistance to a feedback inhibition of much higher levels of free Trp accumulates (Widholm 1971, 1972, 1973; Ranch *et al*. 1983; Brotherton *et al*. 1986; Kang and Kameya 1995).

Although it is possible to select mutants with a high essential amino acid content by a somaclonal variation, mutant frequencies are often lower in plant systems, in part, by inefficient recovery techniques, as well as by the presence of multiple gene copies located on different chromosomes, and by cell-to-cell interactions, in even the most ideal suspension culture systems (Schaffer and Sharpe 1983). The major use of a mutagenic treatment in an *in vitro* culture is in adding one allele at a time to an existing genotype (Nabors 1976; Widholm 1977). This allows for a selection of large numbers and multiplications of the desired genotype in a minimal space and short duration under disease free conditions, and offers an efficient method for improving crop plants. Jacobsen *et al*. (1985) treated N-ethyl-N-nitroso-urea (ENU) for a selection of 5MT resistant cell lines of a dihaploid potato. Jensen (1991) successfully selected barley mutants with an increased content of lysine (Lys) through a treatment with gamma-rays and chemical mutagens such as EMS and NaN3. Lee and Kameya (1991) used ethylene imine (EI) for the selection of a 5MT resistant rice mutant. *Arabidopsis thaliana* mutant to α-methyltryptophan (αMT), an analog structually similar to tryptophan, was selected by using EMS (Kreps and Town 1992).

The purpose of this study was to investigate the effect of a gamma irradiation and tissue culture an the selection of mutant cell lines resistant to 5-methyltryptophan (5MT) with three different rice cultivars.

MATERIALS AND METHODS

Callus induction from rice embryo

The calli were initiated from embryos of hulled seeds of *Oryza sativa* L. Japonica cvs. Dongjinbyeo, Donganbyeo and Jakwangdo that were sterilized in 80% ethanol for 30 seconds and subsequently in 10% sodium hypochlorite with 2～3 drops of tween 40 for 30 min. The embryos of the seeds were rinsed 3～4 times with sterile distilled water and placed on callus induction medium which was N6 basal medium (Chu *et al*. 1975) supplemented with 2 mg/l 2,4-D (2,4-dichlorophenoxyacetic acid), 0.1 g/l myo-inositol, 0.25 g/l L-proline, 1 g/l casein hydrolysate and 30 g/l sucrose solidified with 0.4% (W/V) phytagel, and the pH was adjusted to 5.8 before autoclaving. All cultures were incubated in a growth chamber at 25˚C in the dark until calli were obtained.

Mutagen treatment and selection of 5MT resistant cell lines

The induced calli were plated on the media including various 5MT concentrations (0, 1.125, 0.25, 0.5, 0.75, and 1 mM) to determine the optimum concentration for the selection of 5MT resistant cell lines. The callus survival

rate was determined 40 days after culture as the number of survived calli per inoculated calli. The effect of gamma rays on the callus growth was also investigated 30 days after a callus treatment with gamma rays of 0, 30, 50, 70, and 90 Gy for 24 hrs generated by a ^{60}Co gamma-irradiator (150 TBq of capacity; ACEL, Canada) at the Korea Atomic Energy Research Institute.

The calli induced from the three rice cultivars were divided into 5 to 10 mg fresh weight pieces after being exposed to gamma rays of 0, 30, 50, 70 and 90 Gy doses and placed on selection medium containing 0.25 or 0.5 mM 5MT as one resistant cell line. The calli that survived from 0.25 mM 5MT were also transferred to 0.5 mM 5MT for the high selection intensity. All the selected 5MT resistant calli were propagated through a subculture at 4 week intervals and transferred to the regeneration medium.

Regeneration of 5MT resistant cell lines

In order to determine the optimum components and quantities of hormones suited to the regeneration of calli, several calli were plated on the regeneration medium containing various hormone combinations that are composed of IAA, BAP (bone alkaline phosphatase) and kinetin. The regeneration efficiency from different hormone combinations was calculated as the percentage of green plantlets to the plated calli.

