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Free and bound amino acids and proteins in developing grains of rice with enhanced lysine/proteins

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Abstract Free amino acids were determined in developing seed of a rice mutant with enhanced grain lysine. This phenotype frequently has enhanced protein. Some free amino acids of developing seed are inversely related to the level of total amino acids in proteins of the mature grain. Amino acids that were enhanced in protein, including aspartic acid, threonine, methionine and lysine, were notably lower in the free amino-acid pool. Our conclusion is that mutant-developing grains process aspartate amino acids more rapidly than the controls. Conversely, arginine, valine and glutamic acid/glutamine accumulate as free amino acids with mutant/control ratios of 1.39, 1.29 and 1.12, respectively. Glutamic acid/glutamine in proteins of mature seeds is lower in the mutant than the control. ^{3}H-lysine incorporation showed enhanced isotope incorporation into at least four proteins. One mutant protein was less actively labelled than analogous controls. The ^{3}H-lysine pattern indicates processing modifications in this useful rice mutant.

Key words Rice · Developing seed · Lysine · Free and total amino acids · H-Autoradiograph · Protein

Introduction

The nutritional quality of cereal grains, including rice, could be improved with enhanced free and/or protein lysine. Lysine is essential for non-ruminants and is considered to be the first limiting amino acid in most cereals. This need has generated large-scale searches for lysine-enhanced germ plasm and has promoted extensive research in biochemical and molecular genetics to isolate or generate mutants that overproduce lysine (Singh and Axtell 1973; Juliano 1985; Schaeffer and Sharpe 1987; Galilli 1995; Ghislain et al. 1995). At this time, there is greater variation for lysine, as well as protein, levels in maize, sorghum and other cereals than in rice, and hence the need is more compelling with this cereal. Additionally, societies with limited dietary options have greater need for enhanced lysine rice than do cultures with a wide variety of food resources. New germ plasm for altered lysine and protein levels has nutritional significance and therefore commercial value. Nutritionally enhanced germ plasm is also valuable for basic studies on abiotic stress, the control of protein/carbohydrate metabolism, and for the regulation of gene expression. We recovered germ plasm from inhibitor selections in vitro with several useful characteristics, including enhanced lysine, and/or protein, and altered expression of stress-related enzymes (Schaeffer and Sharpe 1987; Schaeffer et al. 1992, 1994). Our objective in this paper is to characterize free and bound amino acids and proteins in the developing grain of enhanced lysine/protein rice.

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Materials and methods

Genetic sources

The in vitro inhibitor selections, regeneration and progeny characterizations were as described earlier (Schaeffer and Sharpe 1987, 1990). The original mutant is referred to here as 4C. Segregating populations resulted from the backcrosses 4C female × Calrose-76, the parental source line (Schaeffer et al. 1994). Plants were greenhouse-grown and panicles were tagged at anthesis. Developing seed was harvested at various times after tagging and referred to as days post-anthesis (dpa). The seed was either extracted or quick frozen in liquid nitrogen and stored at −80°C until used.

Free amino-acid extraction and analyses

Free amino acids were extracted essentially as described by Bielinski and Turner (1966). Developing endosperms and embroys were dissected from the florets, weighed, and ground in a mortar and pestle and extracted twice with methanol:chloroform:water (12:5:3, v/v/v). The homogenates were centrifuged at 15 000 g for 15 min to pellet the debris. A one-fourth volume each of chloroform and water were thoroughly mixed with the combined supernatants and the phases were separated by centrifugation at 7000 g for 15 min. The upper phase was removed and bubbled with nitrogen to remove solvents and solution freeze-dried. Samples were solubilized at pH 2.2 in sodium citrate buffer and the amino acids were quantified on a Beckman 119BL analyzer. Hydrolyzate analyses of total amino acids and isolated proteins were as described previously (Schaeffer and Sharpe 1987).

Protein fractionations

Developing endosperm, 12–14 dpa, were dissected from the florets and ground in a mortar and pestle with 0.1 mM Tris, 0.1 mM phenylmethyl sulfonyl floride, 1% beta mercaptoethanol and 50 mM of NaCl, pH 8.3. Extracts were cleared by centrifugation at 15 000 g for 15 min and loaded on a 2.5×20 cm DEAE cellulose column previously equilibrated with 0.1 mM Tris, 50 mM NaCl, pH 6.8, and eluted with equilibration buffer only. Four-milliliter fractions were collected. Fractions were pooled and dialyzed against water for 3 h and then freeze dried. The extractions, chromatography and dialysis were done at 2–4°C. Dried fractions were hydrolyzed and analyzed for amino-acid composition as described above.

