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Selection and Characterization of Ethionine-resistant Alfalfa (*Medicago sativa* L.) Cell Lines

B. Reish, Stanley H. Duke and E.T. Bingham
Department of Agronomy, University of Wisconsin, Madison, Wisconsin (USA)

Summary. Diploid alfalfa (HG2), capable of plant regeneration from tissue culture, was used to select variant cell lines resistant to growth inhibition due to ethionine (an analog of methionine). Approximately 10^7 suspension-cultured cells were mutagenized with methane sulfonic acid ethylester and then plated in solid media containing ethionine. Callus colonies formed on media with 0.02 mM ethionine. Of the 124 cell lines recovered, 91 regenerated plants. After six months growth on media without ethionine, 15 of 110 cell lines of callus grew significantly better than HG2 on 1 mM ethionine. Several ethionine-resistant callus cultures were also resistant to growth inhibition due to the addition of lysine + threonine to the media. High concentrations, relative to unselected HG2 callus, of methionine, cysteine, cystathionine, and glutathione were found in some, but not all, ethionine-resistant callus cultures. Cell line R32, which had a *ca.* tenfold increase in soluble methionine, had a 43% increase in total free amino acids and a 40% increase in amino acids in protein as compared to unselected HG2 callus. Relative amounts of each amino acid in protein were the same in both.

Key words: Methionine – Mutagenesis – Mutant selection – Tissue culture – Variants, Resistant

Abbreviation: LT = lysine + threonine in equimolar concentration

Introduction

Selection for increased concentrations of amino acids in tissue cultures has been the subject of investigation in *Nicotiana tabacum* L., *Daucus carota* L., *Arabidopsis thaliana* (L.) Heynh., *Acer pseudoplatanus* L., *Solanum tuberosum* L., and several cereal species (Maliga 1978, Thomas et al. 1979; Scowcroft 1977; Widholm 1977b). Many cell lines with resistance to growth inhibition by amino acid analogs reportedly produce higher than normal amounts of the corresponding natural amino acid (Maliga 1978), hence, growth on specific amino acid analogs has been used as a selection tool. Amino acid overproduction usually is due to relaxed feedback control of an enzyme involved in its production. Other instances of analog resistance may be due to decreased uptake of the analog, selective incorporation of the natural amino acid into protein, or compartmentalization of the analog within the cell (Widholm 1974, 1977a).

Lysine + threonine inhibition of growth is indicative of a feedback regulation system of two enzymes of the aspartate-derived amino acid family, aspartokinase and homoserine dehydrogenase (Cummings et al. 1979; Shewry and Miflin 1977; Bright et al. 1978). Hibberd et al. (1980) characterized a maize tissue culture line selected for lysine + threonine resistance and found that it contained lysine-insensitive aspartokinase and increased levels of free threonine and methionine. Since mutations for such resistance to feedback control are semidominant (Rasse-Massenguy and Fink 1972), it should be feasible to make selections for methionine overproducers using diploid cell lines.

Based on protein scores which compare the distribution of amino acids essential to non-ruminant nutrition to that of ideal (egg) protein, alfalfa forage is low in methionine (Kaldy et al. 1979). Consequently, we attempted to select alfalfa cell lines for resistance to growth inhibition due to ethionine (an analog of methionine). In addition, since very few cell lines have been selected for ethionine resistance, and since high levels of methionine are of only limited overall importance in alfalfa improvement, we considered this project to be a model system where ethionine resistance could be investigated at both the cellular and whole plant level. In this report, we concentrate on the cellular aspects. Resistance to lysine + threonine growth inhibition and production of free and protein amino acids by several cell lines selected for resistance to ethionine growth inhibition are reported herein.

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Materials and Methods

Culture Media

Culture media for callus growth consisted of either BII (Blaydes 1966) with 1% agar or SHII (Schenk and Hildebrandt 1972) with 0.8% agar. Both media were supplemented with 2 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) and 2 mg/l kinetin. Suspension cultures were grown in LB2D.8K – the same as the modified BII above except for the deletion of agar and reduced kinetin concentration (0.8 mg/l). All media were adjusted to pH 5.9 and autoclaved.

