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Selection of 5-Methyltryptophan and S-(2-Aminoethyl)-L-Cysteine Resistant Microspore-Derived Rice Cell Lines Irradiated with Gamma Rays

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Abstract

Microspore-derived cell lines resistant to 5-methyltryptophan (5MT, a tryptophan analog) or S-(2-aminoethyl)-L-cysteine (AEC, a lysine analog) were selected in rice by *in vitro* mutagenesis. For selection of 5MT or AEC resistant cell lines, suspension-cultured cells were irradiated with gamma rays. Thirteen 5MT resistant cell lines were selected and they were able to grow stably at 2 times higher 5MT concentration. A feedback insensitive form of anthranilate synthase, the pathway specific control enzyme for tryptophan synthesis, was detected from the 5MT resistant lines. Contents of the free amino acids in five resistant lines (MR12-1 to MR12-5) showed a 7.4 to 46.6 times greater level than that in the control culture. Tryptophan, phenylalanine, and tyrosine levels in the shikimate pathway were 28.1 and 22.5 times higher in MR12-3 and MR12-4, respectively, than that measured in the control cells. Four AEC resistant cell lines were isolated from cultures grown on medium containing 1 mM AEC. They were able to grow stably with 2 mM AEC, while sensitive calli were inhibited by 0.5 mM AEC. Aspartate kinase activities of the resistant lines were insensitive to the natural inhibitor, lysine, and accumulated 2.2 to 12.9-fold higher levels of free lysine than that of the control cells. Especially, the levels of aspartate, asparagine, and methionine in the aspartate pathway showed higher accumulation in the AEC resistant lines than that in the control cells.

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Introduction

Enhanced production of specific essential amino acids could have a great impact on solving the world's protein nutrition problem.

Since spontaneous mutations occur with extremely low frequency, *in vitro* mutation induction techniques provide tools for the rapid creation and increase the selection frequencies of cell types for which there is no obvious counterpart in field environments (Carlson 1970; Larkin and Scowcroft 1981). The production of haploids is a very useful and efficient method for obtaining homozygous inbred plants. Anther culture is one of the methods for obtaining haploid production. The use of doubled haploids has already become an integral part of the rice breeding process to save time required for fixation of pure lines. Haploid techniques may speed up mutation breeding since haploid regenerants derived from anther or microspore cultures of M_1 plants with M_2 gametes might allow a direct selection of mutants without going through the gametophyte phase into the next generation (Szarejko et al. 1991).

In addition, the application of mutation in combination with doubled haploid methods seems to accelerate breeding programs from the generation of variability through the selection period to rapid propagation of the homozygous desired genotypes. This method is also useful since both dominant and recessive mutants can be detected directly in the regenerated plants. The haploids produced often exhibit recessive charac-

ters previously masked in the diploid state, and may readily be diploidised to produce homozygous lines. After chromosome doubling, homozygous mutant lines would increase the efficiency of mutant selection due to a better distinction between mutated and non-mutated M_2 plants. Therefore, the combined techniques of anther culture and the use of mutagenesis may enable us to carry out mutation breeding. In rice anther culture, Kinoshita et al. (1989) irradiated both the anthers and anther-derived calli and obtained some useful mutants among the progenies of regenerated doubled haploids. Zapata and Aldemita (1989) also combined rice anther culture and irradiation to induce salt tolerance in high yielding rice varieties.

The use of amino acid analogs in these *in vitro* mutation techniques permits selection within the wild type cell populations for desired resistant phenotypes producing the elevated levels of the corresponding natural free amino acids (Widholm 1972; Ranch et al. 1983). Several mechanisms of amino acid analog resistance are; the altered feedback inhibition under the influence of the altering control enzyme resistant to feedback inhibition by the end product, the decrease of uptake inhibitors by a change in cell membrane permeability, and inactivation of inhibitors (Widholm 1774; 1977). However, in the latter cases, any amino acids are not increased *in vivo*, leading us to mistake them for selected cells rather than true analog resistant mutants. Miao et al. (1988) selected AEC resistant maize (*Zea mays* L.) cells on medium containing 2 mM AEC, but resistance to AEC was caused by decreased AEC uptake by the callus tissue and was not due to increased levels of free lysine.

Selection for resistance to 5MT usually results in cell lines with greatly elevated free tryptophan levels (Ranch et al. 1983; Wakasa and Widholm 1987; Lee and Kameya 1991). In most cases, the resistance of the cell lines was due to an altered anthranilate synthase enzyme, which was less sensitive to inhibition by 5MT and also by tryptophan, the natural inhibitor. The anthranilate synthase enzyme converts the branch-point compound chorismate to anthranilate in the first committed step of tryptophan branch of the biosynthetic pathway for aromatic amino acids and is the site of regulation via feedback inhibition by tryptophan (Widholm 1973). Brotherton et al. (1996) reported that the resistance is correlated with a lessened feedback control through kinetic studies of the anthranilate synthase activities from the 5MT resistant cell lines in *Datura innoxia*. Complete inhibition of the wild type or a 5MT sensitive culture was found with 6 mol tryptophan, while the anthranilate synthase activity of the 5MT resistant lines still retained about 25% with 100 mol tryptophan.