^{3}H lysine incorporation

Developing seed at 14 dpa were surface sterilized in clorox:water (1:4, V/V) and rinsed extensively with sterile distilled water. Embryos and endosperm were dissected out and incubated in half-strength Murashige and Skoog (1962) salts, 10 mM dithiothreitol and 50 mM glutamine, and 120 μCi ^{3}H-lysine per 1.0 ml, pH 6.5. The tissue was incubated for 3 h at 28°C at a rate of 200 mg fresh weight/ml incubation medium. Tissue was washed three times with incubation medium minus isotope and ground with a mortar and pestle in 6 M urea, 2% NP-40 and 5% β-mercaptoethanol at 25°C and then centrifuged at 15 000 g for 10 min. The protein in the supernatant was precipitated for 1 h at −70°C by the addition of 3 vol of acetone. Precipitated protein was pelleted by centrifugation as above and re-solubilized in 2% NP-40. The protein was re-precipitated with 3 vol of absolute ethanol for 1 h at 20°C and repelleted as above. The pellets were again solubilized in 2% NP-40 and subjected to two-dimensional electrophoresis essentially as described by O'Farrell (1975). Gels were fixed in 40% methanol and 10% acetic acid for 1 h, equilibrated in En^{3}hance (an autoradiography enhancer) as recommended by the manufacturer, dried and subjected to fluorography at −70°C.

RNA isolations and in vitro translation

Seedlings were grown in a vermiculite/potting soil mix in growth chambers maintained at 28°C and illuminated with 90–100 $uEm^{-2}s^{-1}$ from cool white fluorescent lamps for 14 h. The temperature during the dark period was 25°C. Etiolated seedlings were grown at the same temperature and time periods but in total darkness. Seedlings were harvested 14 days after sowing and total RNA was extracted as described by Chomzynski and Nicoletta (1987) except that the tissue was ground in liquid nitrogen and homogenized in denaturing solution for 1 min with a Brinkman polytron. Poly(A)$^+$ RNA was enriched by one cycle of oligo(dt) cellulose chromatography using the Stratogene Poly(A) Quik mRNA purification kit. One microgram of poly(A) RNA was translated in vitro with the Promega Flexi rabbit reticulosite lysate system.

Results and discussion

Typically, free amino acids of rice grains constitute less than 1% of the total (free plus hydrolyzed protein) amino-acid content of mature seed. Our previous studies have produced no evidence that these mutants have enhanced free lysine. Data presented here provides evidence that the free amino-acid pool is 75% higher in a 12-dpa developing mutant seed than in the wild-type grains (Table 1). However, free lysine, phenylalanine, methionine and proline are considerably lower in the mutant. The mutant/control ratios for these amino acids is 0.61, 0.74, 0.60 and 0.57, respectively. These amino acids are probably incorporated rapidly into specific classes of storage proteins and particular processing enzymes at 12–14 dpa. The lysine-rich glutelins, which are the major endosperm storage proteins, as well as the lysine-rich albumins, or water-soluble enzymatic fraction, accumulate rapidly from about 7 dpa and reach a maximun around 13 dpa (Juliano 1985; Zhu et al. 1989). The reduced free lysine suggests a higher rate of synthesis of these proteins in the mutants than in the controls. The mutant/control ratio for total hydrolyzate amino acids in 12-dpa developing seed is 1.15. Conversely, the free amino acids most prominently accumulated at 12 dpa are arginine, valine, and glutamic acid/glutamine, showing mutant/

Table 1 Free amino acids of developing seeds at 12 days post-anthesis (dpa) of mutant 4C and control, expressed as a percent of total amino acids detected. Means and standard errors of the means (SEx) are from three analyses

Amino acids	Mutant		Control		Ratios mut/con
	Means	SEx	Means	SEx	
Asp	8.07	± 0.71	9.62	± 0.55	0.84
Thr	3.25	± 0.21	3.46	± 0.11	0.94
Ser	8.18	± 0.41	9.79	± 0.18	0.84
Glu	36.3	± 2.70	32.29	± 0.31	1.12
Pro	1.83	± 0.16	3.22	± 0.06	0.57
Gly	5.41	± 0.08	6.13	± 0.08	0.88
Ala	17.9	± 0.90	16.58	± 0.36	1.08
Val	4.87	± 0.25	3.78	± 0.08	1.29
Met	0.55	± 0.05	0.91	± 0.02	0.60
lle	2.65	± 0.14	2.67	± 0.06	0.99
Leu	1.85	± 0.13	2.16	± 0.03	0.86
Tyr	1.65	± 0.09	1.65	± 0.03	1.00
Phe	0.97	± 0.05	1.30	± 0.02	0.75
His	2.04	± 0.13	1.93	± 0.01	1.06
Lys	1.61	± 0.13	2.66	± 0.13	0.61
Arg	2.75	± 0.14	1.83	± 0.04	1.50
Total	2.545	± 0.26	1.463	± 0.11	1.74

