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Segregation for endosperm lysine in F_2, F_3 and F_4 progeny from a cross of in vitro-selected and unselected cultivar of rice *

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Summary. Lysine is a limiting amino acid for optimal nutritional quality in rice grain. In vitro selections using inhibitory levels of lysine plus threonine or s-aminoethylcysteine allow the predictable recovery of variants with elevated levels of lysine and protein. These methods may generate useful starting germplasm for plant breeders. This study was conducted to define the genetics of lysine mutants in progeny from crosses of mutants derived from cells cultured in vitro in the presence of inhibitory levels of lysine plus threonine and s-(2-aminoethyl)-cysteine. In vitro selections produce a wide range of mutants, including endosperm mutants with elevated lysine and protein levels as well as mutants for high and low seed weights. Mutants were analyzed for lysine content by the endosperm half-seed method in which the halves without the embryo were ground and acid hydrolyzed for amino acid determinations. The halves with the embryos were preserved for later germination. In two different F_2 populations derived from a cross of a selected mutant × M-101, a parental marker, there was an inverse relationship between seed weight and percent lysine in endosperm protein (R^2 0.52 and 0.56). The F_2 segregation patterns show that elevated lysine is inherited as a recessive gene and that increased lysine is correlated with decreased seed size. F_3 and F_4 data provide evidence for the transmission of high lysine genes to advanced germplasm in rice. This work supports our earlier conclusions that high lysine phenotypes can be recovered predictably from in vitro selections. The elevated lysine phenotypes are frequently, but not exclusively, associated with opaque seed. Some segregants from crosses produced increased lysine in plants with near normal seed weight and good fertility.

Key words: *Oryza sativa* L. – Biochemical selections – Analogs – Mutants – Seed size – Opaque

Introduction

It is well established that the nutritional quality of the major cereals would be significantly increased if the lysine content, particularly protein-bound lysine, were increased (Mertz 1976; Mertz et al. 1964). The recovery of mutants for the synthesis or metabolism of lysine would provide useful genotypes for basic and molecular studies and may provide information directly applicable to the improvement of the nutritional quality of rice through traditional breeding methods. Plant regeneration from cells or calli of major crop cultivars opened new avenues of research. One useful approach is the recovery of cells resistant to specific metabolic inhibitors, hence the possible recovery of mutants with specific metabolic functions.

Strategies are available for the recovery of mutants in the lysine biosynthetic pathway in microbial systems (Sano and Shho 1970). Current evidence shows that the β-aspartate pathway exists in higher plants. However, detailed information on this pathway is limited and additional or alternate pathways have been suggested (Wenko et al. 1985). Evidence for the function of alternate pathways is provided by the isolation of the meso-diaminopimelate dehydrogenase enzyme from rice (Wenko and Schaeffer, unpublished results) and soybeans (Wenko et al. 1985). Much additional research is required before the regulation of lysine synthesis and metabolism as well

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as the role of the different pathways is defined in rice or other major crops.

The complex control systems in eukaryotes and the interaction of numerous factors in the control of gene expression in higher plants, along with a lack of basic metabolic information in crop plants, precludes facile recovery and characterization of genes along the lysine pathway. Nonetheless, the use of amino acid analogs, particularly amino-ethylcysteine (AEC) and inhibitory levels of metabolic products such as lysine plus threonine (L+T) have promoted some progress in mutant selections in carrots (Cattoir-Reynaerts et al. 1983; Matthews and Widholm 1978), maize (Gengenbach et al. 1978; Green and Philips 1974; Hibberd and Green 1982), rice (Schaeffer and Sharpe 1981, 1983, 1987; Schaeffer et al. 1986), and tobacco (Negrutiu et al. 1984). Studies on the lysine metabolism and amino acid relationships in plant growth and development have also been reported (Bryan et al. 1970; Furuhashi and Yatazawa 1970; Henke et al. 1974). The use of anther-derived haploid or doubled haploid cells (Schaeffer et al. 1984) along with the application of specific biochemical selection pressures (Schaeffer and Sharpe 1981, 1987) results in the recovery of a broad spectrum of variants in rice. These variants are sometimes beneficial, frequently neutral, and often deleterious to whole plant production. The use of specific inhibitors eliminates incidental mutants not carrying resistance to the selective agents and provides an element of predictability not readily achieved by whole plant selections.

