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Increased Lysine and Seed Storage Protein in Rice Plants Recovered from Calli Selected with Inhibitory Levels of Lysine plus Threonine and *S*-(2-Aminoethyl)cysteine

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ABSTRACT

Experiments were designed to test whether variation in percent lysine in seed proteins could be recovered in plants regenerated from callus subjected to inhibitory levels of lysine plus threonine. Anther-derived callus was subjected to 1 millimolar lysine plus threonine for three successive passages and then once to the same concentration of *S*-(2-aminoethyl)cysteine. Plants were regenerated from the resistant callus. Plants recovered directly from tissue culture were normal in color, size and were 50% or less fertile. Second and third generation plants produced a wide range of variants including albinos, deep green plants both short and tall, and totally fertile as well as partially fertile plants. All regenerated plants produced chalky or opaque seed. One unique second generation line had 14% more lysine in seed storage proteins than the controls. This characteristic was transmitted to the next generation. The high lysine plants had reduced seed size with significantly higher levels of seed storage protein than the controls. The phenotypes recovered provide experimental materials for basic studies in protein synthesis and lysine metabolism and may become a source of material for rice breeding.

The nutritional quality of the major cereal grains is not optimal because lysine and threonine are limiting amino acids in seed storage proteins (16). The efficient recovery of plants with altered lysine levels requires mutants either in the metabolism or catabolism of lysine or in the (de)regulation of pathways leading to lysine and protein synthesis. Strategies exist for the recovery of mutants for lysine synthesis in microbial systems but few clearly defined systems or mutants are available in green plants. Lack of basic metabolic information in crop plants precludes the efficient recovery of single gene lysine mutants. Nonetheless, some progress has been made using tissue/anther culture techniques coupled with the use of amino acid analogs and inhibitory levels of metabolic products, particularly AEC[1] and L+T. Combinations of these compounds provide biochemical selection pressure for altered feedback inhibition or insensitivity to precise control of one or more of the enzymatic reactions associated with the β-aspartokinase pathway for lysine and threonine synthesis.

Fermentation research with microorganisms showed that β-aspartokinase was inhibited by L+T through feedback mechanisms (19). It was clear from early research that AEC weakly inhibited microbial cells, but the addition of threonine produced strong inhibition (22). In 1970 Bryan *et al.* (6) isolated β-aspartokinase from multicellular plants and demonstrated feedback inhibition as well. The effect of L+T was demonstrated in rice tissue culture (8), in barley (4), in *Mimulus* seedlings (12), in maize tissue culture (10), and in carrot suspension cultures (7, 15). More recently, maize plants were recovered from L+T selections and progeny showed elevated threonine levels (13). Even though the β-aspartokinase pathway exists in higher plants there is evidence that alternative pathways may also function (29), but details may not be the same for all plant species. Much additional information is required before the regulation of lysine metabolism is completely defined in the major crop plants.

AEC has been implicated as co-inhibitor for false feedback inhibition in microbial systems. Sano and Shho (22) used this analog with lysine and threonine to recover lysine mutants. Brock *et al.* (5) proposed the use of AEC to inhibit embryos for the recovery of feedback mutants in higher plants. The application of AEC selections to higher plants was further demonstrated in barley and maize (2–4, 9). AEC resistant lines have been developed using cell suspension cultures (30) and anther-derived callus cultures (24). In 1984 Negrutiu *et al.* (20) reported AEC resistant lines from protoplasts of tobacco and characterized progeny as monogenic dominant for resistance to the analog.

The most advanced *in vitro* techniques among the major cereal crops exist in rice, *Oryza sativa*. Rice plants were recovered from anther-derived callus of an indica subspecies cultured three times in the presence of 2 mM AEC (24). Plants recovered from AEC selected cells had increased protein and lysine over the controls (24, 26). Even though substantial infertility was induced by the *in vitro* procedures in rice, segregants with normal seed set from both selfed and crossed progeny had increased seed protein as well as lysine (26, 28).

The experiments described here were designed to recover cells resistant to inhibitory levels of L+T and AEC from anther-derived calli. Another purpose was to characterize the progeny of these regenerated plants, and determine if regenerated plants and selfed progeny expressed the phenotype corresponding to the selection pressure applied *in vitro*. There are no previous examples of the recovery and characterization of rice plants from cells resistant to inhibitory levels of L+T.

MATERIALS AND METHODS

Anther-derived calli of rice, *Oryza sativa*, were subjected to inhibitory levels of L+T followed by AEC. Plants were regenerated and selfed, progeny were characterized for levels of seed storage proteins and percent lysine in the proteins. The plants were also evaluated for overall morphology.

Source. Cell selections were done with the cultivar Calrose 76, subspecies japonica (21). The genotype is widely used as a commercial cultivar and as a source for rice breeding, as well as

[1] Abbreviations: AEC, *S*-(2-aminoethyl)cysteine; L+T, lysine plus threonine.

in vitro studies.