Lysine overproducing mutants resistant to AEC resulting from altered aspartate kinase and dihydrodipicolinate synthase (DHPS), the key enzymes by feedback inhibition in the aspartate pathway, have been reported in rice (*Oryza sativa* L.) (Chaleff and Carlson 1974; Schaeffer and Sharpe 1981) and tobacco (*Nicotiana tabacum* L.) (Widholm 1976). Negrutiu et al. (1984) isolated two AEC resistant protoplast lines mutagenized with ultraviolet (UV) light or ethylmethane sulphonate (EMS) in *Nicotiana sylvestris*, and both lines accumulated free lysine at 10 to 20-fold higher levels than that in controls. In this case, a feedback insensitive form of DHPS was detected in callus cultures and leaf extracts from the resistant lines, but aspartate kinase was unaltered in the mutants. Tobacco lines have been selected, which are resistant to the AEC and delta-hydroxylysine. These lines were able to grow in the presence of over 100 times as much inhibitor as the normal cells (Widholm 1976).

The purposes of this study were to select mutant microspore-derived cell lines resistant to 5MT and AEC which may act by false feedback inhibition by incorporating into protein or by some other mechanisms. For this study, rice anther suspension culture systems with the treatment of gamma rays, and then to identify characteristics of selected cell lines through investigation of the kinetic activity of related key enzymes and free amino acid contents were carried out.

Materials and Methods

Plant materials and callus Induction

Rice (*Oryza sativa* L.) *japonica* cultivar Ilpumbyeo, which is high-yielding with good quality and is also lodging and disease resistance, was used as a plant material for anther culture. Young ears of donor plants with anthers at the uninucleate developmental stage of microspore were collected and pretreated in the dark for 7-8 days at 8°C. Panicles were surface sterilized by 80% ethanol for 30 seconds and followed by rinsing four times with sterile distilled water. Anthers in the middle panicles were aseptically removed and cultured on 1% (W/V) phytagel solidified N6 basal medium (Chu et al. 1975) supplemented with 2 mg/L NAA (-naphthalene acetic acid), 0.25 g/L L-proline, 1 g/L casein hydrolysate, and 50 g/L sucrose at pH 5.8. All cultures were incubated in a dark growth chamber maintained at 25°C until calli were obtained. For making a small uniform size, calli were cultured in AA liquid medium (Toriyama and Hinata 1985) supplemented with 1 mg/L 2,4-D (2,4-dichlorophenoxyacetic acid), 0.1 mg/L GA_3, 0.2 mg/L kinetin, and 30 g/L sucrose, with 120 rpm shaking at 25°C in the dark. Subcultures were performed at 2 weeks interval for 2 months. The effects of 5MT (5-methyltryptophan) and AEC [S-(2-aminoethyl)-L-cysteine] on the growth of cells were investigated by measuring the fresh weight after suspension culture for 4 weeks in the AA liquid medium containing 0-1000 mol 5MT or 0-4 mM AEC.

Mutagen treatment and selection of 5MT and AEC resistant cell lines

Cells with a uniform size (300-400 μM) were used as materials for 5MT or AEC resistant cell selection. The cells were irradiated with 120 Gy or 30 Gy (1 Gy = 1.00×10^2 rad, 1 Krad = 1.00×10^3 rad) gamma rays from ^{60}Co source in the radiation facility at the Korea Atomic Energy Research Institute, Daejeon, Korea, and then cultured on AA selection media with 250 mol 5MT or 1 mM AEC for 2 weeks, respectively. The cells were collected by filtration on miracloth and transferred to the same media solidified with 1% (W/V) phytagel for 40 days. Each of the selected cell lines was respectively transferred to the AA solid media with 500 mol 5MT or 2 mM AEC concentration that completely restrained the growth of the control cells in previous experiments for 5MT or AEC inhibition effects, for 40 days with divided into 5 to 10 mg fresh weight pieces.

Anthranilate synthase assay

Calli were dispersed by homogenization with one volume of extraction buffer [0.2 M Tris-HCl (pH 7.5), 0.2 mM EDTA, 10 mM magnesium chloride, 10 mM glutamine, 60% glycerol, 10 mM dithiothreitol, and 100 mM HEPES] and 2 mg polyvinylpolypyrrolidone per 1 g fresh weight of cells. After removal of cell debris by centrifugation for 10 min at 35,000 g (0°C), one volume of supernatant was mixed with two volumes of saturated ammonium sulfate in water, and centrifuged as described above. The resulting pellet was redissolved in a 0.5 mL extraction buffer and used in the anthranilate synthase assay. Anthranilate synthase activity was measured by the chorismate-dependent production of anthranilate. The resuspended ammonium sulfate pellet was combined with a 1.5 mL of buffer [50 mM HEPES, (pH 7.5), 0.05 mM EDTA, 2 mM magnesium chloride, 10 mM glutamine, 2 mM dithiothreitol, and 5% glycerol], and a 0.2 ml of substrate solution [100 mol chorismate, 15 mM magnesium chloride, and 80 mM glutamine, pH 6.0]. Anthranilate was quantified fluorometrically at 340 nm (exitation) and 400 nm (emission) using Aminco-Bowman (SLM-Aminco, Urbana, Ill., USA) spectrofluorimeter. Formation of anthranilate was followed by continuous fluorometric monitoring of the reaction mixture at 30°C with stepwise addition (0, 5, 10, 50, and 100 mol) of small volumes of stock solution (5 mM) of tryptophan, allowing the reaction to stabilize between each addition.

