The Japanese Society of Plant Physiologists

Plant Cell Physiol. 33(2): 165–170 (1992)
JSPP © 1992

Synthesis of an *S*-(2-Aminoethyl)-L-Cysteine Conjugate in *S*-(2-Aminoethyl)-L-Cysteine-Resistant Adenine-Auxotrophic Cells of *Datura innoxia* Mill

Hitoshi Adachi, Masanori Aso[1], Hiroyoshi Saito[2] and Katsuhisa Furuhashi[3]

Department of Biology, Faculty of Science, Niigata University, Niigata, 950-21 Japan

A line of *S*-(2-aminoethyl)-L-cysteine-resistant adenine-auxotrophic cells (Ad^-AEC^r strain) was isolated from adenine-auxotrophic cells (Ad^- strain) of *Datura innoxia* Mill by a stepwise selection method. Ad^-AEC^r and B1 cells, which were cloned from the original Ad^-AEC^r cells, were able to grow actively on medium that contained 10 mM *S*-(2-aminoethyl)-L-cysteine (AEC), whereas the growth of Ad^- cells ceased completely in the presence of 0.5 mM AEC. The resistant phenotype has been maintained for at least 10 months in culture on medium without AEC.

Levels of free lysine in Ad^-AEC^r and B1 cells were similar to that in Ad^- cells. By contrast, the level of free AEC in Ad^-AEC^r cells was 10-fold lower than in Ad^- cells and no free AEC was detectable in B1 cells. However, acid hydrolysis of extracts from Ad^-AEC^r and B1 cells resulted in a remarkable increase in levels of detectable AEC. This result indicates that conjugated AEC is synthesized and accumulated in the AEC-resistant cells. The level of the AEC conjugate in B1 cells increased with increases in the concentration of AEC in the culture medium, while intracellular levels of AEC were so low as not to be detectable in the case of cells grown on medium supplemented with AEC at less than 1 mM. The AEC conjugate was also detected in Ad^- cells, but at lower levels than in the AEC-resistant cells. In addition, AEC was found to be incorporated into soluble proteins in Ad^- cells.

These results suggest that the resistance of AEC-resistant cells of *Datura innoxia* is accomplished via acceleration of the synthesis of the AEC conjugate which prevents any increase in intracellular levels of free AEC.

Key words: Aminoethyl cysteine-resistant adenine-requiring cells — Conjugated aminoethyl cysteine — *Datura innoxia* — Stepwise selection.

A number of cell lines that are resistant to inhibition of growth by amino acid analogs have been selected from plant cell cultures for use in studies of plant metabolism and genetic manipulations (Flick 1983, Maliga 1984). Biochemical analysis of the mechanism of resistance may furnish important information relevant to potential utilization of the resistant trait as a selectable marker of hybrid cells after attempts at somatic hybridization. However, a clear explanation of the mechanism of resistance has not been obtained in many cases.

Since Widholm (1976) selected a tobacco cell line that was resistant to AEC several lines of AEC-resistant cells and plants have been isolated (Widholm 1978, Bright et al. 1979, Matthews et al. 1980, Jacobsen 1986, Kumpaisal et al. 1988, Miao et al. 1988, Redway and Vasil 1990). Matthews et al. (1980) reported that uptake of [^{14}C]lysine from the culture medium was greatly reduced in a line of AEC-resistant carrot cells. Similar results have been obtained with barley plants (Bright et al. 1979), maize callus (Miao et al. 1988) and wheat cell cultures (Kumpaisal et al. 1988). Therefore, it has been generally accepted that reduced uptake of AEC is the most important mechanism responsible for resistance in AEC-resistant cells. However, it is unclear in many cell lines whether the decreased uptake can

Abbreviations: AEC, *S*-(2-aminoethyl)-L-cysteine; TCA, trichloroacetic acid.

[1] Present address: Institute for Biology and Chemistry, Tsumura Co. Ltd., Inashiki, Ibaraki, 300-03 Japan.

[2] Present address: North Kanto Shop, Sakata Seed Co. Ltd., Saitama, 347 Japan.

[3] To whom correspondence should be addressed.

NII-Electronic Library Service

The Japanese Society of Plant Physiologists

maintain the intracellular level of AEC below a critical value, because the level of AEC in resistant cells has only been determined in barley and maize.