In 1970 Bryan et al. isolated the first enzyme in the lysine pathway, β-aspartokinase, from plants and demonstrated feedback inhibition as one form of metabolic regulation of the isolated enzyme. Several years ago, maize plants from specific germplasm were recovered from L+T selections (Gengenbach et al. 1978; Green and Philips 1974; Hibberd and Green 1982) and progeny had higher threonine levels than the controls, but the lysine levels were not greatly changed (Hibberd and Green 1982). In 1981, Schaeffer and Sharpe reported the recovery of cell lines resistant to AEC from anther-derived calli of Assam 5, a cultivar with indica subspecies characteristics. Plants regenerated from these selections and selfed progeny had improved seed protein lysine as well as improved protein levels (Schaeffer and Sharpe 1981, 1983). Similarly, in 1987 plants were regenerated from cells of Calrose 76, subspecies japonica, resistant to L+T and AEC (Schaeffer and Sharpe 1987). Some mutants had 14% more lysine in seed storage proteins than the control cultivar. Protein levels were also improved as was the case in the Assam selections. Large accumulations of free lysine in seed did not occur. The improved level of bound lysine was transmitted by selfing to S_3 and S_4 generations (Schaeffer and Sharpe 1987). There were no increases in the threonine levels of endosperm proteins. Apparently rice responds differently than maize to the L+T selections or a different type of mutant was recovered. Also, the passage of rice cells through anther and tissue culture and in vitro selection procedures can induce grain chalkiness in regenerated plants (Schaeffer et al. 1986).

The purpose of the experiments reported here was to study the genetics of lysine expression in rice and to define more precisely the relationship between percent lysine, seed size, and chalkiness in F_2 to F_4 populations of crosses between high lysine mutants recovered from in vitro selections and the parental and marker cultivars.

Materials and methods

Biochemical selections

Anther-derived callus was subjected to inhibitory levels of 1 m*M* or higher lysine plus threonine (L+T) followed by S-(2-aminoethyl)-cysteine (AEC) as described earlier (Schaeffer and Sharpe 1987). Plants were regenerated from surviving calli and selfed two or more times and crossed to M-101 with glabrous marker to identify F_2 segregants.

Parents of the cross

Parent 1 (P1) designated 397-2-4C (4C), originally derived from Calrose 76, was selfed 3× after regeneration from callus-resistant to inhibitors and carries the gene(s) for improved lysine described above. One S_3 plant was used as the female parent in the cross (4C × M-101). Parent 2 (P2), the male parent, was M-101, a cultivar registered by Rutger et al. (1978) containing a single recessive marker gene for glabrousness (smooth leaf surface) (Jodon 1977).

Progeny

Two F_1 seeds were recovered from the cross 4C × M-101. During the vegetative phase, the two plants were slightly different in color and leaf width. The plant with somewhat narrower leaves and deeper green color than either parent was designated cross 1 or (G, for deep green color) and the other plant which was morphological more like the parents was designated cross 2 or (N).

Progeny characterizations

The embryo half of the F_2 seeds was germinated and grown to maturity in the greenhouse at Beltsville, Maryland. Whole plant data collected included: seed number/plant, seed weight, and glabrous or nonglabrous (rough) leaf surface. The F_2 population from both the (G) and the (N) cross was subdivided into normal and high lysine subpopulations for further comparisons. The lysine level is considered high if the endosperm protein has 3.55% or more lysine. Data presented for the F_3 of the (N) cross includes two subsets representing totally opaque and nearly clear classification. Single F_2 seeds were selected for opaque and low chalky or clear appearance. These were germinated and grown in the greenhouse. Lysine and protein determinations of seeds representing the F_3 progeny were made on 30 half-seed composite samples representing the endosperm half of each of four plants of the two subsets. Additionally, seeds representing F_4 progeny were analyzed from 13 plants.

Seed chalkiness

Chalkiness was scored from 1–10 by subjective rating of the transmission of fluorescent light through seeds as already described (Schaeffer et al. 1986). A score of 1 represents clear seed which is a characteristic of many cultivars in production, and 10 represents fully opaque seeds. Fully opaque seed with a score of 9 or 10 includes those with soft easily crumbled endosperm and those with irregular shapes. Opaque seeds are frequently shrivelled. These characteristics are often associated with the high lysine phenotype, particularly in the early generations. The opaque designation is limited to seeds with ratings of 9 or 10, with soft endosperm whereas the more general term, chalkiness, includes opaque seeds as well as those with partial irregularities in light transmission, scores 2–10.

