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Modification of amino acid composition of endosperm proteins from in-vitro-selected high lysine mutants in rice

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Summary. Endosperm protein mutants in rice may be recovered by biochemical selections with inhibitory levels of lysine and threonine. Among the phenotypes recovered from in vitro selections are lines with increased protein and percent lysine in the protein. This work was designed to identify changes in proteins of rice mutants and to further our understanding of the mechanisms of lysine plus threonine selections in rice. Among the most obvious amino acid changes in mutants was a higher lysine level in all protein solubility fractions and a decrease in tyrosine. Methionine and glutamate are reduced in some protein fractions. However, methionine is significantly higher in the mutant than the control in the glutelin fraction. Several other aspartate pathway amino acids are higher in the mutant than the unselected controls. Separation of proteins in SDS-PAGE gels showed shifts in the protein profiles in the mutants, including a decrease in the major 30 kDa low lysine globulin component, and an increase in several high-molecular-weight components, approximately 60–100 kDa. Increases in the lysine content of proteins of different solubility classes and different proteins within classes are detailed.

Key words: Biochemical selections – Lysine – Mutants – Proteins – Rice

Introduction

Cells in culture provide some advantages over whole plants for the application of biochemical selection pressure and the recovery of specific metabolic mutants (Cattoir-Reynaerts et al. 1983; Furuhashi and Yatazawa 1970; Green and Philips 1974; Hibberd and Green 1982; Schaeffer and Sharpe 1981, 1987). The growth of rice (*Oryza sativa L.*) cells in vitro as anther-derived callus at inhibitory levels of lysine plus threonine (lys + thr) and other inhibitors permits the selective recovery of plants with altered seed storage proteins (Schaeffer and Sharpe 1981, 1987; Schaeffer et al. 1988). Mutants have increased lysine in endosperm proteins, which is usually accompanied with decreased seed size and chalky endosperm (Schaeffer et al. 1986, 1988). The nutritional quality of cereals could be improved with an increase in free or protein-bound lysine (Mertz 1976; Mertz et al. 1964).

Metabolic regulation and pathway interactions are much more complicated in higher plants than in microbial systems (Sano and Shiio 1970); nonetheless, efforts to use microbial strategies have been applied and are at least partially successful (Gengenbach et al. 1978; Hibberd and Green 1982; Matthews and Widholm 1978; Schaeffer and Sharpe 1987; Schaeffer et al. 1988). Evidence shows that the β-aspartate pathway exists in higher plants but the nature of its regulation is still largely obscure. 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 enzymes. Some years back maize plants from specific germ plasm were recovered from lys + thr feedback inhibitor selections, and it was found that progeny from these selections had higher threonine levels than the controls, but that the lysine levels were not greatly changed (Hibberd and Green 1982). Several years ago we showed the recovery of cell lines resistant to AEC from anther-derived calli of Assam 5, a near-Indica subspecies. Plants regenerated from these selections and their progeny had improved seed protein lysine as well as improved protein levels. Also, in

1987 we reported the regeneration of plants from anther calli of Calrose 76, subspecies Japonica, resistant to lys + thr and AEC. Since then we have demonstrated that elevated lysine is inherited as a recessive character and is accompanied in some genetic backgrounds by infertility and abnormal seed fill characteristics (Schaeffer et al. 1988). Fully fertile lines have now been recovered and grown in the field.

The purpose of this communication is to define more precisely the changes in the levels of lysine and other amino acids of rice seed by characterizing different endosperm protein fractions of the mutant and control lines.

Materials and methods

Two types of experiments were done to distinguish the in-vitro-selected mutants from unselected controls; (a) the amino acid composition of half seeds selected for lysine and protein levels was defined and, (b) the mutant/control ratios for individual amino acids in different protein solubility classes extracted from bulk seed samples of rice grains were established.