In a previous study (Furuhashi and Hosaka 1987), we reported the selection of a line of 5-methyltryptophan-resistant adenine-auxotrophic cells derived from *Datura innoxia* Mill. Recently, we isolated a line of AEC-resistant adenine-auxotrophic cells (Ad^-AEC^r) from the a culture of the same adenine-auxotrophic cells (Ad^- strain). In the present report, we present evidence for the accumulation intracellularly of a conjugated form of AEC, which was discovered during the course of the investigation, in Ad^-AEC^r and B1 cells, as well as evidence for the incorporation of AEC into soluble proteins in Ad^- cells.

Materials and Methods

Plant materials—Ad^- cell line: The adenine auxotrophic cell line of *Datura innoxia* Mill (Ad^- cell line) used in this experiment was originally isolated by King et al. (1980) and was made available to our laboratory in 1982. The strain has been subcultured and maintained on B5 solid medium supplemented with 80 mg liter^{-1} of adenine at 26±1°C in the dark.

Ad^-AEC^r cell line: Selection of AEC-resistant cells was carried out in a step-wise manner. Cells that could grow in the presence of 0.3 mM of AEC and 80 mg liter^{-1} of adenine were subcultured on the same medium for two months (two transfers) and then transferred to medium that contained 0.5 mM AEC. After selection by culturing on the same medium for two to three months, adapted cells were transferred to media that contained higher and higher concentrations of AEC. Finally, cells adapted to the presence of 4 mM AEC were isolated and maintained on B5 solid medium that contained 4 mM AEC and 80 mg liter^{-1} of adenine at 26±1°C in the dark.

B1 cloned cell line: Ad^-AEC^r cells that had been cultured on medium without AEC for two passages (4 weeks) were used for cloning. Small aggregates of Ad^-AEC^r cells were selected using a stainless-steel mesh (25 mesh) and incubated in the following solution of enzymes (pH 5.5) at 26°C for 12 hours. The solution consisted of 2.0% (w/v) cellulase RS, 0.2% (w/v) Driselase, 0.1% (w/v) Pectolyase, 0.6 M of glycine-betaine, 3.0% (w/v) sucrose and the mineral salts of V-47 medium (Binding 1974). The incubated solution was filtered through a stainless-steel mesh (300 mesh) to remove undigested aggregates of cells. The protoplasts were sedimented by centrifugation at 100×*g* for 5 min. The sedimented protoplasts were washed twice with potassium phosphate buffer (pH 5.5, 10 mM) that contained 0.5 M mannitol (washing buffer). The preparation of protoplasts suspended in the washing buffer was loaded on a 17% (w/v) solution of sucrose, and centrifuged at 100×*g* for 5 min. The purified protoplasts were collected from the interface between the washing buffer and the 17% sucrose solution and then they were suspended in V-47 medium supplemented with 10 mg liter^{-1} each of adenine and cysteine.

Culture of protoplasts was carried out according to the method of Kyozuka et al. (1987). About 2×10^4 protoplasts were embedded in 1 ml of 1% agarose gel. One fourth of the agarose gel was placed in 3 ml of V-47 liquid medium supplemented with 10 mg liter^{-1} each of adenine and cysteine in a Petridish and incubated with a nurse callus (Ad^-AEC^r) at 26±1°C under diffuse light. On the third day after initiation of the culture, 2 ml of B5 medium supplemented with 80 mg liter^{-1} of adenine and 10 mg liter^{-1} of cysteine were added to supply nutrients and to reduce the osmotic pressure of the medium. In this culture 15% of the protoplasts underwent cell division and a considerable number of colonies grew up from the dividing cells. Several colonies which were growing rapidly were picked and subcultured on B5 agar medium that contained 80 mg liter^{-1} of adenine. The B1 clone, the most rapidly growing colony on AEC medium, was isolated from these colonies.

Culture medium—Callus cells were cultured on 25 ml of the solidified culture medium described below, in 50-ml Erlenmyer flasks. The culture medium consisted of B5 mineral salts, the vitamins found in B5 medium, 80 mg liter^{-1} of adenine, 10^{-6} M 2,4-D, 20 g liter^{-1} of sucrose, 9 g liter^{-1} of agar (Wako, Japan) and AEC at various concentrations. The medium was adjusted to pH 5.6 with NaOH and autoclaved at 1 kg cm^{-2} at 120°C for 15 min.