control ratios of 1.39, 1.29 and 1.12, respectively. Glutamic acid is generally reduced in mutant proteins of mature seed with enhanced lysine (Schaeffer and Sharpe 1990). Hence, some free amino acids of developing seed are inversely related to the level of total amino acids in the proteins of mature grains.

Amino-acid analyses of developing seed proteins separated by anion exchange chromatography show three very distinct fractions eluting with loading buffer only. Fraction one has 6.43 and 6.48% lysine in mutant and control proteins, respectively. Fraction two shows 4.30% for the mutant and 3.45% for the control, and fraction three has 3.30% in the mutant and 3.39% lysine in the control. The largest difference in percent lysine (mutant/control = 1.25) and arginine (mutant/control = 1.22) is in fraction two. The mutant/control ratios for threonine (1.18), proline (1.25) and valine (1.20) are also highest in fraction two. Aspartic acid is elevated in fractions two and three, and glutamic acid is reduced in fraction two but elevated in fraction three. Tyrosine is also significantly lower in fractions two and three. These data point to specific protein enrichments/depletions in developing mutant seed at 12 dpa and illustrate a focal point at which to study specific regulatory events. Mutant proteins different from the controls may be useful in future transformation experiments.

Developing grains become lysine-enhanced with age. The mutant/control ratio for the percent lysine in total hydrolyzates is 0.87, 0.89, 0.97 and 1.09, for 9, 12, 15 and 18 dpa, respectively. The mutant/control ratio for fully mature seed is 1.15 (Schaeffer et al. 1994). We do not know the mechanisms for this pattern at this time. However, this may be due to a reduction in catabolic activity in the mutant, or perhaps to an extension of the temporal sequence of events during which lysine-rich proteins are being synthesized and packaged. Changes in the time frames of events, or altered regulation of protein synthesis, is demonstrated by interesting phenomena observed in F_2 segregants. The recovered segregants: (1) have a fully chalky endosperm with enhanced lysine and elevated protein, (2) are partially chalky, similar to the white belly phenotype, with normal lysine and enhanced protein, or (3) have vitreous grains with normal lysine and enhanced protein. Thus, enhanced lysine is linked with chalkiness and there exists a lysine/protein inverse relationship in which high-protein lines have a normal percent lysine but enhanced total lysine by virtue of the elevated protein. The mutant/control protein ratios for chalky, white belly and vitreous endosperm are 1.37, 1.39 and 1.40 respectively. The mutant/control ratios for percent lysine in the elevated proteins were 1.04, 0.87 and 0.90 for the chalky, white belly and vitreous segregants respectively.

Data from the in vitro translation of purified mRNA of vegetative tissue supports the concept illustrated above that this rice mutant has the capacity for enhanced protein synthesis. The mean mutant/control ratio for total isotope incorporated using ^{3}H-leucine is 1.50 ± 0.04 SEx for etiolated seedlings and 1.53 ± 0.13 or the leaf tissue of mature greenhouse-grown plants. A ratio of 1.65 ± 0.17 is seen for etiolated seedlings using ^{35}S-methionine. Autorads of SDS PAGE-separated products from in vitro translations indicate increases in the entire protein complement. This pattern is consistent, but as yet unexplained.

The rate of ^{3}H-lysine incorporation into proteins of developing seeds of the mutant and control separated two-dimensionally was similar. Notable exceptions are marked by arrows in Fig. 1. The most obvious difference was the extensive incorporation into a large basic polypeptide, kDa >92. This may be a precursor for smaller peptides and could be due either to increased synthesis, protease resistance, or to higher percent lysine in this protein. Additionally, the incorporation pattern shows several acetic proteins to be more prominent in the mutant than in the control at kDa72, kDa40, kDa28, and kDa14–18, as well as reductions at kDa52–56 and kDa16–18. This pattern of lysine incorporation at 14 dpa further identifies the approximate time of grain development as one during which up- and down-regulated proteins may be studied.