Forty seeds were selected on the basis of lysine and protein content from an F_2 population of 299 seeds. Ten seeds from each of two controls were selected at random from greenhouse-grown seed. Seeds were weighed and rated for chalkiness, and individual amino acid levels of half seeds were determined and expressed as a percent of total amino acids in acid hydrolyzates. For each amino acid the data was analyzed as a 2×2 plus 2 controls analyses of variance (ANOVA). The mean amino acid percentages were compared using Fisher's protected LSD.

For the protein fractionation experiment, 21 g of defatted rice flour was fractionated into five solubility classes and the amino acid composition of each fraction was determined. The extractions were replicated four times and the mean mutant/control ratio for each amino acid was calculated. The hypothesis that the mean ratio equals one was tested using a t-test within a pooled estimate of variance.

Genetic sources

Biochemical selections, parents of crosses, progeny descriptions and progeny characterizations, description of seed chalkiness and methodologies in amino acid analyses, and electrophoresis were the same as those described earlier (Schaeffer et al. 1988). The mutant line is referred to as *4C* and the control cultivars as C. The data from crosses refers to *4C* ♀ × M-101 ♂, designated (4C × M-101).

Protein fractionations

All bulk protein fractionations were done with brown rice with aleurone and embryos intact. In contrast, single-seed analyses for lysine and protein levels were done with the endosperm half of the dehulled seed, i.e., the embryo half was saved for germ plasm development (Schaeffer et al. 1986, 1988).

For bulk analyses, dehulled whole seeds were ground in a mortar and pestle and screened through a 40-mesh sieve. The ground samples were defatted twice with 10 vol. of acetone at room temperature for 2 h. The residue was collected on Whatman no. 2 paper in a Buchner funnel and air dried. Seed storage proteins were fractionated into solubility classes with procedures modified from that described by Luthe 1983. All solvents had 1 m*M* phenylmethylsulfonylfloride (PMSF) and were buffered with 10 m*M* TRIS at pH 7.5. The defatted flour was extracted twice with each solvent at room temperature in the following order: water to extract the albumins; 1.0 *M* NaCl to extract the globulins; 70% EtOH to extract prolamins; 0.5% sodium docdecylsulfate (SDS) and 1% β-mercaptoethanol to extract glutelins. The extracts were filtered through Whatman no. 2 paper and processed as follows. Albumins were precipitated with 2 vol. of acetone at $-20\,^{\circ}$C overnight and centrifuged, and the precipitate was solubilized in water and freeze-dried. Globulins were dialyzed against 1 m*M* aqueous PMSF overnight at 4 °C and centrifuged. The globulin precipitate, salt soluble but water insoluble, was suspended in water and freeze-dried. The proteins in the water-soluble supernatant of this globulin fraction were precipitated and freeze-dried in the same way the albumins were processed above. This water-soluble globulin or residual, salt-extractable albumin subfraction is abbreviated Rsealb. Prolamins were precipitated with cold acetone and processed as described above. Glutelins were dialyzed and processed in the same way as the globulins except for the Rsealb partitioning.

Analyses of the extracts

Amino acid analyses and SDS polyacrylaminde gel electrophoresis (PAGE) were done as previously described (Schaeffer and Sharpe 1987).

Protein determinations

Protein levels are expressed as the sum of amino acids recovered from acid hydrolyzates and also by the Pierce BCA protein assay reagents and methologies. Values for aspartic and glutamic acid after acid hydrolysis include the corresponding amides. Cysteine was very low in most samples and was not used in some calculations.

Grouping of F_2s

High lysine, high protein (HLHP): a class of seeds with endosperm lysine equal to or greater than 3.55% of the total amino acids in acid hydrolyzates (protein), and total acid hydrolyzates equal to or greater than 1.2 μmol/unit hydrolyzate. High lysine, normal protein (HLLP): a class with lysine greater than or equal to 3.55% and total acid hydrolyzates of 0.995 or less: Normal lysine, high protein (LLHP): a class with normal or control level of lysine equal to or less than 3.2% and total acid hydrolyzates equal to or greater than 1.2. Normal lysine, normal protein (LLLP): A class with normal lysine equal to or less than 3.2% and normal total acid hydrolyzates equal to or less than 0.995.