Amino acid and protein determinations

The following were split transversely into nearly equal portions (Schaeffer and Sharpe 1987): 555 seeds from two F_1 plants representing F_2 populations and 30 half-seed composite samples from eight plants representing F_3 progeny. Seven or more half-seeds from each of 13 plants representing F_4 progeny were similarly processed. The endosperm portion of the dehulled half-seeds was ground in a mortar and pestle and a 5 mg sample of brown rice endosperm was hydrolyzed in 6 N HCl under N_2. The amino acids were separated with W3H cation exchange resin and quantified with a Beckman 119BL[3] analyzer (Schaeffer and Sharpe 1987). Protein levels are expressed as the sum of all amino acids (AA) in micromoles in the acid hydrolyzates detected with the amino acid analyzer, which was periodically calibrated with external standards. The AA values can be converted to μmol/mg of endosperm by a multiplication factor of 0.693.

Results

F_2 segregants for lysine and seed weight

Crosses made with the improved lysine line, 4C, show that the high lysine phenotype is inherited as a recessive trait and is associated with reduced seed weight. F_2 populations consist of distinct segregants for seed weight with a breakpoint of 20.5 mg and lysine level with a breakpoint of 3.55% (Figs. 1 and 2). Most seeds weighed from 25–29 mg and had from 2.5%–3.5% lysine in endosperm protein. Plants with seed weights ranging from 13–20 mg constitute the principal high lysine population, which average 3.8% lysine. The two major groups of plants are cleary distinguishable with the small-seeded, high lysine plants forming a skewed portion of the overall distribution (Fig. 2). The distribution for percent lysine and for seed weights form similar but reciprocal histograms (Figs. 1 and 2). Seventy-one seeds of the (G) cross were classified high lysine, i.e., greater than 3.55%, and 228 were normal. The highly significant negative correlation $R^2 = 0.56$ ($P < 0.001$) of lysine level with seed weight is illustrated in Fig. 3. The high lysine types may have either elevated or normal protein levels. In the (G) and (N) crosses, the high lysine phenotype is associated with opaque seed and soft, easily crumbled endosperm as well

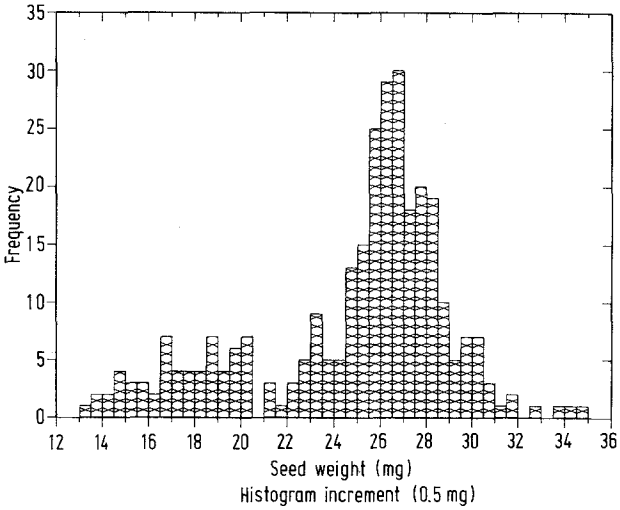

Fig. 1. Histogram of single seed weights of F_2 population of brown rice from 4C × M-101 (G). Mean seed weight of Calrose 76, 4C and M-101 is 19.3, 18.1, 20.3, respectively. N = 299. Bar width represents 0.5 mg

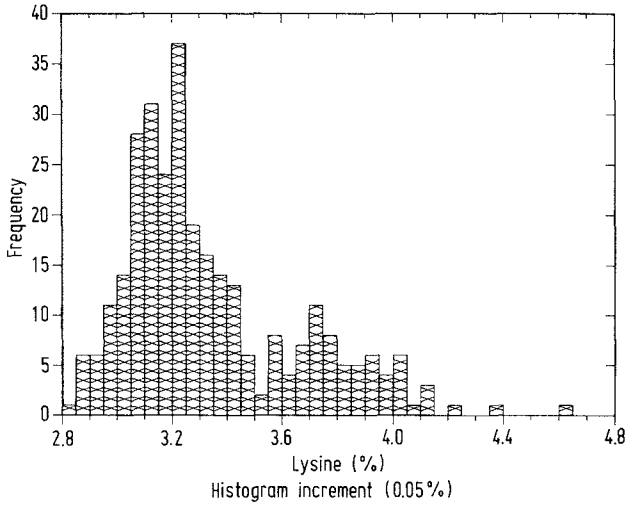

Fig. 2. Histogram of percent lysine in endosperm protein of single seeds of F_2 population representing the cross 4C × M-101 (G). Mean percent lysine of Calrose 76, 4C, and M-101 is 2.87, 3.57, and 3.22, respectively. N = 299. Bar width represents 0.05%

as reduced seed size. There is a significant correlation, $R^2 = 0.51$ ($P < 0.001$), between reduced seed weight and increased lysine in the (N) cross. The overall results for the F_2 populations from the (G) and (N) crosses were remarkably similar.