Anther Culture. The anther culture procedures were those described earlier (24) and modified (27) for Calrose 76. Anthers with uninucleate microspores were given a cold temperature shock at 7°C for 7 d. Florets were excised aseptically and 25 anthers were placed on agar slants in 25 × 150 mm test tubes. Individual calli were lifted from the anthers after 6 to 8 weeks of growth and placed on a callus increase medium for 4 to 5 weeks.

Media. The medium for rice anther culture was a modification of the medium used for anther culture earlier (11). The medium contains Blaydes inorganic salts (1), 150 ml/L coconut milk and 1 g/L yeast extract. Sucrose was added at 3% w/v and agar at 1% w/v. The auxin, 2,4-D, was added at 2 mg/L.

The tissue increase medium was similar to the anther culture medium but had no coconut milk and only 250 mg/L yeast extract. The 2,4-D level was 1.0 mg/L. Murashige-Skoog (MS) (18) inorganic salts from GIBCO[2] were substituted for the Blaydes salts.

For rice regeneration, a simplified medium was used consisting of MS inorganic salts and vitamins, glycine 2 mg/L, *myo*-inositol 100 mg/L, glutamine 146 mg/L, and sucrose 3% w/v. IAA and kinetin (Sigma) were added at 1 mg/L each. The medium was solidified with 1% w/v agar. Developing plantlets were separated from the body of the callus to prevent inhibition of subtending growth centers and promote greater plantlet development. Plants 3 to 10 cm tall were then removed from the agar medium and placed into a liquid rooting medium and maintained on a gyratory shaker at 100 rpm for 10 d to promote tillering and root development. This liquid rooting medium (23) consisted of MS inorganic salts and vitamins, *myo*-inositol 100 mg/L, glutamine 146 mg/L, sucrose 3% (w/v), IAA 5 mg/L, and kinetin 0.1 mg/L.

Generations and Selfings. R_o refers to the vegetative portions of plants regenerated from tissue culture and 10 R_o plants were recovered from callus resistant to L+T and AEC. S_1 refers to the seeds from the 1st meiosis on the R_o plants.

S_1 Population. Seed of 10 R_o plants were analyzed for lysine levels and 8 for protein levels.

S_2 Population. Eleven plants were started from one of the 10 S_1 seed lots, *i.e.* lot No. 2, which had the highest seed lysine value. Three sister lines derived from seed lot No. 2 were designated 1C, 4C, and 7C.

S_3 Population. Fifteen plants each from 1C, 4C, and 7C were grown for S_3 seed production.

S_4 Population. Plants from S_3 seed lots were grown for S_4 seed production: one set of 10 plants was from 1C and 5 sets of 10 plants each from 4C. Two plants from 1 set of 4C did not survive. Plants with 1000 or more seeds were considered normal and analyzed for protein and lysine levels presented in Figure 2.

Controls. Two types of controls were used. (a) Controls that grew with S_4 populations were from bulk Calrose 76 selfed three times in the greenhouse, and (b) controls from four different experiments grown at different times and these represent materials selfed from one to five times. These controls have no *in vitro* history.

Biochemical Selection. Several protocols were used to recover cells insensitive to inhibitory levels of L+T. Calli were fragmented and sized to 860 microns or less by passage through stainless steel sieves. For three different selections sized calli were plated on agar in 5 cm plastic Petri dishes containing inhibitors in the following combinations: Protocol A, one passage on 1 mM L+T, two more passages on 2 mM L+T and two passages on 1 mM AEC. The final passage was on the regeneration medium. Protocol B, three passages on 1 mM L+T, one passage on 1 mM AEC, and the final passage on the regeneration medium. Protocol C, two passages on 2 mM L+T and the final passage on the regeneration medium. The regeneration medium did not contain inhibitory selective agents in any protocol. The primary data presented later in this report came from selection protocol B.

Amino Acid and Protein Determinations. Seed storage proteins were hydrolyzed in 6 N HCl under N_2. The amino acids were separated with W3H cation exchange resin and quantified with a Beckman 119BL analyzer as described earlier (24, 26). Amino acid determinations were done on half seeds, whole seeds and 30 seed composite samples from single plants. Seed protein was calculated from total seed nitrogen determined by the Kjeldahl method. These analyses were done commercially at the Ohio State University research extension analytical laboratory, Wooster, OH. Samples were 30 seed composites from single plants.

For the lysine determination of individual whole or half seeds the grain was split transversely into nearly equal sections. Thus, half was endosperm only and the remaining half was endosperm and embryo. The endosperm half was used for amino acid analyses and SDS-PAGE profiles of storage proteins while the embryo half was planted for germplasm increase.

Other Progeny Characterizations. Greenhouse grown plants were characterized for yield as seeds/plant, seed weight was determined by a 30-seed sample, and tillers were counted at harvest. Plant height was measured as the distance from the soil level to the tip of the mature panicle. Plants were subjectively scored for color with a rating of 1 for light green plants to 5 for intensely green types. Controls usually scored 2 or 3. Seed chalkiness was scored from 1 to 10 by estimating the light transmission from fluorescent lamps through the seeds. A score of 1 represents clear seeds which was usually the case in the control cultivar, and 10 represents completely opaque seeds (28). The chalky index is the chalky rating multiplied by the percent of the seeds in a 30-seed sample showing the chalky phenotype.