Determination of intracellular levels of free amino acids and the AEC conjugate—Harvested cells were washed with potassium phosphate buffer (20 mM, pH 5.5) and moisture was removed. One gram of cells was extracted with 100 ml of 80% ethanol overnight. After collection of the first extract, the cells were homogenized with 20 ml of 80% ethanol and a second extraction was performed. The extracts were pooled and concentrated under a vacuum. The concentrated aqueous solution (ca. 10 ml) was treated with diethylether to remove lipids substances. The aqueous fraction was concentrated to a small volume. Half of this solution was used for quantitation of basic amino acids. The remainder was passed through a column of anion-exchanger resin (Dowexl-X8, CH_3COO^--form) for adsorbing dicarboxylic amino acids and the column was washed with distilled water. The eluate was concentrated to 1.0 ml and hydrolyzed in 10% HCl at 95°C for 2 hours to convert glutamine and asparagine into glutamic acid and aspartic acid, respectively. After the HCl had been removed by evaporation under a vacuum, the concentrated aqueous solution was used for the quantitation of neutral amino acids. Dicarboxylic amino acids adsorbed to the Dowex column were eluted with 2 M acetic acid and the eluate was concentrated to a small volume for quantitation

NII-Electronic Library Service

The Japanese Society of Plant Physiologists

of glutamic acid and aspartic acid.

Amino acids were quantitated with an automatic amino-acid analyzer (KLA-3B; Hitachi, Japan). To separate AEC from lysine, basic amino acids were fractionated on a long column (0.9 cm i.d. × 19 cm) with a buffer (pH 6.10±0.01) that consisted of 40.2 g of sodium citrate $2H_2O$, 1 ml of phenol, 5 ml of benzyl alcohol and 2 ml of concentrated HCl in 1 liter of aqueous solution.

The AEC conjugate was hydrolyzed by heating in 10% HCl at 95°C for 2 hours.

Quantitation of AEC incorporated into soluble proteins—Ad^- and B1 cells that had been cultured on medium that contained 0.1 mM or 0.3 mM AEC were homogenized in 50 mM potassium phosphate buffer. Extracted proteins were precipitated with TCA. After washing with 4% TCA solution, the precipitated proteins were further washed 3 times with acetone. Five mg of protein from which acetone had been evaporated were sealed with 6 N HCl in an ampule under a vacuum and hydrolyzed at 110°C for 24 hours.

The AEC content of the protein hydrolysate was determined with an automatic amino-acid analyzer in a manner similar to that described above for the free amino acids.

Results

Resistance of the Ad^-AEC^r strain and the B1 clone to AEC and other amino-acid analogs—The adenine-requiring phenotype of the newly established strain, Ad^-AEC^r, and of the B1 clone remained stable through out the selection process.

The Ad^-AEC^r strain and the B1 clone were found to be resistant to as much as 10 mM AEC, whereas the growth of the Ad^- strain, the parental cells, ceased completely at 0.5 mM AEC (Fig. 1). The Ad^-AEC^r strain was also resistant to δ-hydroxyl lysine but not to 5-methyl-DL-tryptophan or to ethionine (data not shown). The resistant phenotype has been maintained at least for 10 months (20 transfers) in culture on medium without AEC.

Intracellular levels of free amino acids, AEC and conjugated AEC in Ad^-, Ad^-AEC^r and B1 cells—Mutants that are resistant to amino acid analogs sometimes accumulate the corresponding natural amino acid in order to avoid toxic effects of the analog by diluting it below a critical level. Therefore, we examined intracellular levels of free amino acids in Ad^-, Ad^-AEC^r and B1 cells. When Ad^- cells were cultured on medium that contained 1 mM AEC, levels of amides and basic amino acids increased (Table 1). By contrast, no increases in levels of these amino acids were found in Ad^-AEC^r cells. The level of lysine in Ad^-AEC^r and B1 cells was similar to that in Ad^- cells, whereas the level of free AEC in Ad^-AEC^r cells was 10 times lower than that in Ad^- cells (Table 2). However, acid hydrolysis of the preparation for amino acid analysis resulted in an approximately 180-fold increase in the level of AEC in the preparation from Ad^-AEC^r cells (Table 2). These results indicate that an acid-labile conjugate of AEC is synthesized in Ad^-AEC^r cells. The AEC conjugate was also detected in Ad^- cells, but at considerably lower concentrations than in Ad^-AEC^r cells.