Table 1 illustrates minima, maxima, and means for the parental lines, complete F_2 populations, and the normal and high lysine components of the F_2 populations. The high lysine component of the F_2 shows that some seeds were heavier than the parental type but less so than

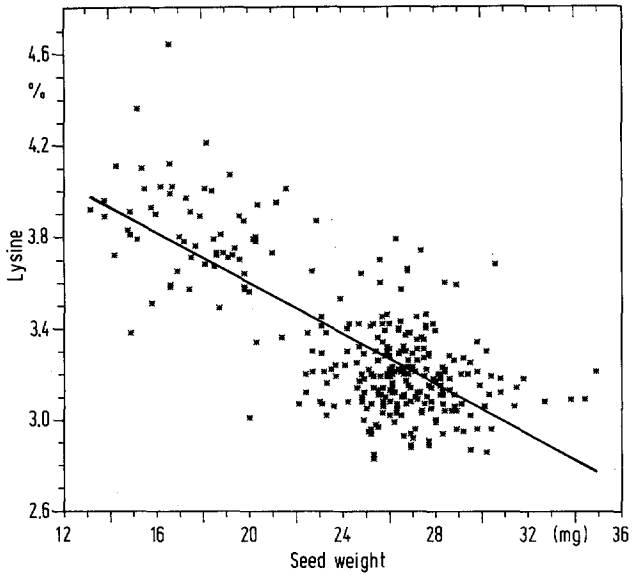

Fig. 3. Scatter plot and regression line for percent lysine in endosperm protein and seed weight of F_2 population from the cross 4C × M-101 (G). N = 299

some normal lysine segregants within the F_2 population. The smallest seeds recovered were in the high lysine population and the largest in the normal component of the F_2 population. The means reflect heterosis for seed size from the cross. The data for lysine levels are clear with the tissue culture-derived parent and the small seeded segregants producing the highest means.

Lysine and protein relationships

Both the high lysine F_2s and to some extent the M-101 parent had high protein levels. High protein values, 1.2 μmol amino acids/seed or more, for the F_2 segregants may be due in part to reduced fertility in the high lysine plants. Differences in seed number per plant make between-plant comparisons for protein levels difficult. However, this is not the case with lysine under normal growth environments. In these experiments, between-plant variation is eliminated by the within-plant half-seed method. Physiological components of the lysine/protein relationship should be nearly constant in this within-plant procedure; hence, the probability for the recovery of true genetic contributions is enhanced. Of the high lysine seeds, 25% had low to normal protein (1.0 μmol AA/seed or less) and 75% had normal to high protein levels.

The relationship between percent lysine and endosperm protein was nonsignificant ($R^2 = 0.001$). This relationship is nonsignificant in both the high and low lysine subsets of F_2 seeds in both crosses. In cultivar comparisons there is often a negative correlation between percent lysine in protein and the level of endosperm protein in cultivars with less than 10% protein (Juliano 1985). High protein cultivars may have a positive correlation (Juliano 1985).

Table 1. Characteristics of Calrose 76, PI (4C), P2 (M-101), F_2 population, lysine and subpopulations representing normal lysine and selected high lysine (equal to or greater than 3.55%) segregants. ±SE represents standard error

Genotype	N	Minimum	Maximum	Mean ±SE
Seed weight (mg)				
Control-Cal 76	20	14.3	22.5	19.3 ±0.439
P_1 (4C)	15	15.9	20.6	18.1 ±0.347
P_2 (M-101)	15	15.4	25.0	20.3 ±0.684
F_2 Total (G)	299	13.2	34.9	24.8 ±0.247
Normal lysine	228	14.9	34.9	26.6 ±0.167
High lysine	71	13.2	30.6	19.3 ±0.475
F_2 Total (N)	256	13.5	32.6	25.6 ±0.237
Normal lysine	186	17.2	32.6	25.3 ±0.187
High lysine	70	13.5	26.6	19.1 ±0.316
Lysine, (%) of total amino acids in seed hydrolyzates				
Control-Cal 76	20	2.62	3.44	2.87±0.084
P_1 (4C)	15	3.36	3.71	3.57±0.115
P_2 (M-101)	15	2.93	3.65	3.22±0.106
F_2 Total (G)	299	2.83	4.64	3.33±0.018
Normal lysine	228	2.83	3.53	3.18±0.010
High lysine	71	3.56	4.64	3.82±0.024
F_2 Total (N)	256	2.80	4.32	3.39±0.020
Normal lysine	186	2.80	3.54	3.22±0.012
High lysine	70	3.56	4.32	3.84±0.022
Protein, total amino acids (μmol)/analyzer sample				
Control-Cal 76	20	0.58	1.55	1.04±0.043
P_1 (4C)	15	0.84	1.13	1.00±0.054
P_2 (M-101)	15	1.02	1.66	1.26±0.052
F_2 Total (G)	299	0.52	1.70	1.09±0.010
Normal lysine	228	0.58	1.62	1.07±0.010
High lysine	71	0.52	1.70	1.16±0.026
F_2 Total (N)	256	0.66	1.69	1.08±0.010
Normal lysine	186	0.66	1.69	1.07±0.012
High lysine	70	0.67	1.50	1.04±0.021