Electrophoresis of Seed Storage Proteins. The electrophoresis procedures used were modifications of those described by Laemmeli (14). Samples of half seeds, whole seeds, or multiple seed composites were weighed and ground into dry powder with a mortar and pestle. Proteins were denatured and extracted with 3% SDS and 5% β-mercaptoethanol at 40°C for 2 h followed by heating at 100°C for 3 min. Thirty μl samples were electrophoresed in 8% or 12% polyacrylamide gels (w/v) (SDS-PAGE) for 4 h at 200 V. The gels were fixed and stained in methanol-acetic acid-H_2O (45-45-10) containing 0.2% Coomassie blue R overnight, then destained with the same acidic methanol solvent. For improved sensitivity, the proteins were silver stained accord-

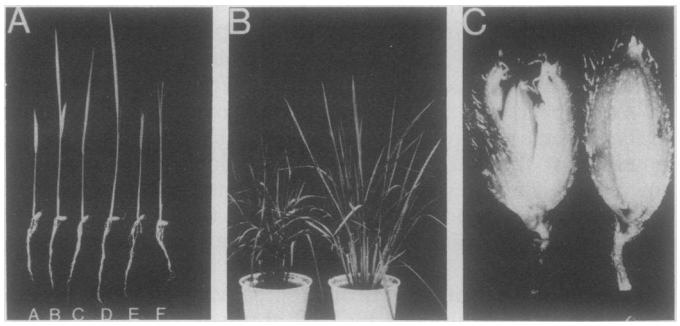

FIG. 1. Variations in progeny from L+T and AEC selections. A, Albino (plants A and B), normal (plants C and D), and short deep green phenotypes (plants E and F) recovered from S_1 seed; B, deep green phenotype (left) and normal green plant (right); C, abnormal endosperm development and lethal seed from homozygous deep green plants (left) and normal seed (right).

[2] Mention of a trademark or proprietary product does not constitute a guarantee of warranty of the product by the United States Department of Agriculture, and does not imply its approval to the exclusion of other products that may also be suitable.

Table I. *Means and Data Ranges of Whole Seed Characteristics of Selfed Plants Regenerated from Anther Calli Resistant to Inhibitory Levels of L + T and AEC*

Generations (Selfings)	N	Seed/Plant		Lysine		Protein	
		Mean	Range	Mean	Range	Mean	Range
				% of total AA		%	
S_1	10	114	44–342	3.13	2.87–3.45	13.4	12.9–13.7
S_2	11	1038	924–1628	3.74	3.40–4.33	9.2	8.1–11.2
S_3	15	916	80–1487	4.09	3.69–4.57	10.9	9.0–12.6
S_4	48	570	50–1050	3.72	3.44–4.11	12.3	10.0–15.4
Control[a]	20	1038	799–1349	3.38	3.03–3.70	9.86	8.8–12.0
Control[b]	50	1196	581–1582	3.51	3.03–3.96	9.16	7.8–12.0

[a] Controls grown at same time and conditions as S_4 population. [b] Composite controls from four different experiments in time and conditions, including the S_4 controls.

Table II. *Percent Lysine in Acid Hydrolysates of Protein from the Endosperm Half of Single Seeds of Rice, cv Calrose 76*

Plants were regenerated from anther calli resistant to inhibitory levels of L + T followed by AEC.

	S_3 Generation		S_4 Generation	
	Control	4C-3-1	Control	4C-3-2
	2.72	3.56	3.16	2.87
	2.94	3.76	3.05	3.68
	2.89	3.69	2.96	3.5
	3.06	3.51	2.85	3.5
	3.16	3.64	2.94	3.31
	3.03	3.65	2.84	4.07
	2.7	3.74	3.14	3.46
	3.01	3.7	2.99	3.3
	2.65	3.4	2.92	3.53
	2.77	3.83	2.95	3.69
		3.5	2.98	3.25
		3.81	2.96	3.88
		3.84	2.97	3.51
		3.47	3.12	3.57
		3.62	3.08	3.45
Mean	2.893	3.648	2.994	3.505
SE	0.175	0.137	0.098	0.278

ing to procedures supplied by ICN Biochemicals, the manufacturer of the ICN reagent kit.

RESULTS

The first plants regenerated (R_o) from L+T selections in three different experiments were normal in color, size, and tillering characteristics. All R_o plants had normal seed weight but were less that 50% fertile. The S_1 seed from R_o plants produced a wide range of plants (Fig. 1) including tall and short albinos and tall and short deep green-colored plants with narrow leaves. Some plants were normal in height, color, and fertility, The plant segregation pattern from S_1 seed for the albino character suggests that it is caused by a single recessive gene. Backcrosses have not yet been made to define precisely the genotype for plant color. Homozygous deep green lines have been produced in S_3 populations and these are over 95% infertile. The abnormal endosperm is illustrated in Figure 1 (right panel, open seed on left).