5MT resistant calli induced from embryos were transferred to the optimum regeneration medium, which was MS (Murashige and Skoog 1962) basal medium supplemented with 0.1 mg/l IAA (Indole-3-acetic acid), 5 mg/l kinetin and 30 g/l sucrose solidified with 0.5% (W/V) phytagel at pH 5.8. All the cultures were maintained in the culture room under the condition of 27˚C light (16 hr)/25˚C dark (8 hr) regime and sub-cultured at every 4 week interval.

The plantlets were grown further on 1/2 MS medium without hormones. When the plants reached about 15～20 cm in height, half of the leaf blades to keep the plants from excessive evapotranspiration (Oono 1981) and raised in the 'Yoshida' culture solution (Yoshida *et al*. 1976) under continuous dark condition at 27˚C until roots were developed. Plants, which survived were transplanted into soil in pots in the greenhouse and were grown to maturity. For the investigation where a 5MT resistance was inherited or not to the next generation, harvested seeds from each 5MT resistant regenerated plant were re-screened and raised in the 'Yoshida' culture solution with 0.5 mM 5MT.

RESULTS

Effect of 5MT and gamma rays on callus growth

In order to determine the optimum 5MT concentration for a resistance selection at a callus level, calli were induced from the embryos of the seeds of Japonica cvs. Dongjinbyeo, Donganbyeo and Jakwangdo (Fig. 2A). They were inoculated on the selection media supplemented with various 5MT concentrations (0, 1.125, 0.25, 0.5, 0.75, and 1 mM) for 40 days. All calli inoculated on the 5MT-free media were in a good state of development, however, the fresh weights of the calli surviving at each 5MT concentration were declined with an increased 5MT concentration. In all the cultivars, the survival rates of the calli were less than 10% at a concentration of 0.125 mM 5MT. Cultivar Dongjinbyeo showed the highest survival rate, and the development levels of calli were also satisfactory. Dongjinbyeo produced two resistant cell lines even at 0.75 mM 5MT and the calli were well grown. On the other hand, the rate of callus formation in cv. Jakwangdo was as low as 4.0% and 1.7% at 0.25 mM and 0.5 mM 5MT, respectively. The callus survival rate of cv. Donganbyeo fell between those of cvs. Dongjinbyeo and Jakwangdo. However, all the inoculated calli at 1 mM 5MT were necrotised in three the cultivars (Table 1).

To test the radiation sensitivity of callus, calli induced from three cultivars were irradiated with gamma ray of 0, 30, 50, 70, and 90 Gy doses, and the fresh weight was measured 30 days after an inoculation on N6 basal medium. As the radiation doses increased, the fresh weight of the callus per clone was gradually reduced, and RD_{50} (50% reduction on fresh weight) of the callus was 71.2, 64.0, and 68.2 Gy in the cvs. Dongjinbyeo, Donganbyeo and Jakwangdo by each exponential function, respectively (Fig. 1). The appearance of 5MT resistant calli was associated with the combined effects between the radiation dose used and the 5MT concentration supplemented in the culture medium. Therefore, both the radiation dose and 5MT concentration should be considered for the selection of 5MT resistant calli.

Table 1. Survival rates of calli induced from rice embryo on medium supplemented with various 5MT concentrations

5MT conc. (mM)	Cultivars	No. of inoculated calli	No. of survivals (%)†
0	Dongjinbyeo	150	150 (100)
	Donganbyeo	150	150 (100)
	Jakwangdo	150	150 (100)
0.125	Dongjinbyeo	150	13 (8.7)
	Donganbyeo	150	9 (6.0)
	Jakwangdo	150	11 (7.3)
0.25	Dongjinbyeo	150	9 (6.0)
	Donganbyeo	150	8 (5.3)
	Jakwangdo	150	6 (4.0)
0.5	Dongjinbyeo	150	4 (2.7)
	Donganbyeo	150	3 (2.0)
	Jakwangdo	150	1 (0.7)
0.75	Dongjinbyeo	150	2 (1.3)
	Donganbyeo	150	0 (0.0)
	Jakwangdo	150	0 (0.0)
1	Dongjinbyeo	150	0 (0.0)
	Donganbyeo	150	0 (0.0)
	Jakwangdo	150	0 (0.0)