Callus Initiation

Callus cultures were initiated from diploid HG2 alfalfa which has a high frequency of plant regeneration from suspension-cultured cells (McCoy and Bingham 1977), and its tetraploid counterpart, HG2-4X, derived by spontaneous chromosome doubling in tissue culture. Whole racemes were washed in 95% ethanol for 1.25 minutes and in 1.25% (v/v) sodium hypochlorite for 1.25 minutes and then rinsed three times in sterile distilled water. Ovaries were excised from buds (field grown plants) or open flowers (greenhouse grown plants) after surface sterilization. Excised ovaries were transferred aseptically to 21 X 70 mm screw-topped vials (seals were removed from vial tops) containing 10 ml each of semi-solid medium. Three ovaries were cultured per vial. Callus cultures were subcultured every 28 days.

Callus Growth Studies

DL-ethionine, L-threonine, L-lysine, L-methionine, and DL-homoserine were obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.). Callus samples were placed on BII or SHII with various concentrations and combinations (see Fig. 1 and Table 1) of lysine, threonine, homoserine, methionine, and ethionine, and incubated for 28 days at 27 ± 3°C under a LD 16:8 photoperiod with 60 μ Einsteins m^{-2} sec^{-1} (cool white fluorescent lamps). Initial fresh weights of callus ranged from 59 to 77 mg (Fig. 1, except for both treatments with ethionine, and Table 1). Mean initial weight for Figure 1 treatments with ethionine was 160 mg. All mean fold increase values were based on 11, 4, and 6 observations in Figure 1, Table 1 (ethionine data) and Table 1 (lysine + threonine data), respectively. Callus for the lysine + threonine (LT) and ethionine inhibition studies in Table 1 was derived from buds regenerated from callus.

Variant Selection Experiments

Liquid cultures were initiated by transferring 1.50 ± 0.25 g of diploid HG2 callus, initiated from ovaries 4 weeks earlier, to a 125 ml Erlenmeyer flask containing 35 ml LB2D.8K. Cultures were incubated under room light (10-15 μ Einsteins m^{-2} sec^{-1}, cool white fluorescent lamps) on a reciprocal shaker at 105-110 rpm at 27 ± 3°C. Suspensions were subcultured once to separate finely divided cells from larger aggregates. Subculturing was done by allowing cells to settle for 30 seconds and then taking 10 ml aliquots from the top layers of cells and transferring them to new flasks containing 17 ml of fresh medium. Transfers were made using 10 ml disposable syringes with 3.75 cm 18 gauge needles. Cells were both mutagenized and plated 8-12 days following the first subculture during the late log phase of growth. Cells were mutagenized in 0.25% (v/v) methane sulfonic acid ethylester (EMS) for 1.75 hours. Over all experiments, death due to EMS was estimated to be 50.7% as determined by a reduction in colony formation. Following mutagenesis, HG2 suspensions were rinsed three times in LB2D.8K and plated on 100 X 15 mm sterile plastic Petri plates with 25 ml of BII medium plus 0.02, 0.10, or 0.24 mM ethionine. Cell density at the time of plating was adjusted to 2 X 10^4 cells/ml (ascertained by packed cell volume). To prevent dessication, cells were suspended in a liquified agar medium at 38-40°C which gelled within 30 seconds. All plates were incubated in a growth chamber on a LD 16:8 photoperiod with 45 μ Einsteins m^{-2} sec^{-1} (cool white fluorescent lamps) at a constant 26°C.

Amino Acid Extraction and Analysis

Three callus cultures of each selected cell line were used for analysis of free amino acids 2 weeks following subculture. Tissue (200-250 mg) was ground in a Virtis 60K homogenizer at 40,000 rpm for 1 minute with 10 ml of methanol-chloroform-water (12:5:3, v/v) containing 1.0% (v/v) thiodiglycol to prevent methionine oxidation. Amino acids were separated from other organic compounds and analyzed as described before (Duke et al. 1978). Tryptophan was not calculated due to its co-chromatographing with a buffer contaminant. Ninhydrin-reactive compounds were identified by co-chromatography with known compounds or by retention times on two amino acid analyzers (Technicon TSM and Durrum D-500) utilizing two differing buffer programs (citrate and lithium buffers). Protein amino acid concentrations were determined by precipitating and washing callus protein in 50% (w/v) trichloroacetic acid two weeks following subculture. Washed samples were hydrolyzed in 6N HCl with 0.2% (w/v) phenol for 20 hours at 110°C in evacuated ignition tubes. Hydrolysates were flash evaporated to dryness, dissolved in a lithium citrate buffer (pH 2.2, 4% w/v) and analyzed on a Durrum D-500 amino acid analyzer.