Aspartate kinase assay

Aspartate kinase enzyme was assayed by modification of the method of Negrutiu et al. (1984). Cells were harvested by vacuum filtration on miracloth and were homogenized by a motor and pestle with one volume of 100 mM potassium phosphate buffer, pH 8.0, containing 1 mM EDTA, 10 mM 2-mercaptoethanol, 10 mM diethylcarbamate, and 20% (V/V) glycerol. For aspartate kinase assay, the extraction mixture was added with 1 mM threonine, and centrifuged for 10 min at 12,000 rpm. One volume of supernatant was mixed with two volumes of ammonium sulfate, saturated at room temperature in water, and centrifuged as mentioned before. The resulting pellet was redissolved in a small volume 50 mM phosphate buffer, pH 7.5, containing 1 mM EDTA, 2 mM 2-mercaptoethanol, and 10 % (V/V) glycerol. All procedures were conducted at 4°C. Protein concentrations were determined using a protein dye-binding assay kit (Bio-Rad Laboratories, Hercules, CA).

Aspartate kinase activity was determined by colorimetric dosage of aspartly hydroxamate with $FeCl_3$ reagent (Bryan et al. 1970). The assay mixture was contained 10 mM Mg-ATP, 0.5 mM hydroxylamine and 100 mM K-aspartate.

Free amino acid analysis

The free amino acid contents within seeds of resistant plants were measured by the Pico-Tag method (Waters) (Tarr 1986). Free amino acids were extracted by homogenizing a 30 mg fresh weight seed with a mortar and pestle. Homogenate was dried under vacuum and redried completely after adding 10 L of MWT (methanol : water : triethylamine, 2/2/1, V/V/V) under vacuum. Derivatized free amino acids by 20 μL of MWTP (methanol : water : TAE : phenylisothiocyanate, 7/1/1/1, V/V/V/V) were applied to a 30 cm Pico-Tag Free Amino Acid Analysis column equilibrated with 140 mM sodium acetate (6% acetonitrile) and equipped with a Waters HPLC system, and eluted with a linear gradient composed by 60% acetonitrile (0, 14, 20, 46, and 100%) at a flow rate of 1 mL/min at 46°C. Absorbance was measured at 254 nm.

Results and Discussion

5-Methyltryptophan (5MT) resistance

Selection of 5MT resistance cell lines

Anther culture was used in these experiments because microspore-derived callus grows more rapidly during tissue increase and maintains its morphogenetic potential longer than callus derived from mature embryos (Schaeffer and Sharpe 1981). To screen for 5MT resistance, 100 mg fresh weight of late log phase cells were inoculated into 5 mL of liquid AA medium (Toriyama and Hinata 1985) with over a wide range of 5MT concentrations and the fresh weight was determined after

4 weeks when the control without 5MT approached the stationary phase. The I_{50} (inhibitor concentration at which cell growth is inhibited by 50%) could be determined at 100 μM 5MT and concentration 375 mol or over completely inhibited the cell growth with amending calli's color brownish (Figure 2A). But, for the selection of accurate 5MT resistant cells, it was finally determined in this study, that the optimal 5MT concentration for selection of resistant cell lines is 250 μM in rice anther culture (Figure 1A, indicated by arrow). This concentration was similar to 300 μM 5MT, normal inhibitory concentration used for selection from rice embryo culture reported by Wakasa and Widholm (1987), and 50 mg/L (about 230 mol) 5MT for selection from anther derived callus of rice (Lee et al. 1992).

For the purpose of increasing the selection efficiency, the cells were irradiated with gamma rays. Practically, only one cell line resistant to 5MT was selected from non-mutagenized populations and became extinct during several subcultures not long after (Figure 2B). The 5MT resistance cells were propagated through prosperous cell divisions, on the other hand, sensitive cells were dead without any divisions (Figure 2C and D). Although, it is possible to select mutants with high essential amino acid content by somaclonal variation, mutant frequencies are often lower in plant systems, in part, by cell-to-cell interactions in the most ideal suspension culture systems, as well as by the presence of multiple gene copies located on different chromosomes, and by inefficient recovery techniques (Schaeffer and Sharpe 1983). Widholm (1977) reported that the frequency of carrot cell resistant to 5MT could be increased 10 to 20 fold by treating the cells with EMS or UV light. Jacobsen et al. (1985) tried N-ethyl-N-nitroso-urea (ENU) for selection of 5MT resistant cell lines of a dihaploid potato. Jensen (1991) successfully selected barley mutants with an increased content of lysine through treatment of gamma rays