† Percentage of survived callus

Selection of 5MT resistant cell lines

Calli induced from the three cultivars were irradiated with 30, 50, 70, and 90 Gy gamma rays and cultured on 0.25 mM and 0.5 mM 5MT, which might be considered as an optimum concentration for the 5MT resistance selection at a callus level (Table 2). The average frequencies of the irradiated calli surviving from 0.25 mM 5MT were 0.90% and 0.85% in cvs. Dongjinbyeo and Donganbyeo, respectively. Although cv. Jakwangdo did not show a difference in the callus survival rate with the other cultivars in the non-irradiated calli, it had a high survival rate throughout all the radiation treatments. The survival rates of cv. Jakwangdo with a gamma irradiation were 14.8%, 16.6%, and 15.6% at 50 Gy, 70 Gy, and 90 Gy, respectively. Calli treated with 30 Gy and 90 Gy did not survive in cv. Donganbyeo. Fig. 2B represents the 5MT resistant colony cultured for 40 days on the selection medium containing 0.25 mM 5MT. The fresh weight calli pieces divided into 5 to 10 mg, which were selected from 0.25 mM 5MT treatment were transferred to the same medium supplemented with 0.5 mM 5MT to provide a higher selection intensity. Table 3 shows the survived calli rate after being transferred from the 0.25 mM 5MT to 0.5 mM 5MT medium. The survival rates of the irradiated calli in cv. Jakwangdo were higher than that

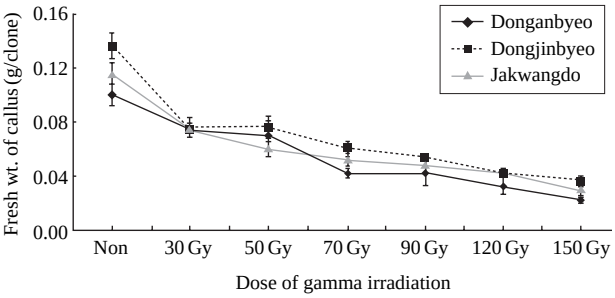

Fig. 1. Effect of gamma irradiation on the growth of calli in 3 rice cultivars for 30 days after culture.

Table 2. Selection of 5MT resistant callus lines irradiated with various gamma ray doses

Radiation dose	No. of calli transferred to 5MT medium	Cultivars	No. of survived calli†	
			5MT conc. (mM)	
			0.25	0.5
Non-irra.	500	Dongjinbyeo	30 (6.0)‡	0 (0.0)
		Donganbyeo	28 (5.6)	0 (0.0)
		Jakwangdo	23 (4.6)	0 (0.0)
30 Gy	500	Dongjinbyeo	2 (0.4)	0 (0.0)
		Donganbyeo	0 (0.0)	0 (0.0)
		Jakwangdo	7 (1.4)	0 (0.0)
50 Gy	500	Dongjinbyeo	4 (0.8)	0 (0.0)
		Donganbyeo	9 (1.8)	2 (0.4)
		Jakwangdo	74 (14.8)	0 (0.0)
70 Gy	500	Dongjinbyeo	5 (1.0)	0 (0.0)
		Donganbyeo	8 (1.6)	8 (1.6)
		Jakwangdo	83 (16.6)	2 (0.4)
90 Gy	500	Dongjinbyeo	7 (1.4)	0 (0.0)
		Donganbyeo	0 (0.0)	0 (0.0)
		Jakwangdo	78 (15.6)	0 (0.0)