Results

Lysine + Threonine Inhibition of Tissue Culture Growth

Lysine was not inhibitory to callus growth whereas threonine caused 38% growth inhibition relative to the control (Fig. 1). The addition of both lysine and threonine to the medium caused nearly complete growth inhibition. Inhibition was reversed by the addition of either methionine (1 mM) or its precursor, homoserine (2 mM), to the medium. BII medium with the addition of either methionine or homoserine inhibited callus growth.

Selection for Ethionine Resistant Cell Lines

The addition of 1 mM ethionine caused a significant reduction in growth which was reversed by the addition of 4 mM methionine (Fig. 1). The reversal of ethionine phytotoxicity by exogenous methionine suggests that callus cells naturally producing high levels of methionine should grow on ethionine-containing medium.

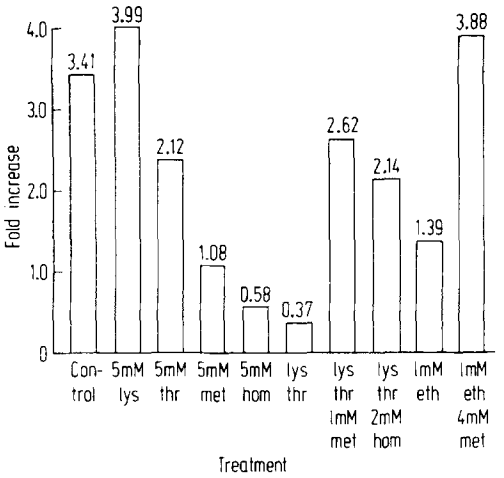

Fig. 1. Fresh weight fold increase of HG2-2X callus on BII and BII with the additions of lysine (lys), threonine (thr), methionine (met), homoserine (hom), and ethionine (eth) as indicated. Measurements were recorded after 28 days of growth on SHII medium at 27 ± 3°C. $LSD_{0.05} = 0.72$

Normal and mutagenized suspensions were plated on media containing 0.02, 0.10, and 0.24 mM ethionine, however, colonies were recovered only from the 0.02 mM ethionine treatment. In 13 plates with normal cells, 13 colonies formed, of which one regenerated plants. Among 52 plates with mutagenized cells 124 colonies appeared and were subcultured in the absence of ethionine after two months in selection. Ninety-one of the 124 colonies formed callus cell lines which regenerated plants.

One-hundred ten of the 124 cell lines were tested for resistance to ethionine after 6 months of growth on non-selection medium. Following this test, sectors of several cell lines with enhanced growth rates on ethionine were separated as sub-lines. Such lines were designated *el* for elite.

Fifteen of the 110 cell lines tested for ethionine resistance had significantly greater fold increases on ethionine than the HG2-4X control (Table 1). The controls, HG2-2X and HG2-4X, had 0.18 and −0.04 fold increases, respectively, on 1 mM ethionine whereas variant cell lines had up to a 1.89 fold increase on ethionine. In a later test, callus culures started from plants regenerated from several of these cell lines exhibited high levels of ethionine resistance (Reisch et al. 1980). Among the other cell lines tested and not listed in Table 1, all but 13 were more than 90% inhibited by 1 mM ethionine.

In a separate test, four variant selections and HG2-2X (control) were tested on SHII and SHII plus ethionine (1 mM). While HG2 and three of the variants did not grow, fresh weights of R32 increased 0.29 fold after 28 days on ethionine. Later testing showed that R32 grew significantly better ($p = 0.01$) than HG2-2X and HG2-4X on 1 mM ethionine (Reisch et al. 1980). R32 was saved and later tested for free methionine content.

Table 1. Growth of control and selected callus lines in the presence of ethionine (1 mM) or lysine + threonine (5 mM each). Fold increase was measured after 28 days of growth on SHII medium at 27 ± 3°C. Values are fold increase.