Selfed progeny from L+T selected lines produced plants with higher protein and lysine levels than the controls. Composite seed samples from single plants often mask individual seed values, particularly during the first generation in which carryover effects from tissue culture are most pronounced. For example, the highest value among 10 S_1 seed lots from individual plants was 3.45% lysine (Table I). However, seed from individual S_2 and S_3 plants had lysine of 4.3 and 4.57%, respectively. Additionally, combined results from the half seed analyses of all the S_1 plants derived from protocol B produced two classes of seeds. Approximately 50% were normal or below normal in lysine content and 50% had levels higher than the maximum (3.16% lysine) recovered in the half seeds of S_4 controls (Table II), the only controls for which half seed analyses were done.

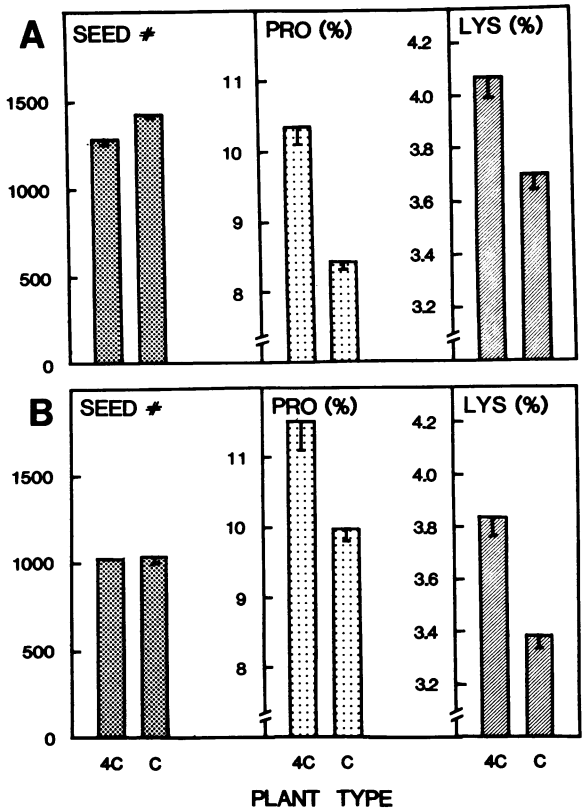

FIG. 2. Percent protein in rice seeds and percent lysine in proteins of S_3 (top) and S_4 (lower) plants selected for normal seed set defined as having 1000 or more seeds per plant. 4C designates the *in vitro*-selected variant and C designates the controls.

Seed storage protein levels are frequently negatively correlated with seed number in cereals and therefore the most valid comparisons would be between plants of equal or near equal seed number. Such a comparison is illustrated in Figure 2 in which only plants with seed number of 1000 or more are represented. The results clearly demonstrate improved protein and lysine levels in normal 4C plants from the L+T/AEC selections. There was a 12 and 14% increase in percent lysine in protein in the

Table III. *Seed Number per Plant, Protein and Lysine Levels from S_2 Whole Seeds of Rice Plants Regenerated from L + T Followed by AEC Selected Calli Initiated by Anther Culture*

The control is from Calrose 76 with no *in vitro* history and 397-4C represents the *in vitro*-selected variant. Protein and lysine were determined from subsamples of a 30-seed composite sample from each plant.

	397-4C			Control Each Plant		
	Seed/Plant	Protein	Lysine	Seed/Plant	Protein	Lysine
	No.	*% Wt/Wt*	*% of total amino acids*	*No.*	*% Wt/Wt*	*% of total amino acids*
	1240	10.5	4.26	1392	8.1	3.87
	150	12.1	4.57	1487	8.3	3.77
	1411	10.2	3.93	1430	8.8	3.41
	632	11.7	4.18	1392	8.8	3.61
	1031	9.0	4.21	1468	8.8	3.32
	1240	10.7	4.08	1449	8.1	3.56
	1487	11.2	4.34	1487	8.1	3.63
	80	12.6	3.96	1430	8.1	3.80
	936	10.5	4.03	1430	7.9	3.74
	594	11.9	4.07	1411	9.0	3.47
	1240	11.0	3.88	1468	7.8	3.96
	110	11.9	4.32	1297	8.6	3.78
	955	10.5	3.81	1582	8.3	3.64
	1354	9.8	3.69	1240	9.3	3.49
	1278	10.0	4.06	1335	8.6	3.86
Mean	916	10.9	4.10	1420	8.4	3.66
± SE	126	0.26	0.06	22	0.44	0.05

selected material over the control in S_3 and S_4 generations, respectively (Fig. 2). The increase in lysine from *in vitro* selections is further illustrated by the single half seed analyses of plants selfed three and four times (Table II). The data show the range of values and low variation in single seed analyses of selected and control materials. The highest lysine value in the control endosperm was a 3.16%, whereas the lowest value for the S_3 selected line was 3.4%. Similar results were obtained with the S_4 generation except the range of variation was wider and only 1 out of 15 seeds had a value less than 3.16%, the highest value of the controls.