The single-seed analyses of F_2 seeds produced a 3:1 ratio of low or normal lysine to high lysine (228:71) and a Chi square of 0.28 ($P < 0.90$ and > 0.50) for that ratio. The distribution for seed weights, illustrated in Fig. 1, does not fit precisely the 3:1 segregation pattern but resembles more closely a 13:3 ratio characteristic of a two-gene system with a dominant inhibitor or modifier. The Chi square for the 13:3 goodness of fit is 0.34 (P approximately 0.90). Additional biochemical evidence is needed to define the role of inhibitors in the genetics of seed size, but the primary conclusion that lysine is inherited as a recessive character in clear.

The values of seed chalkiness, seed weight, percent lysine and protein levels are given in Table 2. The high lysine seeds were predominantly but not exclusively in the highest chalkiness classes, ratings 9–10. These classes also had seeds with the highest protein levels. There was no significant difference among classes 1–8 in seed weight, percent lysine, and protein level.

F_3 progeny

The inheritance of the opaque character, the lysine level, and seed weights in F_3 progeny is shown in Table 3. The range in seed number/plant was the greatest in the opaque or chalky class with 1 plant producing the normal number and 1 plant only 110 seeds, approximately 1/10 of normal. Seed weights were lowest and percent lysine highest in progeny from opaque seed. Some seeds rated as low chalky, rating 4, produced high lysine F_3 progeny; thus, the opaque characteristic is not a requirement for improved lysine. The relationship between seed weight and percent lysine was confirmed in the second cross (N) with near normal seed set. One line had normal seed weight, reduced chalkiness, and high lysine (Table 3, plant no. 6). The small-seeded types, less than 20 mg/seed, represent primarily the opaque population and those larger than 20 mg/seed represent the non-opaque classes (Fig. 3). The R^2 value for the negative correlation of percent lysine and seed weight for this population of 256 plants of the (N) cross was 0.52 ($P<0.001$). Percent lysine, seed weight, and the opaque seed characteristics are genetically conditioned and expressed as illustrated in the F_2 and F_3 populations.

Table 2. Segregation of F_2 population (F_2 seed on F_1 plant) and means and standard errors of associated characteristics from single half-seed analyses of the cross 4C × M-101 (G) for opaque/chalky endosperm. Rating of 10 is fully opaque and rating of 1 represents clear seed. N = 299

Opaque/chalky		Seed weight (mg) ±SE	Lysine (%) ±SE	Protein (μmol) ±SE
Rating	N			
10	67	20.1±0.69	3.71±0.04	1.17±0.02
9	22	23.3±0.98	3.39±0.08	1.15±0.05
8	30	26.2±0.33	3.20±0.03	1.06±0.03
7	30	26.7±0.37	3.28±0.04	1.05±0.03
6	23	27.2±0.40	3.21±0.03	1.08±0.03
5	21	26.1±0.44	3.16±0.03	1.08±0.02
4	25	29.9±0.35	3.20±0.05	1.07±0.03
3	36	26.7±0.33	3.16±0.03	1.06±0.12
2	32	26.0±0.38	3.23±0.03	1.07±0.03
1	13	25.6±0.55	3.22±0.03	1.01±0.07

Fertility of F_2 and F_3 progeny

The progeny from the (G) and (N) cross had characteristics not found in the parents of the cross: for example, some segregants had incomplete panicle exsertion from the leaf sheath and some segregants appeared less determinate in growth habit than controls. Another form of variation was a high level of infertility, particularly during winter months, in the F_2 plants and hence reduced seed yield from both the (G) and (N) cross. M-101 also produced some abnormal plants with infertility during this winter growth cycle. Also, germination of the F_2 seed was only 83%, and 37% of the plants recovered were totally infertile during the winter months in Maryland. Of the plants that produced seed, 22% were from high lysine seed (3.55% or higher) and 78% were seed containing low lysine (3.54% or less). The infertility was not limited to the high lysine phenotype but was more intense than in low lysine types. F_2 segregants from the (N) cross produced fully fertile high lysine types with 1430 seeds/