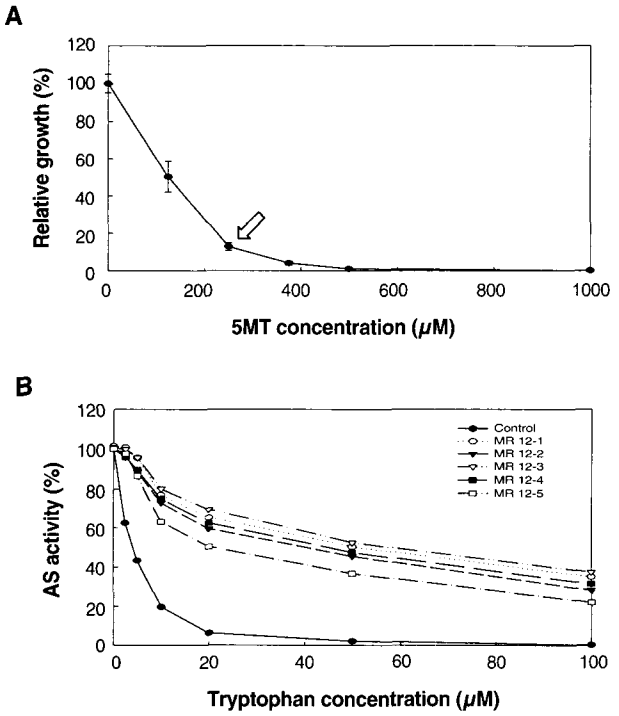

Figure 1. Growth inhibitions of control rice microspore-derived cells on media containing various concentrations of 5MT and the kinetic studies of anthranilate synthase in control and 5MT resistant cell lines. (A) Non-tolerant calli were cultured on 5MT containing media for 4 weeks after which fresh weights were measured. The fresh weight of callus grown on the media without 5MT was 146.4 mg, and was considered as 100%. Arrow indicates the 5MT concentration for selection of resistant cell lines. (B) Inhibition of partially purified anthranilate synthase activity from suspension cultures by tryptophan.

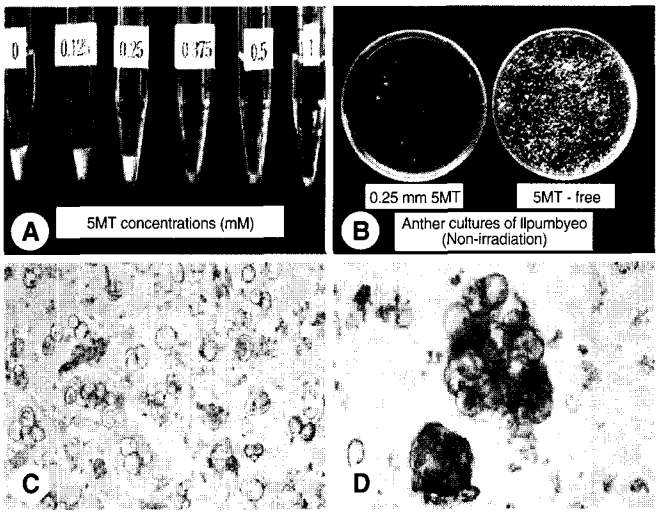

Figure 2. Growth of Ilpumbyeo anther cultures on AA liquid media supplemented with various 5MT concentrations (A) and solid media with or without 5MT (B), and microphotograph of microspore-derived 5MT-sensitive (C) and resistant (D) cells from rice anther culture (× 400). States in 14 days after inoculation of late log phase cells into the 5 mL liquid AA medium.

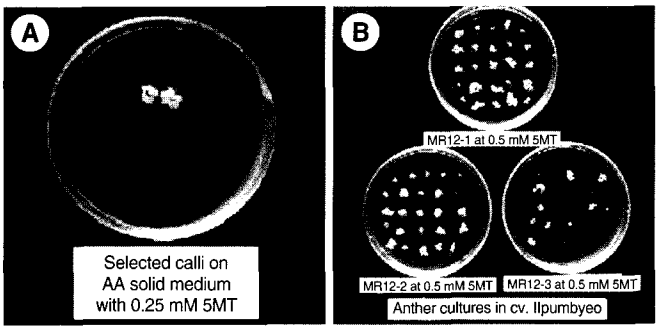

Figure 3. Selection of 5MT-resistant microspore-derived cells from rice anther cultures. (A) 5MT resistant cells selected from AA selection medium containing 250 μM 5MT after irradiation with 120 Gy gamma rays. (B) Re-screening of microspore-derived 5MT-resistant cell lines selected from 250 μM 5MT at 500 μM 5MT.

and chemical mutagens such as EMS and NaN_3.

Thirteen cell lines out of about 1×10^7 cells were selected from 120 Gy irradiation treatment on the AA solid selection medium containing 250 μM 5MT for 40 days and designated as MR12-1 to MR12-13 in the order of their appearance (Table 1, Figure 3A). The first number is the radiation dose (1 Krad = 10 Gy) of gamma rays and the second is the number of the cell line. In order to verify the stability of 5MT resistance in irradiated cell lines, they were divided into 5 to 10 mg fresh weight pieces, and the selected cell lines were transferred onto the same medium containing 500 μM 5MT for 40 days before counting resistant calli. The selection conditions appear to be very stringent as no false positives were observed. Except MR12-3 (65.1%), the survival rate of 5MT resistant cell lines was over 70%, while the control cells showed little or no growth at this concentration (data not shown). These results indicated that 5MT resistance trait was safely maintained at twice the 5MT concentration (Table 1, Figure 3B). It has been reported that the concentration of 5MT required to reduce growth by 50%, was about 100 times higher for resistant cells than for normal cells (Widholm, 1977).