Results

Lysine, protein, and seed size relationships

The average lysine content of rice endosperm protein (half seed excluding the embryo) of Calrose 76 ranges from 2.8 to 3.2% of total amino acids in greenhouse-grown plants. Mutant lines range from 3.2 to 4.2%. Crosses of the mutants to the original cultivars or other control lines produce F_2 segregants with high lysine, reduced seed size, and seeds with high or intermediate levels of chalkiness, characterized by soft or crumbly endosperm, which is opague to light. Figure 1 illustrates the relationships among lysine content, protein levels, and

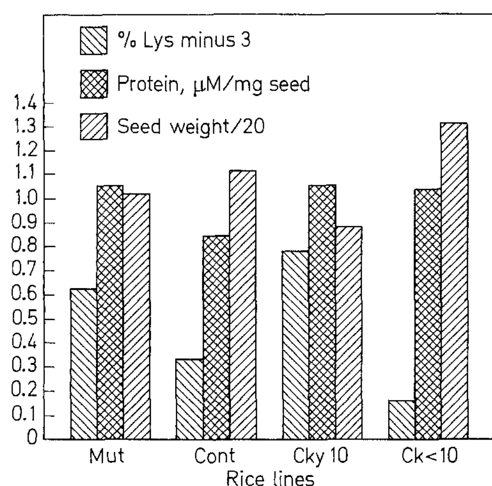

Fig. 1. Relationships among seed weight, protein content, lysine level, and chalkiness in endosperm half seeds of *in-vitro-selected* mutant and non-selected control rice. Absolute values are modified to bring the different parameters into the same scale. Percent lysine in acid hydrolyzates is expressed as percent lysine minus 3%. Protein is expressed as total micromoles amino acids/mg seed. Seed weight is expressed as mg/seed divided by 20

seed size in lines with different levels of chalkiness. The controls and the low chalky F_2 segregants had the lowest lysine levels. The highest seed weight recovered was in the chalky classification less than ten. Table 1 also shows the relationship of lysine and protein levels and seed characteristics, including chalkiness, with assigned statistical parameters to lysine and protein groupings. The mutant seeds with the greatest lysine and protein have the highest chalky ratings and seed weight is frequently smaller. On the other hand, most F_2 segregants with a chalkiness rating close to nine not only had high lysine but could be high or normal in protein levels and low and normal in seed weights, respectively. The low chalky F_2 segregants had larger seeds than the controls. This is further documentation of results reported earlier (Schaeffer et al. 1988).

Amino acids in F_2 classes

The amino acid analyses of seeds from F_2 populations, grouped according to lysine and protein levels (Table 1), show several interesting relationships. The high lysine types also had increased alanine and glycine as well as histidine and arginine, two basic amino acids. Methionine was highest in the LLLP group and lower in both components of the HL group. One hydrophobic amino acid, tyrosine, was lowest in the HL groups and significantly lower than both controls. Glutamic acid was more than 5% lower in the high lysine population and lowest in the HLLP subclass. There appear to be shifts between HP and LP within the high lysine classes (HL). For example, aspartic acid appears to be higher in the high protein subclasses compared with the low protein. Differences are less obvious in the normal lysine, LL, classes. There may be metabolic shifts in pathways not directly

Table 1. Relationships of lysine and protein levels to mean seed weight, chalky rating, and percent amino acids in the endosperm of selected F_2 seed. Twenty seeds each representing high (HL) and low lysine (LL) were separated equally into high (HP) and low (LP) protein subgroups. Calrose 76 and M101 represent control lines. Subscripts attached to control means represent differences from the lysine and protein selected subgroups. Sdwt = seed weight, and Cky = chalkiness rating