Table 3. Characteristics of F_3 progeny from single F_2 seeds selected for opaque and low-chalky appearance. F_2 seeds came from the cross 4C × M-101 (N). Lysine and protein determinations were made from a 30 seed composite sample (endosperm half only) of each plant. Plants grown in Summer/Fall of 1987. SE ± standard error

Plant no.	Seeds/plant	Mean seed wt. (mg)	Chalky rating	Lysine (%)	Protein (μmol AA)
Opaque (A)					
1	110	15.6	10	3.99	1.08
2	1,430	18.8	10	3.84	1.41
3	264	17.7	10	4.04	1.24
4	924	18.0	10	3.93	1.20
Means ±SE	682±305.5	17.53±0.68	10	3.9±0.04	1.2±0.08
Low chalky (B)					
5	924	24.8	2	3.55	0.96
6	726	21.1	4	4.15	0.82
7	770	25.3	3	3.31	1.00
8	616	23.6	3	3.42	1.14
Means ±SE	759±63.8	23.7±0.94	3	3.60±0.19	1.00±0.07

plant and also produced types 90% infertile (Table 3). The recovery of fertile segregants shows that the infertility can be genetically uncoupled from the high lysine phenotypes. In addition, the single segregant in the low chalky group with 4.15% lysine has normal seed weights. Even though this weight is the smallest among the low chalky group, its seed weight was equal to that of Calrose 76 (Tables 1 and 4). F_2 seed of the cross grown in the greenhouse in 1987–88 winter were highly atypical in fertility, floral morphology, and plant form and segregation patterns for percent lysine could not be reliably established. Random samples of the F_3 seed showed all lysine values shifted downward by more than 5%, i.e., 3.39%–3.20%. Protein values were shifted upward by as much as 40%. Even so, a segregation pattern close to 3:1 could be established based on seed size and the upper range of lysine values for 19 F_2 progeny bearing F_3 seed. The R^2 value for the negative correlation of F_2 percent lysine with the next generation seed weight was 0.54 ($P<0.001$).

F_4 progeny

The average percent lysine (average of seven half-seeds) for the nine plants which make up this group of F_4 progeny were 3.75, 3.73, 3.70, 3.63, 3.62, 3.60, 3.41, 3.28, and 3.27. Two are considered control level and seven had lysine values greater than the source mean of Calrose 76 or M-101. The line with the highest average percent lysine gave individual half-seed values of 4.04, 3.90, 3.86, 3.81, 3.71, 3.58, and 3.38. The second highest gave half-seed responses of 3.93, 3.90, 3.78, 3.70, 3.67, 3.63, and 3.56. Clearly the responses of six out of the nine advanced lines were greater than the original source by 0.3%–0.6% lysine. This represents a 9.3%–18.6% improvement of lysine over the M-101 control and 10.3%–20.5% improvement over the Calrose 76 control, the starting material for the in vitro selections and the source of 4C. Figure 4 shows the distribution of percent lysine in four populations of plants including F_4 progeny, cross 4C × M-101(N); 4C-mutant selfed; M-101, the male parent of the cross; and Calrose 76, the cultivar used for the in vitro selections. The means for these four populations are given in Table 4. Also shown in Table 4 are high lysine progeny (F_3 plant no. 2) representing more F_4 progeny. The small F_4 population with a mean of 3.56% lysine, plant no. 6, had segregants for both high and low lysine showing that line 6, one source of the F_4 population, was not yet fully homozygous or fully stable. Nonetheless, high lysine segregants were recovered. Three of the F_4 lines produced uniformly low lysine seed, two had intermediate lysine levels and four had uniformly high lysine seed. The F_4 population showed the typical negative correlation between seed weight and percent lysine, $R^2=0.65$ ($P<0.050$ and >0.010).

Table 4. Mean seed weight, percent lysine and protein levels from half-seed analyses of seed having lysine values 3.55% or higher and F_4 progeny of seven half-seeds from each of nine lines derived from plant 6, Table 3, and 7–11 half-seeds from each of four lines derived from plant 2, Table 3. Progeny from the cross, 4C × M-101 (N), are compared with three controls: Calrose 76, M-101 and selfed mutant 4C. Means of the three controls are composites from several experiments. SE represents standard error of the means, AA = amino acids

Genotype	N	Seed weight (mg ±SE)	Lysine (%) ±SE	Protein (μmol AA) ±SE
Calrose 76	40	19.6±0.11	2.92±0.011	1.14±0.015
M-101	69	20.5±0.11	3.22±0.009	1.27±0.006
4C mutant	105	17.9±0.02	3.57±0.002	1.13±0.003
F_4 (F_3 plant 6)	63	20.2±0.15	3.56±0.010	1.51±0.011
F_4 (F_3 plant 2)	31	16.5±0.38	3.68±0.032	1.62±0.043

Composite samples of S_2 and S_3 plants of the 4C-mutant had mean lysine values of 3.57% with 71 out of the 105 entries greater than 3.55%. Both Calrose 76 and M-101 had low lysine means, 2.92 and 3.22, respectively. The M-101 has a wider range of variation in seed lysine than Calrose 76. This may be due to different photosensitivities and due in part to environmental interaction expressed during the winter growth cycle.