† The number of propagated calli was recorded after 40 days
‡ Percentage of survived calli

of the non-irradiated calli, and approximately three times higher than those of the other cultivars. The radiation dose of 70 Gy showed the highest survival rate in all the cultivars, which indicated that 70 Gy might be used efficiently for selecting resistant calli. Because there were no selected cell lines at 30 Gy and 90 Gy in cv. Donganbyeo, we could not select them at 0.5 mM 5MT (Table 3). The selected 5MT resistant calli were transferred to the same selection medium, and propagated by two sub-cultures (Fig. 2C)

Regeneration of 5MT resistant cell lines

To investigate the effect of growth regulators on a regeneration, calli of three cultivars irradiated with 50 Gy were

cultured on four hormone combinations including one auxin (IAA) and two cytokinins (BAP and kinetin). The number of green plantlets was 1.8～2.1 times higher on the media with IAA and kinetin than that of IAA and BAP. The regeneration rate of cv. Donganbyeo was 14.8%, which was 3.5 times and 2.6 times higher than cv. Dongjinbyeo (4.2%) and cv. Jakwangdo (5.6%), respectively (Table 4). However, these regeneration rates were much lower than that of the calli without the inhibition effects by 5MT. The reason was considered to be that the development of the calli was affected by a continuous 5MT inhibition for a long culture period.

Propagated 5MT resistant calli were transferred to the optimum regeneration medium supplemented with 0.1 mg/l IAA and 5 mg/l kinetin. Two weeks after a calli transfer, green spots were observed on the calli, and green plantlets and albinoes were continuously differentiated. Fig. 2D shows the green plantlets differentiated from the 5MT resistant calli after a 4 week culture on the regeneration medium. The regeneration rates were varied depending on the cultivars, radiation doses and the developmental degree of the calli.

Regenerated plantlets were transferred to a 1/2 MS basal medium for the development of roots (Fig. 2E) without 5MT. All the regenerants were transplanted to pots filled with soil and grown to maturity in a greenhouse (Fig. 2F). In order to test the inheritance of a 5MT resistance, surface-sterilized seeds of two M_2 populations induced from the 5MT resistant regenerated plantlets were germinated on germination paper moistened with a nutrient solution containing 0.5 mM 5MT. Although, two populations (M_2-1 and M_2-2) showed a high 5MT resistance, this 5MT concentration severely inhibited the growth of the control plants (Fig. 2G). This result shows that the 5MT resistance character is inherited to the next generation and is expressed in the germinating M_2 seeds and would appear to be a dominant trait.

Table 3. Survival rates of 5MT resistant callus lines selected from 0.25 mM 5MT after being transferred to 0.5 mM 5MT medium

Radiation dose	No. of inoculated calli	No. of survived calli		
		Dongjinbyeo	Donganbyeo	Jakwangdo
Non-irra.	300	22 (7.3)†	10 (3.3)	9 (3.0)
30 Gy	300	3 (1.0)	0 (0.0)	12 (4.0)
50 Gy	300	5 (1.7)	4 (1.3)	10 (3.3)
70 Gy	300	7 (2.3)	13 (4.3)	16 (5.3)
90 Gy	300	2 (0.7)	0 (0.0)	11 (3.7)

† Percentage of survived calli

DISCUSSION

Amino acid analogs may function as feedback inhibitors

Table 4. Effects of growth regulators on regeneration from 5MT resistant rice calli irradiated with 50 Gy gamma ray