Callus line	SHII	SHII + ethionine	SHII	SHII + lys/thr
HG2-2X	2.43	0.18	4.20	0.36
HG2-4X	1.72	−0.04	6.21	−0.06
R39	5.65	1.57**	–	–
R39*el*	–	–	7.11	−0.09
R41	4.93	1.35**	3.77	2.33**
R47	2.72	1.09**	4.63	1.84*
R59	2.95	1.59**	6.44	1.62*
R64	2.06	0.86**	15.47	2.62**
R71	3.89	0.72*	–	–
R82	3.44	1.28**	4.83	3.04**
R86	4.72	1.89**	4.97	2.10**
R90	1.98	0.55	–	–
R92	2.16	0.91**	5.80	1.22
R96	3.13	0.78*	–	–
R97[a]	1.30	1.65**	3.95	−0.12
R97*el*	–	–	3.07	1.20
R115	2.14	0.74*	–	–
R140	3.44	0.90**	–	–
R142	3.42	1.39**	6.42	3.19**
R105*el*	–	–	4.48	3.18**
R110*el*	–	–	3.65	0.86
R144	6.99	1.12**	–	–
$LSD_{.05}$		0.65		1.60
$LSD_{.01}$		0.86		2.13

* Significantly different from HG2-4X at $p = 0.05$

** Significantly different from HG2-4X at $p = 0.01$

[a] Upon subsequent testing, R97 was found to grow more rapidly on SHII than on SHII + ethionine

Test for Lysine + Threonine Resistance

The nine best callus lines from the ethionine resistance test, as well as the *el* series, were tested for LT resistance. HG2-2X and -4X were 91 and 101% inhibited, respectively, by 5 mM lysine plus 5 mM threonine (Table 1). Growth inhibition due to LT among the variant cell lines ranged from 29 to 103%. The most resistant line was R105*el*. Two other *el* lines, from R97 and R110, were also resistant to LT growth inhibition although R39*el* was completely inhibited by LT. While R97 had no LT resistance, its derivative R97*el* had a moderate level of resistance. In many instances, ethionine resistance was accompanied by some level of LT resistance. However, cell lines with the greatest amounts of ethionine resistance exhibited both high and low levels of LT resistance.

Free Amino Acids

Callus cultures of HG2-2X and -4X (controls), and the variant selections R59, R64, and R90 contained relatively

Table 2. Concentrations of free amino acids and other ninhydrin-reactive products in callus cultures of control and several variant callus lines. Concentrations reported in the Table represent the mean of 3 replications

Amino acid	HG2-2X	HG2-4X	R32	R39	R59	R64	R90	R97
	nmoles/g fresh weight[a]							
cysteic acid	2^{B}	1^{B}	24^{A}	5^{B}	0.3^{B}	2^{B}	0^{B}	2^{B}
glutathione	6^{C}	13^{BC}	29^{AB}	23^{ABC}	37^{A}	16^{BC}	16^{BC}	14^{BC}
met sulfoxide	8^{CD}	5^{D}	20^{BC}	27^{AB}	34^{A}	12^{CD}	8^{CD}	13^{CD}
asp	240^{AB}	279^{AB}	387^{A}	373^{A}	323^{AB}	246^{AB}	199^{B}	317^{AB}
thr	254^{B}	512^{A}	487^{A}	355^{AB}	351^{AB}	317^{AB}	193^{B}	360^{AB}
asn, gln, ser	661^{B}	631^{B}	935^{A}	720^{AB}	827^{AB}	565^{B}	689^{AB}	811^{AB}
glu	242^{B}	323^{B}	710^{A}	421^{B}	929^{A}	310^{B}	300^{B}	219^{B}
pro	129^{DE}	290^{BC}	779^{A}	146^{DE}	119^{DE}	362^{B}	193^{CD}	157^{DE}
cys	0^{C}	0^{C}	11^{C}	61^{A}	30^{B}	4^{C}	0^{C}	0^{C}
gly	453^{AB}	417^{B}	638^{A}	465^{AB}	460^{AB}	339^{B}	340^{B}	439^{AB}
ala	824^{A}	894^{A}	705^{AB}	733^{AB}	757^{AB}	451^{BC}	797^{A}	584^{AB}
cystine	3^{A}	0^{A}	12^{A}	15^{A}	17^{A}	5^{A}	7^{A}	2^{A}
val	188^{BC}	212^{BC}	368^{A}	313^{AB}	227^{BC}	134^{C}	217^{BC}	191^{BC}
met	9^{F}	14^{DE}	114^{A}	26^{B}	8^{F}	12^{EF}	18^{D}	22^{C}
cystathionine	51^{C}	37^{C}	65^{C}	31^{C}	11^{C}	46^{C}	244^{A}	144^{B}
ile	74^{BC}	104^{BC}	219^{A}	111^{BC}	86^{BC}	69^{BC}	112^{BC}	132^{B}
leu	93^{BC}	110^{BC}	201^{A}	123^{ABC}	91^{BC}	69^{BC}	130^{ABC}	142^{AB}
tyr	16^{AB}	10^{B}	50^{A}	13^{AB}	13^{AB}	9^{B}	19^{AB}	15^{AB}
phe	131^{C}	111^{C}	24^{E}	179^{B}	245^{A}	131^{C}	40^{DE}	194^{B}
lys	86^{AB}	107^{A}	95^{AB}	78^{AB}	88^{AB}	72^{AB}	78^{AB}	101^{A}
his	82^{AB}	81^{AB}	135^{A}	82^{AB}	62^{B}	84^{AB}	82^{AB}	60^{B}
arg	46^{ABC}	61^{ABC}	92^{A}	36^{BC}	41^{BC}	69^{AB}	16^{C}	32^{BC}
Total[b]	3528	4156	5950	4235	4657	3243	3423	3776