Anthranilate synthase assay

To determine whether the anthranilate synthase enzymes of 5MT resistant cell lines were altered in sensitivity by tryptophan feedback inhibition, anthranilate synthase enzyme activities in control and 5MT resistant cell lines, MR12-1 to MR12-5, were measured in the presence of increasing concentration of tryptophan. Anthranilate synthase activity of all five 5MT resistant cell lines tested was less sensitive to tryptophan inhibition than that from the control cells (Figure 1B). For the resistant cell lines, anthranilate synthase enzymes required about 6.3 to 18.5 times greater concentration of either inhibitor in order to be inhibited to the same extent as the control cell enzymes. The apparent *Ki* values for tryptophan, estimated from the tryptophan concentration causing 50% inhibition, were 65, 48, 74, 55, and 25 μM for MR12-1 to MR12-5, respectively, and 4 mol for the control cell. These differences were larger than those found with anthranilate synthase activity in normal and 5MT resistant carrot cells where the *Ki* values were 3.3 and 17 μM, respectively (Widholm 1972).

In most cases, the resistance of the cell lines was due to an altered anthranilate synthase enzyme, the key feedback control enzyme in the tryptophan biosynthetic branch of the shikimate pathway, which was less sensitive to inhibition by 5MT (Widholm 1973). This resistance mechanism led to the increase of corresponding free tryptophan level *in vivo*. The altered anthranilate synthase enzymes in the 5MT resistant cell lines were confirmed through the above kinetic studies of the anthranilate synthase enzyme.

Table 1. Selection of 5MT resistant microspore-derived cell lines in AA medium supplemented with 250 μM 5MT and confirmation as resistants by a second transfer on 500 μM 5MT.

Radiation Dose	Selection frequency of 5MT resistant cell	Selected lines at 250 μmol 5MT	Survival rates of selected cell lines at 500 μmol 5MT (%)	Resistance or Sensitive
120 Gy	13×10^{-7} cells	MR12- 1	92/101 (91.1)	R
		MR12- 2	86/101 (81.2)	R
		MR12- 3	67/103 (65.1)	R
		MR12- 4	83/101 (82.2)	R
		MR12- 5	85/100 (85.0)	R
		MR12- 6	76/100 (76.0)	R
		MR12- 9	76/100 (76.0)	R
		MR12-10	83/100 (83.0)	R
		MR12-11	43/50 (86.0)	R
		MR12-12	12/15 (80.0)	R
		MR12-13	9/10 (90.0)	R

Analysis of free amino acids

Five cell lines (MR12-1 to MR12-5) out of 13 selected 5MT resistant cell lines were analyzed for free amino acid contents. Analysis of free amino acids showed a 7.4 to 46.6 times higher levels in the resistant lines than that in the control cultures. As compared with other selected lines, tryptophan levels in MR12-3 and MR12-4 were 23.2 and 16.3 times greater than the control. Free phenylalanine and tyrosine levels involved in the shikimate pathway in company with tryptophan in MR12-3 and MR12-4 were also 28.1 and 22.5 times as great as the control cells, respectively (Table 2). These phenylalanine and tyrosine increases could be due to the stimulation of the phenylalanine and tyrosine feedback control enzyme, chorismate mutase, by tryptophan (Gilchrist and Kosuge 1980). Wakasa and Widholm (1987) reported that the mean levels of free tryptophan and phenylalanine in the 5MT resistant rice calli were respectively 87 times and 8.4 times higher than in the sensitive lines.

Free amino acids in the aspartate pathway (aspartate, asparagine, methionine, threonine, lysine, and isoleusine) were also significantly increased. Lysine level that is remarkably low amino acid in rice was 59.5, 14.1, 105.9, 78.9, and 74.2 times greater in MR12-1 to MR12-5 than that in the control culture, respectively (Table 2).

Levels of the free amino acids in 5MT resistant cell lines were increased. Alanine level whose metabolic precursor is pyruvate was increased remarkably from 61.7 nmol for the control to 3442.7, 8691.7, 3364.6, and 5995.7 nmol per g fresh weight for MR12-1, MR12-3, MR12-4, and MR12-5, respectively. These results indicated that additional factors also might be affecting the levels of other amino acids, with those carrying

Table 2. Free amino acid profiles of control and 5MT resistant cultures.

Amino Acid	Contents in 5MT resistant cell lines (n mole g^{-1} f.w.$^{-1}$)					
	Control	MR12-1	MR12-2	MR12-3	MR12-4	MR12-5
Cys	24.31	184.70	60.00	440.69	393.55	321.52
Asp	31.10	851.35	311.08	2051.68	1028.60	987.00
Glu	88.92	1892.79	223.39	2536.84	1910.13	2315.82
Asn	37.49	456.44	975.75	55.11	1114.85	705.26
Ser	34.40	842.44	588.20	2618.80	993.91	1132.79
Gln	19.59	530.52	170.83	605.62	419.25	720.85
Gly	41.80	623.05	292.75	935.02	695.64	998.70
His*	59.06	769.15	289.67	303.39	787.86	1658.64
Arg	19.93	765.95	95.50	2159.71	1297.51	1517.59
Thr*	26.33	707.93	248.10	702.52	689.61	837.02
Ala	61.74	3442.68	308.94	8691.73	3364.64	5995.74
Pro	46.34	751.20	153.37	1031.64	575.05	1344.36
Tyr	14.39	263.07	125.07	576.06	450.48	295.90
Val*	31.25	731.94	246.54	1248.64	909.45	1012.46
Met*	7.18	314.27	60.54	837.18	529.89	407.26
Ile*	16.67	350.93	68.66	752.16	544.73	437.99
Leu*	33.43	599.90	65.91	2057.84	1228.55	504.94
Phe*	29.58	354.28	249.95	748.52	652.61	384.79
Trp*	18.49	93.15	60.78	428.19	301.30	87.17
Lys*	14.09	838.13	199.22	1491.69	1111.95	1045.68
Total	650.26	15363.78	4794.26	30273.06	18999.56	22711.51
Ratio[a]	1.00	23.63	7.37	46.56	29.22	34.93

*Essential amino acid.
[a]Ratio of MR12 lines to control cells.