Cultivars	Growth regulators (mg/l)			No. of plated calli	No. of green spots†	No. of green plantlets (%)‡	No. of albino
	Kinetin	BAP	IAA				
Dongjinbyeo		3	0.2	300	24	3 (1.0)	1
		5	0.1	300	48	14 (4.7)	2
	3		0.2	300	42	12 (4.0)	0
	5		0.1	300	34	24 (8.0)	0
Total				1200	148	50 (4.2)	3
Donganbyeo		3	0.2	300	86	42 (14.0)	2
		5	0.1	300	44	21 (7.0)	1
	3		0.2	300	138	63 (21.0)	2
	5		0.1	300	108	51 (17.0)	3
Total				1200	376	177 (14.8)	8
Jakwangdo		3	0.2	300	68	14 (4.7)	1
		5	0.1	300	84	9 (3.0)	0
	3		0.2	300	42	23 (7.7)	1
	5		0.1	300	108	21 (7.0)	1
Total				1200	302	67 (5.6)	3

† The numbers were calculated 2 weeks after culture on the regeneration medium

‡ Regeneration rates were calculated as the percentage of plated calli forming green plantlets after incubation for 40 days

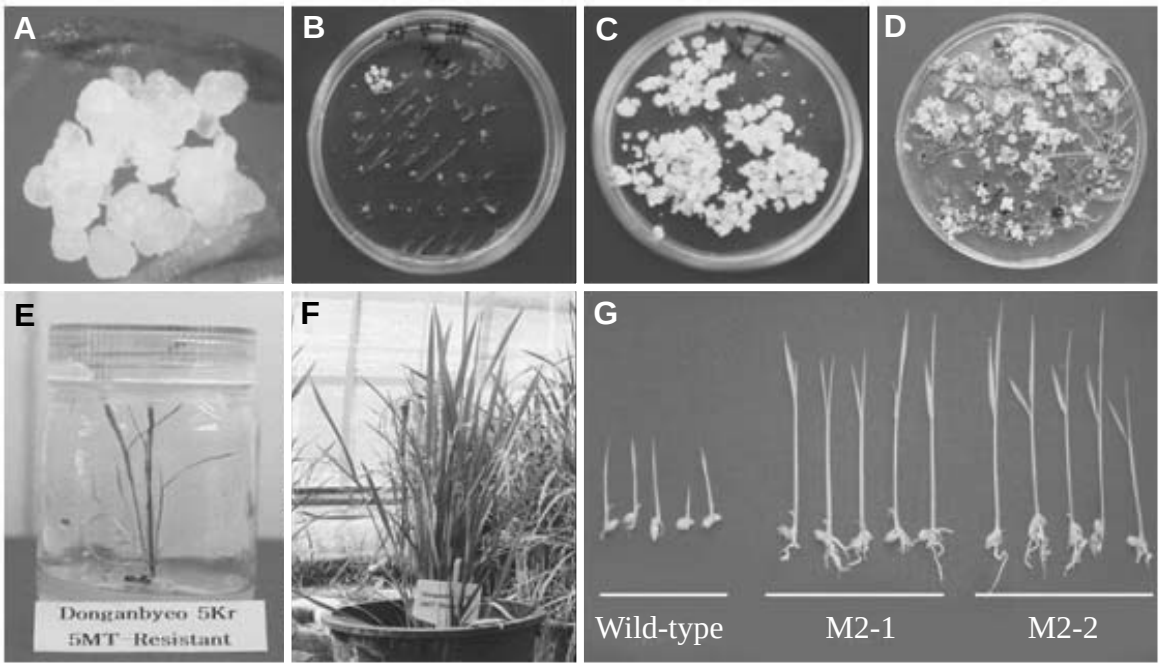

Fig. 2. Selection and regeneration of 5MT resistant cell lines in cv. Donganbyeo. A: Callus induction from rice embryo, B: 5MT resistant colony cultured on selection medium containing 0.25 mM 5MT for 40 days, C: Propagation of 5MT resistant calli, D: Differentiation of plantlets from embryo-derived callus after 4 weeks of culture on the regeneration medium, E: Normal green plantlets from 5MT resistant cell lines after transfer to 1/2 MS medium, F: 5MT resistant mutants transferred to pot in greenhouse, G: Conformation of 5MT resistance in M_2 next generation.