The overall results demonstrate the value of the half-seed method for the recovery of within-cultivar variation for lysine and possibly protein, and illustrate the potential for the recovery of other biochemical variants in this and other cultivars not previously selected. Additionally, many of the lines are apparently improved in protein content. We made no attempt to prove this point because seed number/plant in many segregants was lower than the control making objective conclusions about protein genetics impossible. Nonetheless, individual plants with excellent seed number were recovered with elevated lysine and protein (Table 3).

Segregation of marker genes

Segregation data for the glabrous marker used in the cross gave the expected 3:1 ratio for the single recessive gene character which provides evidence for normal meiotic processes. Adult plants scored produced 180 non-glabrous types and 67 glabrous types, Chi-square = 0.595 ($P<0.90$ and >0.50). The average lysine value for the seeds which carried the non-glabrous and glabrous alleles was 3.31% and 3.30%, respectively.

Discussion

Two unique features reported earlier showed that high lysine phenotypes were recovered predictably from anther-derived callus with inhibitors such as lysine plus

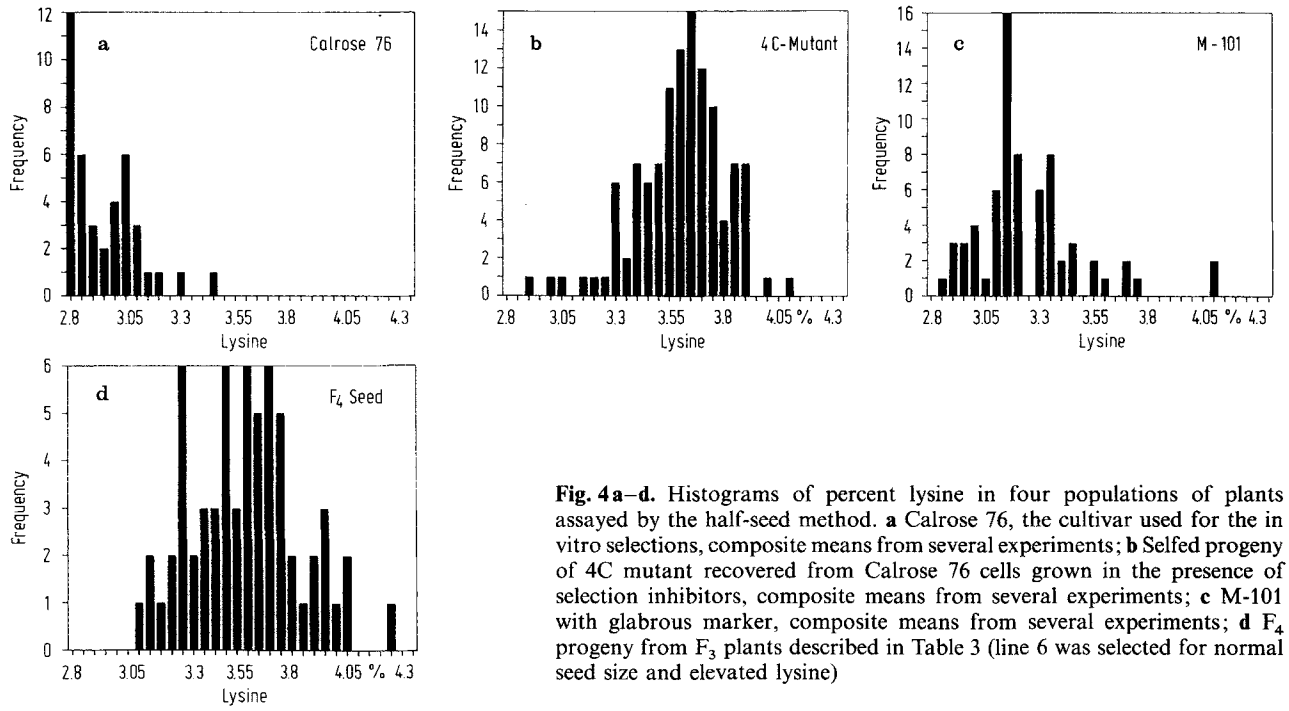

Fig. 4a–d. Histograms of percent lysine in four populations of plants assayed by the half-seed method. **a** Calrose 76, the cultivar used for the in vitro selections, composite means from several experiments; **b** Selfed progeny of 4C mutant recovered from Calrose 76 cells grown in the presence of selection inhibitors, composite means from several experiments; **c** M-101 with glabrous marker, composite means from several experiments; **d** F_4 progeny from F_3 plants described in Table 3 (line 6 was selected for normal seed size and elevated lysine)