[a] Means across rows followed by the same letter are not significantly different at $p = 0.05$ by Duncan's multiple range test

[b] Totals include only amino acids found in protein

low concentrations of free methionine (Table 2). In contrast, R32, R39, and R97 had significantly higher methionine concentrations than the controls, with R32 having a ca. tenfold increase in free methionine. Although thiodiglycol was added to amino acid extraction solvents, elution solutions, and buffers, methionine sulfoxides were present in all samples. Both R32 and R59 were especially high in methionine sulfoxide concentration. The origin of methionine sulfoxides in our samples is unclear.

Little or no free cysteine was found in either controls or R90 and R97 callus tissues. Low but measurable levels of free cysteine were in the other variant selections. Cultures R39 and R59 were the only selections with higher concentrations of free cysteine than free methionine.

High but variable concentrations of the cysteine-containing peptide, glutathione, were found in tissues of R32, R39, and R59. Cystathionine is either absent or at a very low concentration in free amino acid profiles from leaf tissues of plants regenerated from tissue cultures (S. Duke, unpublished results). Cystathionine was found in highest concentrations in R90 and R97 variant cultures.

Free threonine was twice as high in concentration in HG2-4X as in HG2-2X cultures. Most of the variant cultures were intermediate in threonine concentration. There were no great differences among cultures in lysine concentration. There were no positive correlations between free threonine or lysine concentrations and free methionine concentrations.

Protein Amino Acids

Cell line R32 had 43% more total free amino acids and had *ca.* ten times more methionine than HG2-4X; hence, it was examined for protein amino acids. Comparisons were made between levels of protein amino acids in HG2-4X and R32 since, after 18 months in culture at the time of analysis, R32 callus was presumed to be polyploid. HG2-4X and R32 had a total of 572.6 and 799.5 μmoles of amino acid per gram dry weight, respectively, representing a 40% increase in R32 over HG2-4X (Table 3). On a mole-percent basis, the ratio of amino acids remained

Table 3. Amino acid content of HG2-4X and R32 callus protein extracted from 5-7 mg of tissue (dry weight) grown for 2 weeks on SHII medium. Concentrations reported in the Table represent the mean of 2 replications. Values are mole-percent.

	Cell line	
Amino acid	HG2-4X	R32
asx	11.8	12.0
thr	6.0	5.4
ser	8.8	8.1
glx	8.7	9.2
pro	6.5	6.9
gly	9.5	9.2
ala	7.3	7.8
cystine	0.17	0.07
val	7.3	7.2
met	1.36	1.41
ile	4.3	4.1
leu	7.1	7.3
tyr	4.7	4.1
phe	3.9	3.8
lys	5.8	7.1
his	2.4	2.4
arg	4.5	4.0
Total[a]	572.6	799.5

[a] μmoles/g dry weight

essentially unchanged with the possible exception of lysine which increased from 5.80% in HG2-4X to 7.08% in R32. Methionine was measured to be 1.36 and 1.41% in HG2-4X and R32, respectively (Table 3).

Discussion

The reversal of LT inhibition by the addition of methionine or its precursor is consistent with previous reports on callus of *Z. mays* (Green and Phillips 1974), *D. carota* and *N. tabacum* suspension cultures (Widholm 1976) and *Oryza sativa* L. callus (Furuhashi and Yatazawa 1970). Apparently, LT inhibition is due to methionine starvation. The presence of an LT feedback regulation mechanism would make HG2 alfalfa suspension cultures a suitable system for the selection of feedback inhibition-resistant cultures. Since lysine or threonine alone do not cause complete inhibition, a mutation at the allosteric regulatory site for either amino acid on aspartokinase or homoserine dehydrogenase should be detectable as a methionine overproducer. Having two mutations to choose from should increase the chances of finding an altered enzyme mutant which overproduces the aspartate amino acid family.