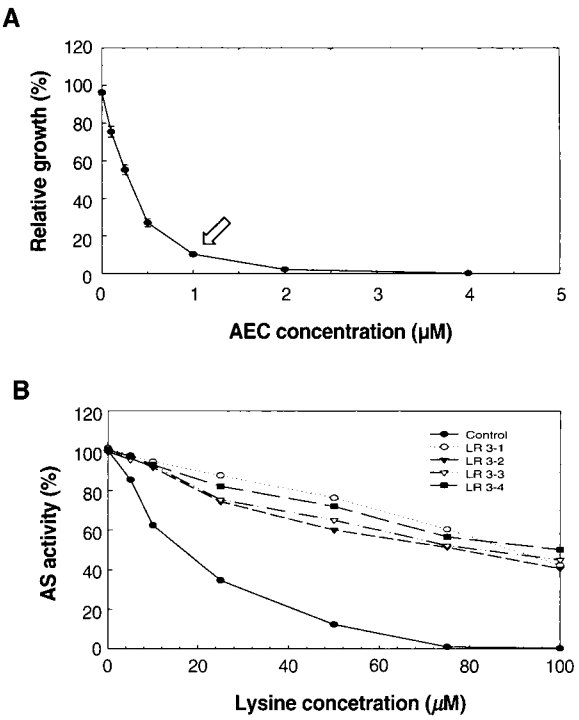

Figure 4. Growth inhibitions of rice microspore-derived cells on media containing various concentrations of AEC and the kinetic studies of aspartate kinase in control and AEC resistant cell lines. (A) Non-tolerant calli were cultured on AEC containing media for 4 weeks after which fresh weights were measured. The fresh weight of callus grown on the media without AEC was 127.3 mg, and was considered as 100%. Arrow indicates the AEC concentration for selection of resistant cell lines. (B) Inhibition of partially purified aspartate kinase activity from suspension cultures by AEC.

5MT resistance being higher than the control. Considering essential amino acid levels, analyzed 5MT resistant lines were 6.3 (MR12-2) to 36.3 (MR12-3) times higher than the control culture (Table 2).

S-(2-Aminoethyl)-L-Cysteine (AEC) resistance

Selection of AEC resistant cell lines

The selection of AEC resistant microspore-derived cell lines was done with rice anther-derived cells in an attempt to select lysine, which is considered to be deficient in rice, overproducing cultures. In the investigation of the sensitivity to AEC inhibition, the I_{50} could be determined at 0.25 mM AEC, and the growth of the control cells was completely inhibited at 1 mM concentration. A four-fold higher concentration was needed to reach equal inhibition levels to the 5MT case, considering the concentration of resistance selection (Figure 4A).

The microspore-derived cells were irradiated with 30 Gy gamma rays and subjected to selection against 1 mM AEC, which is completely inhibitory to the growth of the control cells, approximately two weeks after the initiation of the cultures. Subsequently, they were incubated on AA solid medium containing 1 mM AEC.

Four AEC resistant cell lines were isolated from the transferred cells with the selection frequency of 4×10^{-6} and designated as LR3-1 to LR3-4. The first number in Table 3 is the radiation dose (Krad) of gamma rays and the second number is the number of the selected cell line. The selected calli resistant to AEC had several unique characteristics to grow more rapidly and to be more friable than the control calli without inhibitor. These morphological characteristics were similar to AEC resistant rice callus reported by Schaeffer (1981).

The resistant calli were subcultured twice at 4 weeks interval for the propagation and then plated on 2 mM AEC media by dividing into 5 to 10 mg fresh weight pieces for further confirmation as resistants. As the survival rates of them were from 77.5% (LR3-2) to 87.5% (LR3-4), AEC resistance trait was successfully maintained in all AEC resistant cell lines at twice the AEC concentration (Table 3) and thus, the survived calli were used for aspartate kinase kinetic studies and free amino acid analysis. Schaeffer and Sharpe (1981) selected rice callus resistant to 1 mM AEC that is inhibitory to the growth of control

Table 3. Selection of AEC resistant cell lines in AA medium supplemented with 1 mM AEC and confirmation as resistants by a second transfer on 2 mM AEC.

Radiation dose	Selection frequency of AEC resistant cell	Selected lines at 1 mM AEC	Survival rates of selected cell lines at 2 mM AEC (%)	Resistance or Sensitive
30 Gy	4×10^{-6}cells	LR3-1	102/120 (85.0)	R
		LR3-2	86/111 (77.5)	R
		LR3-3	95/121 (78.5)	R
		LR3-4	105/120 (87.5)	R

rice callus, and recovered 34 lines after three subcultures.