threonine and s-(2-aminoethyl)-cysteine (Schaeffer and Sharpe 1987), and that the single half-seed method is an excellent tool for the evaluation of progeny from single plants for lysine and protein levels as well as SDS-PAGE electrophoretic profiles. The seeds on F_1 plants represent the full F_2 genetic potential; nonetheless, these seeds carry some F_1 components in the form of the aleurone cells with unique characteristics (Juliano 1972). Since the volume of the aleurone layer is small relative to the volume of the endosperm and since the seeds with the increased lysine are frequently opaque, an endosperm characteristic of most of our high lysine lines, we are confident that the presence of the aleurone layer does not interfere with the classification of the segregants for opaque endosperm, which is often associated with high lysine in these experiments. Just as the F_1 provides the genetic potential for the F_2, so the single seeds on F_2 plants represent the range of genetic potential for the F_3 endosperm characteristics. Demonstrated here is the application of the half-seed method for the characterization of F_2, F_3, and F_4 progeny from selfed seeds of F_1 plants for lysine in protein and other characteristics. Others have recently demonstrated the single seed method for the genetic analyses of amylose content (Kumar and Khush 1987) in rice.

The inheritance of elevated lysine in rice fits the single recessive gene model even though modifiers of seed size probably play a significant synergistic role. Since protein levels are modified by seed number and many of the high lysine plants were partially infertile, we do not provide information on the genetics of protein levels in these progeny even though the high lysine plants could be either normal or above normal in protein level. Those seeds with exceptionally high protein levels, 1.5–2 times the control level, often had displaced SDS-PAGE protein profiles and did not germinate. There is no evidence that percent lysine in endosperm protein is greatly altered by changes in seeds/plant. Photosynthetic rates, photoperiod, and temperature may influence percent lysine in rice grains.

One unusual finding in these experiments was that all of the in vitro L + T selections produced plants in the S_2 that had chlorophyll variants (Schaeffer and Sharpe 1987). Also, extensive infertility was observed in low and high lysine plants but particularly in the high lysine populations. The infertility was most obvious in those plants that had been selfed several times and had some chlorophyll, leaf size, and shape deviations from normal. The infertility is most intense in completely homozygous material, whereas the heterozygous forms show better fertility. In the homozygous form some plants have very abnormal flowers which are completely sterile and often have multiple stigmas. This abnormal flower development was most severe during winter when photon flux was low and photoperiod short. These plants may be manually cross-fertilized but with greater difficulty than normal plants, and this suggests that the infertility is at least partially caused by both male and female compo-

nents. These abnormal but open flowers may also promote involuntary outcrossing.

Even though the genetic results show distinct segregants for increased lysine from the in vitro-recovered mutants, we do not yet know the biochemical nature of the mutants. Experiments to define the mutants are now in progress. In spite of predictable recovery of potentially valuable germplasm by in vitro selection, the phenotypes in their present form are not now of cultivar quality. Cultivar development was not the primary objective of this study. Nonetheless, some segregants have good overall characteristics and appear promising under greenhouse conditions.

Occasionally, high lysine seeds produce plants with low lysine progeny. This variable response in specific genotypes may be due to one or a combination of several causes: (1) unstable germplasm, perhaps due to the presence of transposable elements; (2) outcrossing due to open male sterile flowers; and (3) alternative expression of high lysine phenotypes as altered protein levels, i.e., high percent lysine with normal protein, or low lysine with high protein, or intermediate levels of both percent lysine and total protein. One consistent observation had been the occurrence of chlorophyll and fertility variants among all the high lysine lines and our current speculation is that these variants are conditioned by unstable genetic elements of the transposable type with integration sites in organelle and nuclear DNA. These alternatives will be examined in future research.

Our current experiments were designed firstly to recover lysine mutants and secondly to develop germplasm with modified lysine. The germplasm recovered from in vitro procedures has increased lysine and/or protein levels above the starting cultivar, Calrose 76 (Rutger et al. 1977), extensively grown in the past.

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