Line 4C and some other *in vitro* selected lines do not display the inverse protein/lysine relationship. The controls and some segregants such as 7C express the inverse protein/lysine relationship. The high lysine phenotype recovered in line 4C has lower seed weights than the control. The average seed weights for lines 1C, 4C, 7C, and the controls were 23, 18, 23, and 22 mg/seed, respectively. A cross of 4C to a derivative of Calrose 76 with a glabrous marker produced segregants with high lysine and low seed weight. The R-square regression value of percent lysine and seed weight was 0.54 from a population of over 200 F_2 seed.

Analyses of single seeds of the S_1 generation demonstrate within plant variation for lysine levels. The S_1 plants derived from selection protocol B can be grouped into two classes: (a) plants with only low lysine levels, and (b) mixed plant types with both high and low seed lysine. The range in percent lysine in seed proteins from 10 S_1 plants was 2.87 to 3.45 with a mean of 3.13%. The range for protein values was 12.9 to 13.7% and the mean was 13.4% (Table I). Segregants in the next generation, S_2, produced one line identified as 4C which showed distinctly higher percent protein and lysine than the control line (Table III). However, this 4C line produced plants with both low and near normal seeds per plant. The controls were uniform in seed number. The elevated protein levels in 4C occurred with both average and below average seed set. The percent lysine level was also independent from seed set and was consistently high. The comparison of 4C to its sister lines and controls is illustrated in Figure 3. The higher lysine level occurs in the more advanced generation as well.

Single seed analyses showed 7C to be uniform for normal lysine and hence could serve as an internal control whereas 1C was heterozygous and had seeds with the elevated as well as near normal range.

The average percent lysine in seed endosperm protein based on single half seed analyses for 1C, 4C, 7C, and the controls was 3.24, 3.64, 3.11, and 2.99%, respectively. Whole seeds including the embryo have 0.2 to 0.4% higher lysine than the endosperm half only.

The initial analyses of S_3 plants recovered from selection protocols A and C in separate experiments ("Materials and Methods") confirm the recovery of plants with reduced seed weights, chalky seeds, and improved lysine similar to the progeny from 4C recovered from protocol B. Since protocol C did not include AEC in the cell selection process we conclude that the L+T selection pressure with Calrose 76 is required for the recovery of the small-seeded high lysine mutants.

The SDS PAGE profiles for seeds from a highly infertile plant show abnormal seed storage protein patterns (Fig. 4A, vertical arrows). The high mol wt material is decreased and higher quantities of low mol wt proteins appear. The 4C profiles (Fig. 4B) came from a plant with normal seed number and the profiles are nearly as uniform as the controls. The SDS PAGE profiles of storage proteins from single seeds of the 4C line is not greatly different from the control when visualized with Coomassie blue (not shown here) but does show shifts in seed protein patterns (horizontal arrows) with the more sensitive silver stain (Fig. 4B).

In vitro culture of rice produces chalky seeds (28). The 4C line has a high chalky index and high seed protein. Histograms for chalky index and protein levels for the controls and three selected sister lines from a common parent form very similar profiles (Fig. 3), reflecting some association of chalkiness to seed storage protein levels. The 4C line had the highest seed storage protein levels as well as the highest chalky index.

DISCUSSION

The mean lysine levels, expressed as percent of all amino acids in seed protein hydrolyzates, in first generation selected plants were nearly the same as the control means. However, depressed