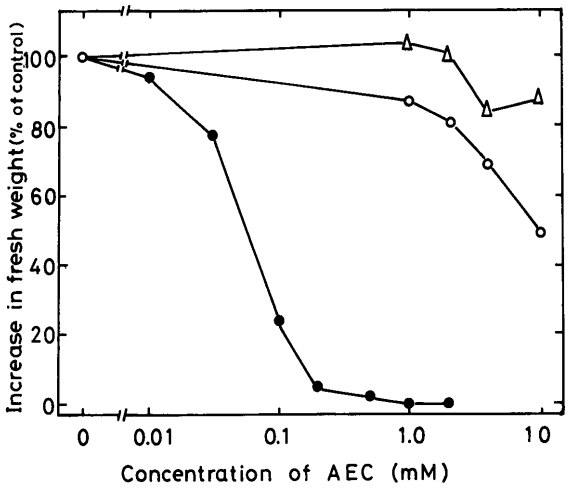

Fig. 1 Effects of AEC on the growth of Ad^-, Ad^-AEC^r and B1 cells. Callus cells (0.2 g) were divided into four clusters and inoculated on 25 ml solidified culture medium in a flask. Cell clusters were harvested after 2 weeks. Average fresh weights of Ad^-, Ad^-AEC^r and B1 cells cultured on medium without AEC were 1.8, 1.6 and 1.8 grams per flask, respectively. Each point represents the average of results from at least 5 flasks. The culture methods are described in Materials and Methods. —●—, Ad^- cell line; —○—, Ad^-AEC^r cell line; —△—, B1 cell line.

In order to obtain more information about the synthesis of the AEC conjugate, details of the accumulation of free AEC and of the AEC conjugate were examined. When Ad^-AEC^r cells were cultured on medium that contained 1 mM AEC, the intracellular concentration of free AEC always remained low and no AEC could be detected beyond the 10th day after the initiation of the culture (Fig. 2). In B1 cells, no free AEC could be detected during the entire culture period. By contrast, levels of free AEC in Ad^- cells increased rapidly and AEC accumulated to levels 10 times higher than those in Ad^-AEC^r cells (Fig. 2). The level of the AEC conjugate in Ad^-AEC^r cells increased markedly from the first day and then decreased gradually from the tenth day. Ad^- cells contained a lower concentration of the AEC conjugate than that in Ad^-AEC^r cells.

Intracellular accumulation of the AEC conjugate was affected by the concentration of AEC in the medium. The level of the AEC conjugate in B1 cells increased with increases in the concentration of AEC in the culture medium (Fig. 3). However, the level of free AEC was suppressed to

NII-Electronic Library Service

The Japanese Society of Plant Physiologists

Table 1 Levels of free amino acids in Ad^-, Ad^-AEC^r and B1 cells

Amino acid	Ad^- cells		Ad^-AEC^r cells		B1 cells
	B5A medium[a]	B5A (+1 mM AEC)	B5A medium	B5A (+4 mM AEC)	B5A (+4 mM AEC)
Asp	317 (5.7)[b]	847 (11.8)	342 (8.0)	449 (11.5)	303 (7.0)
Asn	274 (4.9)	429 (6.0)	407 (9.5)	984 (25.1)	208 (4.8)
Thr	173 (3.1)	123 (1.7)	168 (3.9)	157 (4.0)	230 (5.3)
Ser	659 (11.7)	354 (4.9)	403 (9.4)	224 (5.7)	362 (8.3)
Glu	1,257 (22.4)	1,349 (18.7)	686 (16.0)	645 (16.5)	794 (18.3)
Gln	684 (12.2)	1,510 (21.0)	481 (11.2)	240 (6.1)	387 (8.9)
Pro	61 (1.1)	—[c]	—	—	54 (1.2)
Gly	568 (10.1)	274 (3.8)	204 (4.8)	122 (3.1)	255 (5.9)
Ala	796 (14.2)	635 (8.8)	567 (13.2)	235 (6.0)	416 (9.6)
Cys	—	—	—	—	—
Val	150 (2.7)	222 (3.1)	125 (2.9)	51 (1.3)	113 (2.6)
Met	10 (0.2)	11 (0.2)	10 (0.2)	3 (0.1)	13 (0.3)
Ile	88 (1.6)	135 (1.9)	76 (1.8)	44 (1.1)	55 (1.3)
Leu	146 (2.6)	279 (3.9)	198 (4.6)	205 (5.2)	145 (3.3)
Tyr	78 (1.4)	112 (1.6)	121 (2.8)	87 (2.2)	235 (5.4)
Phe	151 (2.7)	396 (5.5)	237 (5.5)	244 (6.2)	255 (5.9)
His	38 (0.7)	129 (1.8)	69 (1.6)	47 (1.2)	144 (3.3)
Trp	14 (0.2)	46 (0.6)	22 (0.5)	trace	44 (1.0)
Lys	72 (1.3)	200 (2.8)	86 (2.0)	102 (2.6)	176 (4.1)
Arg	74 (1.3)	153 (2.1)	93 (2.2)	76 (1.9)	152 (3.5)
Total	5,610 (100)	7,204 (100)	4,294 (100)	3,915 (100)	4,341 (100)