	HL		LL		Controls	
	HP	*LP*	*HP*	*LP*	*CAL76*	*M101*
Sdwt	17.60_d	20.50_c	26.90_b	28.20_a	$20.6_{1,3,4}$	$21.40_{1,3,4}$
Cky	9.20_a	8.80_a	5.50_b	4.80_b	$1.40_{1,2,3,4}$	$3.40_{1,2,3}$
Asp	9.78_a	9.32_b	9.55_a	9.33_b	$9.17_{1,3}$	9.43
Thr	4.10_a	3.95_a	3.97_a	4.03_a	3.99	4.04
Ser	6.75_b	6.52_b	6.92_a	7.23_a	6.79	6.95
Glu	15.44_b	15.12_c	16.14_a	16.32_a	$16.76_{1,2,3,4}$	$16.56_{2,3,4}$
Pro	5.08_a	4.93_a	5.21_a	5.12_a	5.50_2	5.12
Gly	8.76_a	8.82_a	8.15_b	8.13_b	$7.84_{1,2,3,4}$	$8.15_{1,2}$
Ala	8.79_a	8.70_a	8.53_b	8.34_b	8.49_1	$8.41_{1,2}$
Cys	0.01_b	0.07_a	0.06_a	0.01_b	0.03_2	$0.01_{2,3}$
Val	6.81_a	7.01_a	7.18_a	6.96_a	7.29	6.71
Met	1.60_b	1.66_b	1.74_a	1.92_a	1.76	$1.41_{2,3,4}$
Ile	3.73_a	4.30_a	4.12_a	4.15_a	4.00	3.92
Leu	8.54_b	8.28_b	8.85_a	8.81_a	8.84_2	$9.01_{1,2}$
Tyr	3.58_b	3.69_b	3.92_a	3.87_a	$4.00_{1,2}$	$4.11_{1,2,3}$
Phe	4.09_a	4.26_a	4.23_a	4.24_a	4.34_1	4.37_1
His	2.44_b	2.57_a	2.11_d	2.19_c	$2.09_{1,2,4}$	$2.18_{1,2}$
Arg	6.53_a	6.73_a	6.22_b	6.20_b	$6.32_{1,2}$	$6.50_{3,4}$

Means within rows followed by the same letter are not significantly different at the 5% level as determined by Fisher's protected LSD. Numbers following controls indicate the means within the same row (1-HLHP, 2-HLLP, 3-LLHP, 4-LLLP) which are significantly different than that control at the 5% level as determined by Fisher's protected LSD

Table 2. Ratios of amino acids (mutant/control) in five solubility fractions of rice endosperm proteins. Ratios based on percent of individual amino acids among total amino acids in acid hydrolyzates recovered from amino acid analyzer. Means calculated from four separate determinations

	Ratios of amino acids (Mutant/Control) Hypothesis: mutant/control = 1				
	Albumin	*Resealb*	*Globulins*	*Prolamins*	*Glutelins*
AsP	1.012	0.994	1.129 A	1.016	1.007
Thr	1.030	1.454 A	1.035	1.055	0.998
Ser	0.990	1.226 A	0.936	0.984	0.989
Glu	1.024	0.850 B	0.998	0.986	0.995
Pro	0.939	0.988	0.896 B	0.995	1.036
Gly	0.985	0.978	1.017	1.021	1.008
Ala	0.995	1.110 A	0.993	1.012	1.027
Val	1.062	0.977	1.030	0.976	1.053
Met	0.885	0.581 B	0.784 B	0.739 B	1.145 A
Ile	0.952	1.275 A	1.105	1.153	1.058
Leu	1.015	0.896 B	0.962	0.983	1.001
Tyr	0.882 B	0.85 B	0.781 B	1.003	0.945
Phe	1.032	0.767 B	0.990	0.998	0.997
His	1.057	0.803 B	1.460 A	0.909 B	1.006
Lys	1.107	1.148 A	1.352 A	1.213 A	1.125 A
Arg	1.030	0.927 B	1.002	0.999	0.976