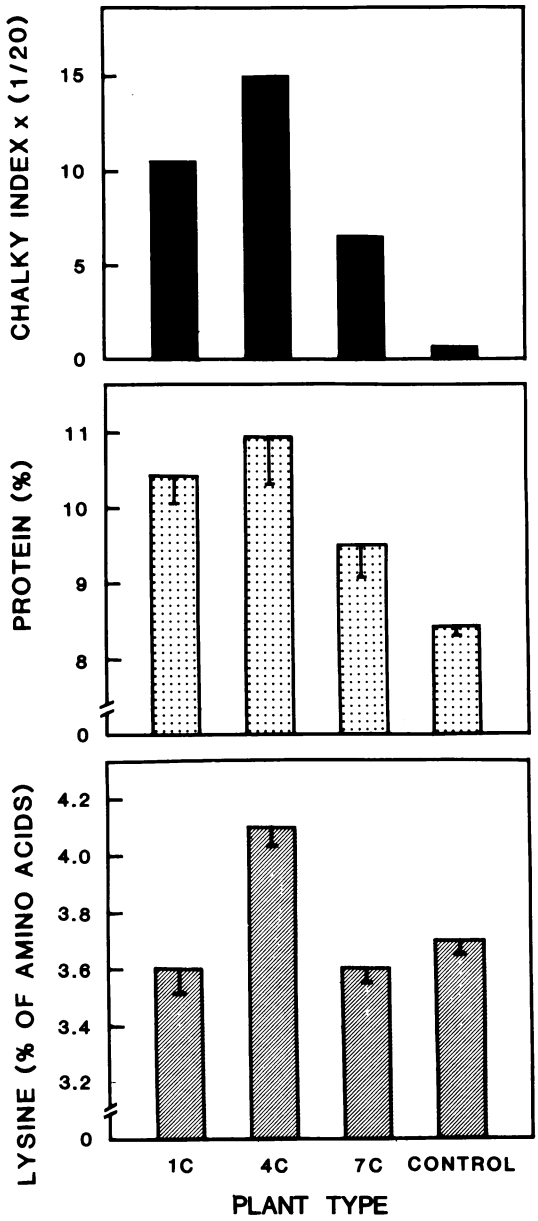

FIG. 3. Chalky index, percent grain protein, and percent lysine in acid hydrolyzed protein of rice seeds, cv Calrose 76 from plants regenerated from anther callus insensitive to exogenous L+T and AEC. Plant types 1C, 4C, and 7C represent three S_3 lines from a single S_2 plant. Line symbols represent the sample SE.

lysine levels may be expected since seed set is frequently reduced in tissue cultured plants. Under conditions of reduced seed set protein levels are frequently increased and lysine expressed as percent of all amino acids is decreased. Nonspecific carryover effects from tissue culture can produce unexpected and artificially high or low lysine values. The negative correlation between percent lysine and percent protein would give a low lysine phenotype even though the genotype is one for altered lysine level. Although the percent lysine is less in 1C and 7C compared with 4C, the means may not reflect fully the lysine potential. There may be several reasons for this. One is that these lines are not yet fully homozygous. The original plants out of tissue culture may be heterozygous or even chimeric for cell types conditioning lysine/protein levels. The data in Figure 1 represent means of 15 individuals in which 7C appears uniformly low compared with 4C. However, line 1C has two categories of seeds; one-third with

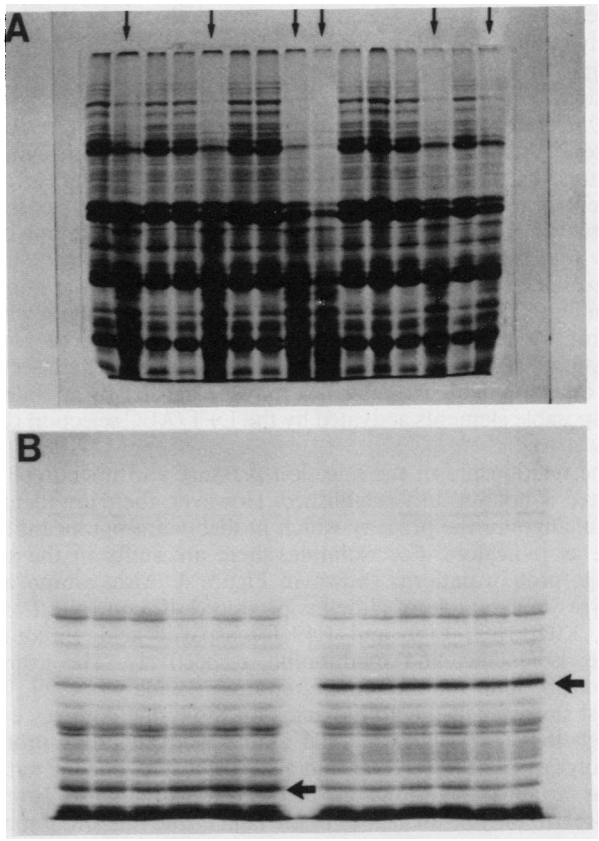

FIG. 4. Variations in SDS PAGE profiles of half-seed storage proteins visualized with Coomassie blue (A) from lines heterozygous for normal and deep green color. Deviations from normal marked with vertical arrows include lethals and seeds which produce deep green phenotypes. Improved lysine line, 4C (left) and controls (right) are shown in the lower gel (B). Six half-seed profiles of controls and normal yielding 4C progeny are visualized with ICN silver staining reagent. Protein shifts are marked with horizontal arrows.

elevated lysine levels and two-thirds with values equal to or lower than the controls.

One of the consequences of rice tissue culture is the production of chalky or opaque seeds (28). It is likely that the passage of rice cells through tissue culture, conditions this seed characteristic. However, there are different types of chalky seeds. The high lysine types have low seed weight and have grinding and shatter characteristics different from large-seeded chalky types and controls. These differences have not been quantified but the powdery rice mutant endosperm seems analogous in some respects to the maize opaques. The opaque seed may adversely affect certain rice quality characteristics but the phenotype may also be beneficially associated with elevated protein levels. The uniformity and intensity of chalkiness in 4C is striking, and therefore this line may be particularly valuable for biochemical and genetic studies.

It is becoming increasingly clear that single plants regenerated from biochemical selection pressure contain mixed cell types, which may be a rich reservoir of variation. Progeny must then be selfed or crossed for the identification of the desired phenotypes. As shown in this report, the most beneficial phenotype was not evident until the second generation was examined. The first generations out of tissue culture may be chimeric as well as heterozygous for some genes. Both forms are possible. The full lysine potential was probably not expressed in the S_1 seed.