Conclusions

We have recovered and partially characterized a rice mutant which has enhanced endosperm lysine and enhanced protein as well. Free amino-acid levels in developing seeds are 75% higher in the mutant than in the control. However, free lysine, phenylalanine, methionine and proline are reduced in the mutant;

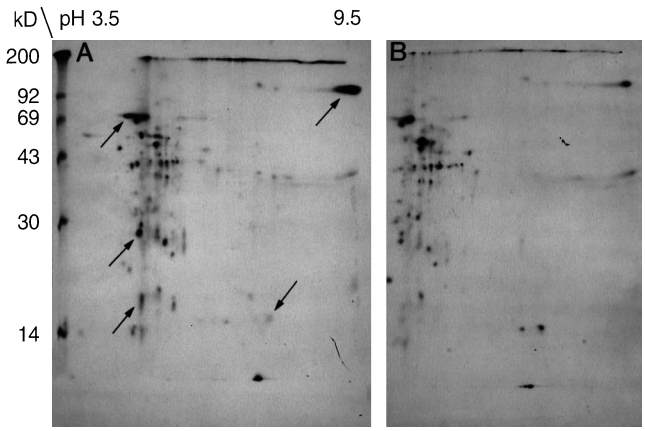

Fig. 1 Autoradiographic profiles of ^{3}H-lysine-labelled proteins in developing rice grains, 14 days post-anthesis, of the mutant (left, **A**) and the control (right, **B**). Proteins were separated in the first dimension by isoelectric focusing, pH 3.5–9.5, and in the second by SDS PAGE. Arrows highlight differences between the mutant and the control. *Arrows pointing up* indicate higher levels in the mutant than the control. The *arrow pointing down* indicates a lower level in the mutant

whereas, free arginine, valine and glutamic acid accumulate in mutant cells. Total amino acids recovered from hydrolyzed mature grain of the mutant typically have enhanced protein over the control, as well as increased lysine and decreased glutamic acid. Proteinaceous lysine increases in the mutant with development of the grain. The highest level of lysine relative to the control is in fully mature endosperm. ^{3}H-lysine incorporation into developing grains demonstrates differential accumulation of a number of proteins. The labelling pattern indicates increases in several proteins of the mutant, as well as a few decreases relative to the control. This pattern suggests changes in protein processing. The in vitro translation of purified mRNA implies that the mRNA itself, or else some tenaciously bound particle, is the cause of the enhanced incorporation of labelled amino acids into the entire complement of translated proteins.

References

Bielinski RL, Turner NA (1966) Separation and estimation of amino acids in crude plant extracts by thin-layer electrophoresis and chromatography. Anal Biochem 17:278–293

Chomzynski P, Nicoletta S (1987) Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156–159

Galilli G (1995) Regulation of lysine and threonine synthesis. Plant Cell 7:899–906

Ghislain M, Frankard V, Jacobs M (1995) A dinucleotide mutation in dihydrodipicolinate synthetase of *Nicotiana sylvestris* leads to lysine overproduction. Plant 8:733–743

Juliano BO (1985) Polysaccharides, proteins and lipids of rice. In: Juliano BO (ed) Rice: chemistry and technology. The American Association of Cereal. Chemists, St. Paul, Minnesota, pp 105–109

Murashige T, Skoog FA (1962) A revised medium for rapid growth and bioassays with tobacco cultures. Physiol Plant 15:473–497

O'Farrell PH (1975) High-resolution two-dimensional electrophoresis of proteins. J Biol Chem 250:4007–4021

Schaeffer GW, Sharpe FT Jr (1987) Increased lysine and seed storage protein in rice plants recovered from calli selected with inhibitory levels of lysine and threonine and s(2-aminoethyl)-cysteine. Plant Physiol 84:509–515

Schaeffer GW, Sharpe FT Jr (1990) Modification of amino-acid composition of endosperm proteins from in vitro-selected high-lysine mutants in rice. Theor Appl Genet 80:841–846

Schaeffer GW, Sharpe FT Jr, Dudley JT (1992) Rice protein mutant expressed in liquid suspension cultures: chitinase, b-glucanase and other proteins. Theor Appl Genet 84:26–32

Schaeffer GW, Sharpe FT Jr, Dudley JT (1994) Registration of five lysine-enhanced rice germ plasm lines: 2K41, 2K539, 2K(C193) 2K497 and 2K601. Crop Sci 34:1424–1425

Singh R, Axtell JD (1973) High-lysine mutant gene (*h1*) that improves protein quality and biological value of grain sorghum. Crop Sci 13:535–539

Zhu ZP, Shen RJ, Tang XH (989) The biosynthesis of storage protein and changes of free amino-acid content during seed development of rice. Chinese J Bot 1:123–130