A = Mutant significantly greater than control ($P = 0.05$)
B = Mutant significantly less than control ($P = 0.05$)

Table 3. Lysine distributions in different fractions of rice, whole-seed proteins isolated from mutant (*4C*) and control (C) lines

Fractions		Protein (mg/21 g seed)[a]	Total AAs (mg/21 g seed)[b]	Lysine (mg/21 g seed)	Ratio (mutant/control) lysine
Albumins	*4C*	278	322	18.3	2.08
Resealb	*4C*	42	32	1.5	1.88
Globulins	*4C*	94	111	3.8	1.9
Prolamins	*4C*	54	113	0.7	1.75
Glutelins	*4C*	827	1,204	43.6	1.16
Albumins	C	155	173	8.8	–
Resealb	C	24	18	0.8	–
Globulins	C	79	75	2.0	–
Prolamins	C	50	77	0.4	–
Glutelins	C	575	1,041	37.7	–

[a] Pierce assay
[b] Based on sum of amino acids (AAs) in micromoles recovered from analyzer

related to lysine synthesis in order to satisfy cell charge or the hydrophobic-hydrophilic balance in seed storage proteins.

Composition of protein solubility fractions

The examination of the changes in amino acid composition of the different solubility classes of rice endosperm proteins (Table 2) provides additional clarification of the data presented above. The protein fractions differing the most from the Calrose 76 control are the Rsealb and globulin fractions, both components of the salt-soluble proteins. Isoleucine, lysine, and threonine, as well as alanine and serine, were significantly higher in the Rsealb fraction of the mutant than the control. All of these amino acids except alanine and serine are aspartate pathway amino acids. Even though seven amino acids were significantly lower in the mutant than the control in the Rsealb fraction, methionine was decreased the most, whereas the mutant/control ratios for glutamic acid, histidine, leucine, and tyrosine were 0.85, 0.80, 0.90, and 0.85, respectively. In the globulin fraction the level of asparatic acid, histidine, and lysine are significantly higher in the mutant. Among the globulin and Rsealb fractions, all the aspartate pathway amino acids are higher in the mutant in one or the other fraction except methionine, which is significantly lower in both fractions. The results suggest that the mutant pathway is feedback insensitive or the branch point enzyme leading to methionine synthesis is modified, thereby favoring the lysine branch, or that both events have occurred.

The overall shifts in lysine, methionine, and tyrosine distribution in the solubility fractions are illustrated in Fig. 2. The mutant/control ratios show that four out of five solubility fractions of proteins had significantly greater lysine in the mutant than the control. The mutant/control ratio for lysine was 1.11, 1.15, 1.35, 1.21, and 1.12 for albumins, Rsealb, globulins, prolamins, and glutelins, respectively (Table 2). However, the ratios for tyrosine were 0.88, 0.85, 0.78, 1.00, and 0.95 for albumins, Rsealb, globulins, prolamins, and glutelins, respectively. Other amino acids in the globulin fraction on the lysine pathway including aspartic acid, threonine, isoleucine, and methionine had ratios of 1.13, 1.04, 1.10, and 0.78, respectively. The level of globulin tyrosine in the mutant was reduced 22%. In the glutelin fraction, which constitutes approximately 65% of the rice endosperm protein, lysine and methionine were increased 13 and 15%, respectively. The glutelin fraction is the most important for seed fill by virtue of its high level in the seeds; however, other fractions such as the Rsealb may be more important than the glutelins in the regulation and expression of the mutant gene(s).

Experiments designed to quantitate proteins and lysine in whole seed on a weight basis as well as a percent basis show that mutant seed had more protein per unit weight of seed. Unimproved mutant seeds are smaller and probably have less carbohydrates than the controls.