if they are stereo-chemically close enough to natural amino acids. This is apparently the case with 5MT, because tryptophan effectively reverses 5MT inhibition. Even though 5MT is incorporated into protein, the analog fits the other criteria, at least hypothetically, as a selective agent for the recovery of mutants which overproduce tryptophan (Schaeffer 1981). Feedback inhibition resistant mutants should behave as dominant ones and therefore can be isolated from the first generation in diploids as heterozygotes. This would significantly reduce the time and population size required for a mutant isolation (Green and Phillips 1974). It is also known that a dominant or a semi-dominant mutant resistant to an amino acid analog accumulates the corresponding free amino acids, while a recessive gene-controled mutant does not accumulate them (Mori *et al*. 1989).

In this study, 5-methyltryptophan (5MT) resistant mutant cell lines were selected through rice embryo cultures after a mutagenic treatment of the calli with gamma rays. As the 5MT concentration and/or radiation dose increased, the survival rate of the calli was reduced, although the callus represented a higher sensitivity to 5MT than the embryo. These results indicated that the appearance of 5MT resistant calli seemed to be dependent on a combination between the radiation dose and the 5MT selection pressure. The optimum 5MT concentration for a resistance selection was 0.25 mM at a callus level.

In the present work, the frequency of the 5MT resistant calli of cv. Jakwangdo was clearly increased after a gamma irradiation, especially at the doses of 50 Gy, 70 Gy and 90 Gy. Similar effects were observed in the ENU treated cell suspensions of a dihaploid potato (Jacobsen *et al*. 1985). However, the frequency of the 5MT resistant calli was lower than the frequency obtained from the untreated material in the other cultivars. This revealed that the treatment of mutagens for the selection of amino acid analog resistant mutants has a different influence among cultivars on the resistance frequency.

Selected calli at an optimum concentration were transferred to higher selection media concentrations to provide a high selection pressure. This style of selection revealed a higher degree of selection efficiency by considering the frequency of the 5MT resistant calli than a direct selection at a

high concentration, in which the survival rate of the calli was not more than 1% of the initially inoculated calli.

In spite of this advantage of the selection method, the regeneration rates of these selected embryo-induced calli were as low as 1～17%. Since the regeneration frequency decreases in a long-term callus culture and a sustained rapid cell division frequently results in a loss of the regenerative potential, when grown on 5MT, it is necessary to balance the selection and regeneration protocols for an optimum production of plants with an improved 5MT resistance and a test of a M_2 generation. Green and Phillips (1974) demonstrated that current cell culture limitations such as the inability to routinely differentiate plants from desired cultures in some crops may hamper mutant recovery and utilization.

The effects of growth regulators on a regeneration were studied by different hormone combinations, and we found that a combination of IAA and kinetin for the regeneration rates resulted in 1.8～2.1 times higher rates than that of IAA and BAP. The addition of kinetin to the medium reportedly stimulates and prolongs the potential to organize shoots (Saka and Maeda 1969). The regeneration rate of cv. Donganbyeo, in particular, showed about 3 times higher than the other cultivars for the media with a combination of 0.2 mg/l IAA and 3 mg/l kinetin.

The isolation and analysis of the feedback inhibition resistant variations is work that must be solved for an improvement of the nutritional quality in rice. 5MT resistant variations selected by an *in vitro* mutagenesis are being cultured in a greenhouse. Their progeny will be tested for the regulatory properties of the key anthranilate synthase, which is the key feedback control enzyme in the tryptophan biosynthetic branch of the shikimate pathway, and amino acid contents within the grain, the edible portion in cereal, will be studied. The 5MT resistant mutants will be useful in molecular and biochemical studies for a regulation of the nutritional quality in rice, and the amino acid analog resistant cell lines may also be used effectively in developing dominant markers for the selection of somatic hybrids.

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ABBREVIATIONS

BAP: bone alkaline phosphatase, MS: Murashige & Skoog, IAA: Indole-3-acetic acid, 5MT: 5-Methyltryptophan

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