One of the striking features of the biochemical selections with L+T is that three separate selections each produced some second

generation plants with a deep green color with narrow leaves and nearly sterile phenotype. The R_o plants in each case appeared normal. Inasmuch as this phenotype was not recovered in hundreds of anther cultured plants (25) of Calrose 76, nor in AEC selected plants using Assam 5, an indica subspecies (26), the results suggest that the deep green phenotype of this cultivar is caused by the L+T selection pressure. However, the elevated percent lysine occurs in both normal fully fertile but possibly heterozygous and deep green partially fertile plants. Selfing data show that the deep green phenotype is homozygous and stable whereas the heterozygous is indistinguishable from the normal green types. One S_3 line produced only striated seedlings in which columns of albino cells were detected within the green blade. The albino sectors were approximately 1 cm long. These sectors and other pigment irregularities may be caused by mobile or transposable elements activated by the L+T/AEC selection pressure.

The relationship of the selection pressure and phenotype recovered is not yet fully established. However, there are identifiable changes in the progeny which probably are significant and serve as indicators. For example, there are shifts in the seed storage protein patterns shown in Figure 4. Also, amino acid analyses of a sample of glutelins produced 4C:control ratios of 1.02, 1.09, 1.33, 1.09, and 1.47 for aspartic acid, threonine, lysine, isoleucine, and methionine, respectively. The average 4C:control ratio for the remaining amino acids was 0.99. The largest shift was in the amino acids on the lysine pathway even though these are values of total amino acid including protein hydrolyzates and not free amino acids only. The opaque nature of the variant seed and the reduced seed weight and the crumbly endosperm may be analagous to the high lysine mutant of maize (17). It is also reasonable that a change in protein composition and/or processing could lead to altered chloroplast function and leaf color. Segregation for leaf color was a common feature in selfed progeny in this work. The free lysine level in rice grain is very low (24). In preliminary assays of lines reported here, we find no clear distinction between L+T selected lines and control lines. Since the free lysine in mature seeds is very low, the levels of free amino acids in seeds may be less significant than the rates of amino acid metabolism or translocation. L+T selected callus had 13% greater free lysine than control callus in a nonreplicated assay.

The selection with AEC only reported earlier (24, 26) produced some different phenotypes than the selections reported here. The high percent lysine in seed proteins was found in plants with excellent seed set, seed weights were normal, and the highest chalky classes had higher seed proteins than the controls. Plant Chl levels of selfed progeny were normal. In contrast, the selection with L+T and L+T + AEC reported here produced high lysine mutants with small seed, high chalkiness with soft and hence crumbly endosperm and a spectrum of Chl mutants. There may be several reasons for the different responses including: (a) the two sets of experiments were done with different cultivars belonging to different subspecies, and (b) the only selection pressure applied to the early work (24, 26) was AEC. In spite of the experimental differences segregants were recovered from selections with AEC only, L+T only, and L+T + AEC with improved percent lysine in the seed proteins. Mutants with small seeds, crumbly or soft endosperm, and elevated lysine as well as the pigment mutants were unique to the L+T containing selections reported here.

Conclusions. The *in vitro* selection of rice cells in the presence of inhibitory levels of L+T produced progeny in the second and third generation after cell culture that had increased seed storage protein levels and increased percent lysine in the protein over controls grown under the same greenhouse environments. The biochemical selection pressure induced intense seed chalkiness and a unique soft and easily crumbled endosperm that is absent in plants regenerated from callus not subjected to inhibitory levels of lysine plus threonine.

A broad spectrum of variation in plant color was recovered from all the lysine plus threonine-containing selections both with and without AEC.

Among the second generation lines selected one had elevated percent lysine, another line had normal as well as high lysine seed, whereas a third line had only normal seed lysine. The improved lysine was transmitted by selfing to the next generation. We believe the *in vitro* selections with inhibitory levels of L+T provide efficient methods for the recovery of unique, specific, and possibly useful phenotypes in rice.