The data summarized in Table 3 give an estimate for the absolute values of protein and lysine in 21 g of mutant and control seed. The values illustrate the relationship between a colorimetric assay for proteins and the amino acid analyzer methods, which gives the sum of all

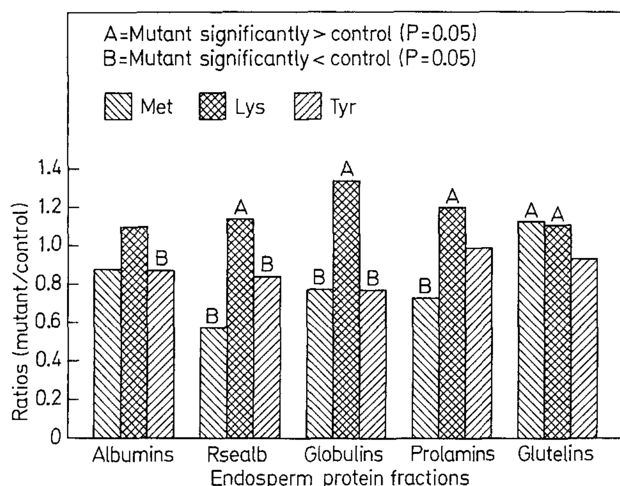

Fig. 2. Mutant/control ratio for percent lysine, methionine, and tyrosine in different protein solubility fractions of rice endosperm half seeds

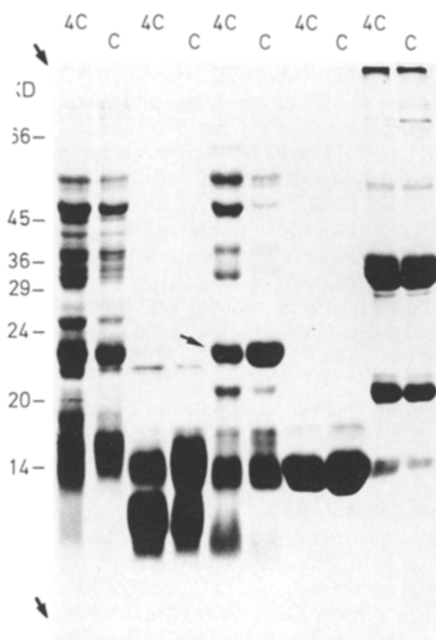

Fig. 3. SDS-PAGE of rice endosperm proteins representing five solubility fractions. Pairs of wells from *left to right* are albumins, Resealb, globulins, prolamins, and glutelins. *Left well* of each pair represents the mutant and the *right well* the control. Proteins visualized with Coomassie blue

measured amino acids. Except for the Rsealb fraction, all analyzer values are higher than the Pierce colorimetric values. In addition, Table 3 shows the relative contribution of individual protein fractions to the total protein content, as well as the total lysine in 21 g of seed. The mutant/control ratio for mg/21 g of seed shows the additive effect of increased proteins, as well as increased lysine in the mutant over the control. The overall lysine distribution in the five fractions of the control is similar to data reported by others (Juliano 1985).

SDS-PAGE work in which equal quantities of protein were loaded per lane shows one major rice protein that is naturally low in lysine (arrow, Fig. 3) in the control at Rf 0.6. This may be replaced or compensated for by a number of proteins with increased lysine, shown in the upper portions of the gel. The decrease in the low lysine 30-kDa protein in the mutant illustrated in Fig. 3 may be similar to the high lysine phenotypes of other cereals in which the relative proportion of lysine-rich proteins is increased due to the loss of a low lysine fraction(s). There are also quantitative differences in the protein banding patterns in other fractions between the mutant and controls, particularly in the albumin and Rsealb fractions.