Cells were cultured on each medium for 6 days and used for amino acid analysis. Levels of amino acids are expressed on a fresh weight basis (nmol (g fr wt)$^{-1}$).

[a] B5A medium: A modified version of B5 medium that contained 80 mg liter^{-1} of adenine.

[b] Percentage of each amino acid relative to the total amino acids.

[c] —, Not detectable.

the point that there was no detectable AEC in cultures grown on medium supplemented with less than 1 mM AEC. Even when cells were grown on medium that contained 10 mM AEC, free AEC at a level of only 80 nmole per gram fresh wt was detected (Fig. 3). However, when Ad^- cells were cultured on medium that contained 10 mM

Table 2 Levels of basic amino acids and AEC in preparations before and after acid hydrolysis of extracts of Ad^- and Ad^-AEC^r cells

	Extract of Ad^- cells		Extract of Ad^-AEC^r cells	
	Before hydrolysis	After hydrolysis	Before hydrolysis	After hydrolysis
His	124	135	58	70
Trp	36	—[a]	18	—
Lys	116	135	145	?[b]
Arg	75	89	110	118
NH_3	1,984	2,852	2,600	3,700
AEC	75	402	8	1,472

Cells cultured on the B5A medium that contained 1 mM AEC for 6 days were used for amino acid analysis. Levels of amino acids are expressed on a fresh weight basis (nmol (g fr wt)$^{-1}$).

[a] —, Not detectable.

[b] ? Lysine content could not be calculated because of the overlap with the very large peak due to AEC on the chromatogram.

NII-Electronic Library Service

The Japanese Society of Plant Physiologists

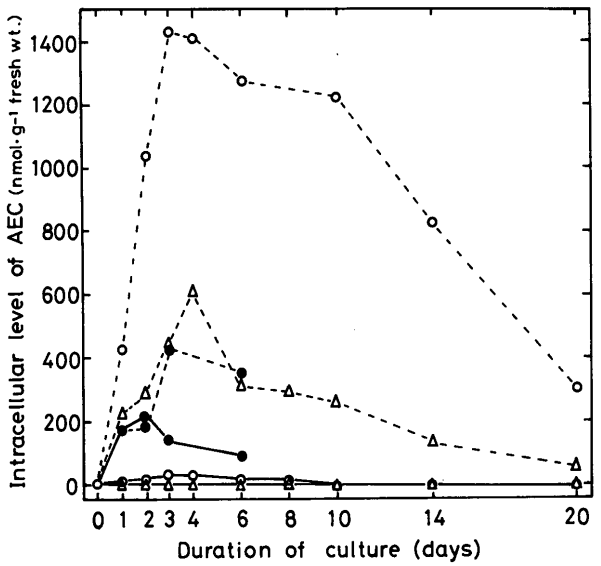

Fig. 2 Accumulation of free and conjugated AEC in Ad^-, Ad^-AEC^r and B1 cells. Callus cells (0.2 g) were inoculated on solidified culture medium that contained 1 mM AEC. Growth of Ad^- cells ceased as a result of necrosis on the fourth day after initiation of the culture. Therefore, determination of levels of AEC was not carried out for cells cultured for more than 6 days. —●—, Free AEC in Ad^- cells; —○—, free AEC in Ad^-AEC^r cells; —△—, free AEC in B1 cells; --●--, conjugated AEC in Ad^- cells; --○--, conjugated AEC in Ad^-AEC^r cells; --△--, conjugated AEC in B1 cells.

AEC, free AEC at 2,150 nmole per gram fresh wt was detected (data not shown).