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LITERATURE CITED

1. BLAYDES DR 1966 Interaction of kinetin and various inhibitors in the growth of soybean tissue. Physiol Plant 19: 748–753
2. BRIGHT SWJ, PR SHEWRY, BJ MIFLIN 1978 Aspartate kinase and the synthesis of aspartate-derived amino acids in wheat. Planta 139: 119–125
3. BRIGHT SWJ, LC FEATHERSTONE, BJ MIFLIN 1979 Lysine metabolism in a barley mutant resistant to *S*-(2-aminoethyl)cysteine. Planta 146: 629–633
4. BRIGHT SWJ, PB NORBURY, BJ MIFLIN 1980 Isolation and characterization of barley mutants resistant to aminoethylcysteine and lysine plus threonine. *In* F Sala, B Parisi, O Ciferri, eds, Plant Cell Cultures. Results and Perspectives. Elsevier/North-Holland Biomedical Press, Amsterdam, pp 179–182
5. BROCK RD, EA FRIEDERICH, J LANGRIDGE 1973 The modification of amino acid composition of higher plants by mutation and selection. *In* Nuclear Techniques for Seed Protein Improvement. International Atomic Energy Agency, Vienna, Austria, pp 193–198
6. BRYAN PA, RD CAWLEY, CE BRUNNER, JK BRYAN 1970 Isolation and characterization of a lysine-sensitive aspartokinase from a multicellular plant. Biochem Biophys Res Comm 41: 1211–1217
7. CATTOIR-REYNAERTS A, E DEGRYSE, I VERBRUGGEN, M JACOBS 1983 Selection and characterization of carrot embryoid cultures resistant to inhibition by lysine plus threonine. Biochem Physiol Pflanzen 178:81–90
8. FURUHASHI K, M YATAZAWA 1970 Methionine-lysine-threonine-isoleucine interrelationships in the amino acid nutrition of rice callus tissue. Plant Cell Physiol 11: 569–578
9. GENGENBACH BG, TJ WALTER, CE GREEN, KA HIBBERD 1978 Feedback regulation of lysine, threonine and methionine biosynthetic enzymes in corn. Crop Sci 18: 472–476
10. GREEN CE, RL PHILLIPS 1974 Potential selection system for mutants with increased lysine, threonine and methionine in cereal crops. Crop Sci 14: 827–830
11. GUHA-MUKHERJEE S 1973 Gametophytic differences in the in vitro formation of embryoids from rice pollen. J Exp Bot 24: 139–144
12. HENKE RR, KG WILSON, JW MCCLURE, RW TREICK 1974 Lysine methionine-threonine interactions in growth and development of *Mimulus cardinalis* seedlings. Planta 116: 333–345
13. HIBBERD KA, CE GREEN 1982 Inheritance and expression of lysine plus threonine resistance selected in maize tissue culture. Proc Natl Acad Sci USA 79:559–63.
14. LAEMMLI UK 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685.
15. MATTHEWS BF, JM WIDHOLM 1978 Regulation of lysine and threonine synthesis in carrot cell suspension cultures and whole carrot roots. Planta 141: 315–321
16. MERTZ TE 1976 Case histories of existing models. *In* Genetic Improvement of Seed Proteins, pp 57–70. Proceedings of Workshop, March 18–20, 1974. Board of Agriculture and Renewable Resources National Academy of Science, Washington, DC
17. MERTZ TE, LS BATES, OE NELSON 1964 Mutant gene that changes protein composition and increases lysine content of maize endosperm. Science 145: 279–280.
18. MURASHIGE T, F SKOOG 1962 A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15: 473–497
19. NAKAYAMA K, H TANAKA, H HAGINO, S KINOSHITA 1966 Studies on lysine fermentation. V. Concerted feedback inhibition of aspartokinase and the absence of lysine inhibition on aspartic semialdhyde-pyruvate condensation in *Micrococcus glutamicus*. Agric Biol Chem 30: 611–616
20. NEGRUTIU I, A CATTOIR-REYNEARTS, I VERBRUGGEN, M JACOBS 1984 Lysine overproduced mutants with an altered dihydrodipicolinate synthase from protoplast culture of *Nicotiana sylvestris* (Spegazzini and Comes). Theor Appl Genet 68: 11–20

21. RUTGER JN, ML PETERSON, CH HU 1977 Registration of Calrose 76. Crop Sci 17: 978
22. SANO K, I SHHO 1970 Microbial production of L-lysine. III. Production of mutants resistant to *S*-(2-aminoethyl)-L-cysteine. J Gen Appl Microbiol 16: 373–391
23. SCHAEFFER GW, PS BAENZIGER, J WORLEY 1979 Haploid plant development from anthers and in vitro embryo culture of wheat. Crop Sci 19: 697–702
24. SCHAEFFER GW, FT SHARPE 1981 Lysine in seed protein from *S*-aminoethyl-L-cysteine resistant anther-derived tissue cultures of rice. In Vitro 17: 345–352
25. SCHAEFFER GW 1982 Recovery of heritable variability in anther-derived doubled haploid rice. Crop Sci 22: 1160–1164
26. SCHAEFFER GW, FT SHARPE JR 1983 Mutations and cell selections: genetic variation for improved protein in rice. *In* L Owens, ed, Proc Symp Genet Engineering: Applications to Agriculture. Rowman & Allanheld, Totowa, NJ, pp 237–254
27. SCHAEFFER GW, FT SHARPE JR, PB CREGAN 1984 Variation for improved protein and yield from rice anther culture. Theor Appl Genet 67: 383–389
28. SCHAEFFER GW, FT SHARPE, HL CARNAHAN, CW JOHNSON 1986 Anther and tissue culture-induced grain chalkiness and associated variants in rice. Plant Cell Tissue Organ Cult 6: 149–157
29. WENKO LK, RW TREICK, KG WILSON 1985 Isolation and characterization of a gene encoding *meso*-diaminopimelate dehydrogenase from *Glycine max*. Plant Mol Biol 4: 197–204
30. 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