Further fractionation of the globulins on SDS-PAGE gels showed several major shifts in the amount of lysine recovered from eluted proteins from the mutant and the control. Proteins recovered from SDS-PAGE showed that the mutant had more protein than controls at Rf 0.1, when expressed as a percent of total recovery from 1-cm gel bands (data not presented). The mutant had less protein than the control in the major globulin band at Rf 0.6. This is confirmation of the electrophoretic pattern shown in Fig. 3. There are also other high-molecular-weight storage proteins, perhaps glycoproteins, in the control, not readily stained with Coomassie blue, that are absent or very low in the mutant. These can be visualized with silver stains on one or two dimensional gels. These data are to be published elsewhere.

Discussion

Clues to the relationship between the lysine-enriched phenotype and the amino acid composition of proteins are provided in the grouping of F_2 segregants into the HLHP, HLLP, LLHP, and LLLP classes. The lysine increase as well as the corollary increases in histidine and arginine are largest in the HLLP class. The decrease in methionine and tyrosine is largest in the HLHP class, whereas the decrease in glutamate, proline, aspartate, and serine is largest in the HLLP class. This suggests that these amino acids could be limiting or particularly critical in protein synthesis, processing, or transport in rice seed. The reduction of leucine and isoleucine in the HLHP could mean that the relationship of hydrophobic amino acids to membrane function is different in the mutant and the control. The increased temperature sensitivity, expressed as infertility, induced by the in vitro environment in some inbred lines may be due, at least in part, to the decrease in these hydrophobic amino acids in the mutant. Additionally, dysfunction in membrane or transport phenomena may predispose mutant proteins to proteolytic alterations and, hence, altered amino acid compositions.

We see similar changes among a large number of experiments with the *4C* mutant in percent lysine in protein hydrolyzates. All solubility fractions, including the nutritionally important glutelins, usually have mutant/control ratios greater than one. However, there is preliminary evidence that the percent lysine in different fractions changes slowly during seed storage. It is possible that proteases function in situ or may function differently during extraction in fresh and aged seeds, and that they thereby alter the solubility characteristics, including the high lysine proteins in the glutelins. If this scenario is correct, the percent lysine in glutelins of seed over 3 years old may not represent a precise estimate for the phenotype. The phenotype would be underestimated.

The *4C* mutant is not an adapted line and the amino acid composition of different fractions probably will be different in improved field lines from crosses, backcrosses, and advanced selfings. Several advanced lines grown in the field in 1989 have near-normal fertility and statistically significant increases in percent lysine in endosperm proteins (G. W. Schaeffer and F. T. Sharpe, Jr. unpublished results). These mutants represent unique and rare sources of genetic material for molecular and biochemical studies in the regulation of nutritional quality of rice. The lines represent starting materials for cultivar development with excellent nutritional quality.

Conclusions

Progeny from plants regenerated from in vitro inhibitor selections with lys+thr and their crosses segregate for different lysine and protein levels. The recessive gene(s) conditioning lysine level changes the proportion of the proteins in different solubility classes, as well as the amino acid composition within classes. Lysine per unit weight of protein is higher in all solubility classes, but the greatest change is in the globulin fraction. The mutant/control ratio for percent lysine is over 1.30. Among the other changes is a decrease in tyrosine and methionine in this fraction. The hydrophilic basic amino acids are increased and the several hydrophobic amino acids are decreased relative to the control in the globulin fraction.

Electrophoretic separation and subsequent isolation of the globulin proteins showed several major changes in the mutant. The major low lysine globulin proteins at 30 kDa was decreased in the mutant relative to the control. Conversely, several proteins partitioning at Rf 0.1–0.3 had increased lysine over the control. Further purifications are underway.

Our conclusion from this work is that in vitro selection may be an effective way to recover specific metabolic variants in major crop plants. The significance is that these lysine phenotypes can be developed in a consistent and predictable manner. However, somaclonal variation induced by the the passage of cells through the in vitro environment is pervasive in rice and may account for a wide range of phenotypic variation, including infertility in progeny of regenerated plants.

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