Aspartate kinase assay

The aspartate kinase enzyme activities in both control and AEC resistant cell lines, LR3-1 to LR3-4, were measured stepwise addition of feedback inhibitor, lysine. As shown in Figure 4B, the enzyme of the AEC resistant cell lines was more resistant to lysine inhibition. The activity of aspartate kinase was inhibited to 50% in the control cells when 16 mol lysine was added to their extracts. Extracts from AEC resistant cell lines required more than five times higher concentration of lysine to cause equal inhibition. The apparent *Ki* values for lysine were about 90, 75, 85, and 100 mol for LR3-1 to LR3-4, respectively. These results showed that the AEC resistance mechanism was on account of the alteration of target enzyme by a feedback inhibitor, lysine. Negrutiu et al. (1984) isolated 10 to 20-fold lysine overproducing mutagenized protoplasts from diploid *Nicotiana sylvestris*, but aspartate kinase enzyme was unaltered in the mutants although a feedback insensitive form of DHPS was determined.

Analysis of free amino acids

Free amino acid composition was analyzed in four AEC resistant cell lines. The free amino acid profiles of the AEC resistant cell lines revealed several interesting patterns, some of which were apparently unrelated to the predictions based on the selection scheme and others appeared related. Although total free amino acids were increased respectively 9.1, 1.8, 2.9, and 3.4 times in LR3-1 to LR3-4 than that in the control cells, the most striking increases in the resistant lines occurred in aspartate, asparagine, and methionine in the aspartate pathway (Table 4). These results implied that AEC resistance in the selected cell lines by an altered feedback regulation might lead to the accumulation of one or more of the end-product amino acids. In absolute values the amount of lysine per g fresh weight was increased also from 11.72-16.46 nmole (the control) to 181.75, 30.44, 46.76, and 52.73 nmole in LR3-1, LR3-2, LR3-3, and LR3-4, respectively (Table 4).

In addition to the free amino acids in the aspartate pathway, free serine level, its molecular precursor is 3-phosphoglycerate, was significantly increased 3.14, 5.23, and 6.17 times in LR3-2, LR3-3, and LR3-4, respectively, and alanine level, its molecular precursor is pyruvate, was increased from 61.74 nmole to 1657.65 nmole per g fresh weight (approximately 26.9 times) in LR3-1 (Table 4). These results showed likewise 5MT that additional factors also seemed to affect the levels of other amino acids with carrying AEC resistance, although free amino acid contents in the AEC resistant cell lines were lower than those in 5MT resistant cell lines. Chaleff and Carlson (1974) were able to select AEC resistant rice cells containing increased levels of protein as well as about twice the free lysine levels of the control cells. The selected cells also contained about three times higher free methionine levels and four and more times higher free isoleusine levels.

The cell lines which were resistant to growth inhibition by tryptophan and lysine analogs were selected. In 5MT and AEC resistant cell lines, the resistant traits were usually stable. The natural free amino acids were accumulated 7.4 to 34.9 times and 1.8 to 9.1 times higher than the control. In the case of lines resistant to 5MT and AEC, the control enzymes in the tryptophan and lysine biosynthetic pathways, respectively, altered

Table 4. Free amino acid profiles of control and AEC resistant anther cultures.

Amino Acid	Contents in AEC resistant cell lines (n mole g^{-1} fw^{-1})				
	Control	LR3-1	LR3-2	LR3-3	LR3-4
Cys	24.31	104.42	27.24	37.98	73.06
Asp	31.10	590.53	53.26	43.95	57.06
Glu	88.92	379.20	125.88	164.61	282.67
Asn	37.49	636.81	72.97	274.63	333.05
Ser	34.40	179.45	108.17	179.93	212.25
Gln	19.59	177.46	34.49	48.72	67.47
Gly	41.80	62.02	118.14	197.39	193.94
His*	59.06	192.16	109.50	160.71	163.82
Arg	19.93	334.07	45.03	35.76	36.99
Thr*	26.33	110.62	72.26	93.58	90.75
Ala	61.74	1657.65	79.80	66.91	100.47
Pro	46.34	198.48	47.15	45.99	75.54
Tyr	14.39	106.21	33.56	65.69	52.90
Val*	31.25	206.75	48.18	85.05	94.14
Met*	7.18	173.34	20.75	34.67	29.58
Ile*	16.67	117.73	19.29	33.84	35.04
Leu*	33.43	324.71	36.01	52.20	55.78
Phe*	29.58	131.34	60.35	162.62	169.18
Trp*	18.49	79.82	20.35	52.14	39.97
Lys*	14.09	181.75	30.44	46.76	52.73
Total	650.26	5944.51	1162.83	1883.13	2216.37
Ratio[a]	1.00	9.06	1.77	2.87	3.38

*Essential amino acid.
[a]Ratio of LR3 lines to control cell.

feedback control properties that apparently allow the overproduction of the end-product amino acids. Thus, a rice anther culture system established in this study could be employed as an efficient method for rice breeding.

The *in vitro* selection of cells provides the sources of specific variability, which are required for a better understanding of genetic and regulatory phenomena, and the 5MT or AEC resistant cell lines may be useful in studies of amino acid biosynthesis.