Whereas ethionine may have multiple toxic effects on alfalfa tissue cultures, so long as its toxicity can be reversed by methionine (Fig. 1) it should be an effective agent in selecting for methionine overproduction. It should be noted that 1 mM ethionine inhibition was not complete in one experiment (Fig. 1) whereas the same concentration of ethionine caused 93% growth inhibition in another experiment (Table 1). This may be explained by the large initial callus fresh weight (160 mg) used in the ethionine treatment in Figure 1 as compared to the 76 mg initial weight used for the experiment reported in Table 1. It might also be noted that 1 mM ethionine was required for callus inhibition whereas the plated cells only grew at 0.02 mM ethionine. It therefore seems likely that with decreasing initial callus weight and increasing direct contact of cells with the medium, ethionine inhibition becomes more severe.

Spontaneous polyploidy is common in suspensions of HG2 (McCoy and Bingham 1977) and is significantly affected by high kinetin levels. Consequently, kinetin in the suspensions was used at the reduced level of 0.8 mg/l. In addition, to maximize regeneration of diploid variants, only one-month-old callus cultures were used to reinitiate suspensions for each selection experiment. A check of regenerated plants showed that 73% were diploids (Reisch et al. 1980).

Only 15 of the 110 cell lines tested grew significantly better than the control on media with 1 mM ethionine. The apparent loss of ethionine resistance may be due to two factors: (1) normal cells surviving selection on inhibitory medium, and (2) epigenetic changes – induced physiological changes causing transient ethionine resistance followed by reversion to the normal phenotype in the absence of ethionine.

Chimerism of normal and mutant cells within a selected cells line, followed by the rapid growth of normal cells and the decline of mutant cells may be considered a special case of factor (1) above. Evidence for chimerism may best be illustrated with R97. Ethionine resistance testing revealed less than a 0.5 fold increase on ethionine in three of four callus samples tested. The fourth sample had a 5.9 fold increase on ethionine and continued growth for a second month on ethionine (R97*el*). In a concomitant test of R97 and R97*el* on LT medium (Table 1) the former culture was 103% inhibited while the latter was only 61% inhibited. The evidence is strongly suggestive of chimeral callus lines.

Fold increase values on SHII averaged much higher in the LT test than in the ethionine test (Table 1). While differences can usually be expected between experiments run at different times, some of the observed differences can be explained by the use of callus 5-6 weeks following the previous subculture to initiate the ethionine test and 3-week-old callus to initiate the lysine + threonine test.

Glutathione has been suggested to be a reservoir for cysteine in some organisms (Tateishi et al. 1977). If this is

the situation in alfalfa tissue cultures, our data showing low to nonexistent levels of cysteine could be misleading. It should be noted that a correlation appears to exist between levels of cysteine and glutathione (Table 2). Cell line HG2-2X with no cysteine had the lowest glutathione level whereas R39 and R59 with high cysteine levels have high levels of glutathione. This suggests that excess cysteine may be shunted into glutathione production or vice versa.

High levels of cystathionine (a precursor of methionine) in tissue cultures as compared to leaf tissues would suggest that in tissue cultures one or more enzymes involved in cystathionine conversion to methionine is inhibited or in low quantity. Low activity of an enzyme such as β-cystathionase could cause a 'bottleneck' in methionine synthesis which does not ordinarily occur in differentiated tissues. On the other hand, cystathionine may serve some function, other than as a methionine precursor, such as storing cysteine, or it could be the result of methionine degradation.

In that we found a positive relationship between high free methionine concentration and resistance to ethionine in some variant tissue culture selections (Tables 1, 2), it appears that as with previous studies (Widholm 1976, 1978), selection for amino acid overproducers with amino acid analogs is a valid procedure. Chaleff and Carlson (1975) have shown that lysine-overproducing cell lines of *O. sativa* have as much as 27% more protein lysine than the normal cell line. However, even though we found higher concentrations of protein methionine in R32 (Table 3), the ratio of methionine to other amino acids was not changed appreciably. This indicates that, at least in this one case, the presence of excess soluble methionine does not necessarily alter the ratio of amino acids in protein.

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Dr. Bruce I. Reisch
Department of Pomology and Viticulture
New York State Agricultural Experiment Station
Cornell University, Geneva, N.Y. 14456 (USA)