Incorporation of AEC into soluble proteins in Ad^- and B1 cells—AEC was incorporated into soluble proteins in Ad^- cells (Table 3). The extent of this incorporation increased with increases in the concentration of AEC in the culture medium. However, no incorporation of AEC into soluble proteins was detected in B1 cells, which can sequester free AEC by forming the conjugate.

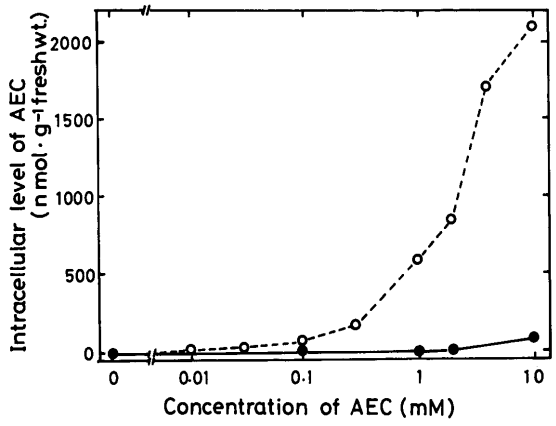

Fig. 3 Increases in intracellular levels of the AEC conjugate with increases in the concentration of AEC in the medium. B1 cells (0.2 g) were inculated on solidified medium and quantitation of free and conjugated AEC was carried out using cells that had been cultured for 3 days. —●—, Free AEC; —○—, conjugated AEC.

Table 3 Incorporation of AEC into soluble proteins in Ad^- and B1 cells

AEC in medium	Ad^- cells (nmol (mg protein)$^{-1}$)	B1 cells (nmol (mg protein)$^{-1}$)
0.1 mM	0.5	nd[a]
0.3 mM	5.9	—[b]

[a] nd, Not determined.
[b] —, Not detectable.

Discussion

Amino acid analogs are toxic as a result of their incorporation into proteins or/and of inhibition of the synthesis of related amino acids (reviewed by Maliga 1980). In the former case, resistance may be acquired via mutations that decrease the rate of uptake or that selectively discriminate against the analog. In the latter case, resistance can result from relaxed control of the amino-acid biosynthetic pathway.

The phenotype of AEC-resistant cells examined to date has been the result of impaired uptake of AEC (Bright et al. 1979, Matthews et al. 1980, Miao et al. 1988, Kumpaisal et al. 1988, 1989). It is apparent that a reduction in the rate of uptake of AEC provides the simplest and most effective mechanism for survival on AEC-containing medium unless AEC is distinguished from its normal cognate amino acid when protein synthesis occurs. However, an example of a different mechanism has been found in *Nicotiana sylvestris*. An AEC-resistant line of cells derived from *Nicotiana sylvestris*, which was isolated by Negritie et al. (1984), has an altered dihydrodipicolinate synthase, and the resistant cells contain free lysine at levels 10- to 20-fold higher than those in controls. These results suggest that the AEC-resistant trait of *Nicotiana sylvestris* is the result of the relaxed control of the biosynthetic pathway to lysine. However, there is no evidence that the lysine deficiency resulted from feeding AEC to the non-resistant parental cells. This relaxed control of the biosynthesis of lysine may be more important for prevention of the incorporation of AEC into proteins than for prevention of a lysine deficiency.

Recently, we isolated a line of AEC-resistant cells from adenine-auxotrophic cells (Ad^- strain) of *Datura innoxia*. In these resistant cells, the level of free AEC is low, while the level of free lysine is similar to that in Ad^- cells

NII-Electronic Library Service

The Japanese Society of Plant Physiologists

which are susceptible to AEC (Table 2). Moreover, a decrease in intracellular levels of free lysine did not occur upon feeding AEC to Ad^- cells even though the proliferation of the cells was inhibited by AEC.