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References

Brotherton JE, Schechter S, Ranch JP, Widholm JM (1996) Inheritance and stability of 5-methyltryptophan resistance in *Datura innoxia* selected *in vitro.* Plant Cell Physiol 37: 389-394

Bryan PA, Cawley D, Brunner CE, Bryan JK (1970) Isolation and characterization of a lysine-sensitive aspartate kinase from a multicellular plant. Biochem Biophys Res Commun 41: 1211-1218

Carlson PS (1970) Induction and isolation of auxotrophic mutants in somatic cell cultures of *Nicotiana tabacum.* Science 168: 487-489

Chaleff RS, Calson PS (1974) *In vitro* selection for mutants of higher plants. In Ledoux L (ed), Genetic manipulation with plant material, pp 351-357. Plenum Press, New York London

Chu CC, Wang CC, Sun CS, Hsu C, Yin KC, Chu CY, Bi FI (1975) Establishment of an efficient medium for anther culture of rice through comparative experiments on the nitrogen sources. Sci Sin 18: 659-668

Gilchrist DG, Kosuge K (1980) Aromatic amino acid biosynthesis and its regulation. In Miflin BJ (ed), The biochemistry of plants, Vol 5. Amino acids and derivatives, pp 507-531. Academic Press, New York

Jacobsen E, Visser RGF, Wijbrandi J (1985) Penylalanine and tyrosine accumulating cell lines of dihaploid potato selected by resistance to 5-methyltryptophan. Plant Cell Rep 4: 151-154

Jensen J (1991) New high yielding, high lysine mutants in barley. In Plant mutation breeding for crop improvement, Vol 2, pp 31-41. IAEA, Vienna

Kinoshita T, Mori K, Takamure I (1989) Mutagenesis by means of anther culture combined with gamma irradiation. Rice Genet Newsl 6: 139-141

Larkin PJ, Scowcroft WR (1981) Somaclonal variation-a novel source of variability from cell cultures for plant improvement. Theor Appl Genet 60: 197-214

Lee HY, Kameya T (1991) Selection and characterization of a rice mutant resistant to 5-methyltryptophan. Theor Appl Genet 82: 405-408

Lee HY, Kim JH, Kim HJ, Kameya T (1992) Selection and rice cell lines resistant to 5-methyltryptophan in cell cultures. Korean J Plant Tissue Culture 19: 289-294

Miao S, Dunkan DR, Widholm J (1988) Selection of regenerable maize callus cultures resistant 5-methyl-DL-tryptophan, S-2-aminoethyl-L-cysteine and high levels of L-lysine plus L-threonine. Plant Cell Tiss Org Cult 14: 3-14

Negrutiu I, Cattoir-Reynearts A, Verbruggen I, Jacobs M (1984) Lysine overproducer mutants with an altered dihydrodipicolinate synthase from protoplast culture of *Nicotiana sylvestris* (Spegazzini and Comes). Theor Appl Genet 68: 11-20

Ranch JP, Rick S, Brotherton JE, Widholm JM (1983) Expression of 5-methyltryptophan resistance in plants regenerated from resistant cell lines of *Datura innoxia.* Plant Physiol 71: 136-140

Schaeffer GW (1981) Mutations and cell selections: increased protein from regenerated rice tissue cultures. Env Exp Bot 21: 333-345

Schaeffer GW, Sharpe FT (1981) Lysine in seed protein from S-aminoethylcysteine resistant anther-derived cultures of rice. *In Vitro* 17: 345-351

Schaeffer GW, Sharpe FT (1983) Mutations and selection: Genetic variation for improved protein in rice. Genetic engineering, application to agriculture; Invited papers presented at a symposium, pp 237-254, May 16-19, 1982, Beltsville

Szarejko I, Maluszynski M, Polok K, Kilian A (1991) Double haploids in the mutation breeding of selected crops. In Plant mutation breeding for crop improvement, IAEA, Vienna, Vol 2, pp 355-378

Tarr GE (1986) Methods of protein microcharacterization. In Shively JE (ed), pp 155-194. Humana Press, Clifton, NJ

Toriyama K, Hinata K (1985) Cell suspension and protoplast culture in rice. Plant Sci 41: 179-183

Wakasa K, Widholm JM (1987) A 5-methyltryptophan resistant rice mutant, MTR1, selected in tissue culture. Theor Appl Genet 74: 49-54

Widholm JM (1972) Anthranilate synthetase from 5-methyltryptophan susceptible and resistant cultured *Daucus carota* cells. Biochem Biophys Acta 279: 48-57

Widholm JM (1973) Measurement of the five enzymes which convert chorismate to tryptophan in cultured *Daucus carota*

cell extracts. Biochem Biophys Acta 320: 217-226

Widholm JM (1974) Cultured carrot cell mutants: 5-methyltryptophan resistant trait carried from cell to plant and back. Plant Sci Lett 3: 323-330

Widholm JM (1976) Selection and characterization of cultured carrot and tobacco cells resistant to lysine, methionine, and proline analogs. Can J Bot 54: 1523-1529

Widholm JM (1977) Selection and characterization of amino acid analog resistant plant cell culture. Crop Sci 17: 597-600

Zapata FJ, Aldemita RR (1989) Induction of salt tolerance in high-yielding rice varieties through mutagenesis and anther culture. In Maluszynski M (ed), Current options for cereal improvement, pp 193-202. Kluwer Academic Publishers, Dordrecht