These data led us at first to conclude that the Ad^-AEC^r cells might have acquired resistance by reduceing the rate of uptake of AEC. However, large amounts of AEC were detected by amino acid analysis of the preparation after acid hydrolysis of the cell extract (Table 2). This result indicates that an acid-labile AEC conjugate was synthesized in the cells. The level of the AEC conjugate was far larger than the amount of free AEC taken up by Ad^- cells. Moreover, the results of Figures 2 and 3 indicate that the synthesis of the AEC conjugate contributes to a decrease in the level of intracellular free AEC in Ad^-AEC^r and B1 cells. As shown in Table 3, AEC was incorporated into soluble proteins in Ad^- cells, indicating that *Datura* cells cannot discriminate between AEC and lysine during protein synthesis. Therefore, the removal of intracellular free AEC may be very important for cell survival. The removal of free AEC can be accomplished via prevention of the influx of AEC or via the metabolic conversion of AEC into other compounds that are not incorporated into proteins. The above mentioned data indicate that survival of the AEC-resistant cells derived from *Datura innoxia* depends on the latter mechanism. This mechanism has not previously been reported as an explanation for the resistance to AEC of cells derived from higher plants. It is conceivable that this resistant trait may be more useful than the resistant trait achieved by reduced uptake of AEC when such a trait is used as a selectable marker of hybrid cells in attempts of somatic hybridization.

We identified the AEC conjugate as ε-N-acetyl AEC, and details of this identification will be published at a later data.

This study was supported in part by a Grant-in-Aid for Scientific Research (no. 01304010) from the Ministry of Education, Science and Culture of Japan.

References

Binding, H. (1974) Regeneration von haploiden und diploiden pflanzen aus protoplasten von *Petunia hybrida* L. *Z. Pflanzenphysiol.* 74: 327–356.

Bright, S. W. J., Featherstone, L. C. and Miflin, B. J. (1979) Lysine metabolism in a barley mutant resistant to *S*-(2-aminoethyl) cysteine. *Planta* 146: 629–633.

Flick, C. E. (1983) Isolation of mutants from cell culture. *In* Handbook of Plant Cell Culture. Edited by Evans, D. A., Sharp, W. R., Ammirato, P. V. and Yamada, Y. Vol. 1. pp. 393–442. Macmillan, New York.

Furuhashi, K. and Hosaka, Y. (1987) Selection of 5-methyltryptophan-resistant adenine-auxotrophic cell line of *Datura innoxia* Mill. *Plant Tissue Cult. Lett.* 4: 90–92.

Jacobsen, E. (1986) Isolation, characterization and regeneration of an *S*-(2-aminoethyl) cysteine resistant cell line of dihaploid potato. *J. Plant Physiol.* 123: 307–315.

King, J., Horsh, R. B. and Savage, A. D. (1980) Partial characterization of two stable auxotrophic cell strains of *Datura innoxia* Mill. *Planta* 149: 480–484.

Kumpaisal, R., Hashimoto, T. and Yamada, Y. (1988) Selection and characterization of *S*-(2-aminoethyl)-L-cysteine-resistant wheat cell cultures. *J. Plant Physiol.* 133: 608–614.

Kumpaisal, R., Hashimoto, T. and Yamada, Y. (1989) Uptake of lysine by wild-type and *S*-(2-aminoethyl)-L-cysteine-resistant suspension cultured cell of *Triticum aestivum*. *Plant Cell Physiol.* 30: 1099–1105.

Kyozuka, J., Hayashi, Y. and Shimamoto, K. (1987) High frequency plant regeneration from rice protoplast by novel nurse culture methods. *Mol. Gen. Genet.* 206: 408–413.

Maliga, P. (1984) Isolation and characterization of mutants in plant cell culture. *Annu. Rev. Plant Physiol.* 35: 519–542.

Matthews, B. F., Shye, S. C. H. and Widholm, J. M. (1980) Mechanism of resistance of a selected carrot cell suspension culture to *S*-(2-aminoethyl)-L-cysteine. *Z. Pflanzenphysiol.* 96: 453–463.

Miao, S., Duncan, D. R. and Widholm, J. M. (1988) Selection of regenerable maize callus cultures resistant to 5-methyl-DL-tryptophan, *S*-2-aminoethyl-L-cysteine and high levels of L-lysine plus L-threonine. *Plant Cell Tissue Organ Culture* 14: 3–14.

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

Redway, F. A. and Vasil, I. K. (1990) Selection of *S*-(2-aminoethyl)-L-cysteine tolerance in embryogenic calli and regenerated plants of *Pennisetum purpureum* Schum. *Plant Sci.* 67: 203–209.

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

Widholm, J. M. (1978) Selection and characterization of a *Daucus carota* L. cell line resistant to four amino acid analogs. *J. Exp. Bot.* 29: 1111–1116.

(Received July 31, 1991; Accepted December 22, 1991)

NII-Electronic Library Service