Theor Appl Genet (1997) 95:230–235

G. W. Schaeffer · F. T. Sharpe

Electrophoretic profiles and amino acid composition of rice endosperm proteins of a mutant with enhanced lysine and total protein after backcrosses for germplasm improvements

Received: 14 October 1996 / Accepted: 8 November 1996

Abstract Seed storage proteins from in vitro-derived rice mutants improved by several backcrosses to 'Calrose 76' and BC_2 and BC_3 were characterized for changes in five different solubility classes. Albumins, rsealb (water-soluble globulins), true salt-soluble globulins, prolamins and glutelins were SDS-PAGE separated in a single dimension, and some two-dimensionally, to identify protein modifications. The genetic transmission of the enhanced-lysine mutants in backcrosses and the linkage of lysine with grain chalkiness were confirmed. Advanced lines had altered globulin profiles similar to those of unimproved lines. Chalky/enhanced-lysine phenotypes had similar prolamin and glutelin profiles in the mutant and controls at the same protein level. Mutants had increased levels of globulins at 50 kDa and 33 kDa but had substantially less protein at 25 kDa than the controls. High protein in the mutant contributed to an increase in prolamins and the major storage proteins in both the globulins and glutelins. A significant decrease in low-molecular-weight, 15- to 18-kDa albumins was associated with the chalky/enhanced-lysine mutant phenotype. Two proteins in the 15- to 18-kDa group were amino acid sequenced, and database comparisons identified these proteins as allergens. Advanced lines down-regulated for allergens and with enhanced-lysine/-protein but with normal fertility and seed weight should be useful in breeding programs for nutritional quality.

Key words Lysine · Mutants · Rice proteins · 2-D electrophoresis · Globulins · Allergens

Communicated by H. F. Linskens

G. W. Schaeffer (✉) · F. T. Sharpe
USDA, ARS, PSI, Beltsville Agriculture Research Center,
Plant Molecular Biology Lab, Beltsville, MD 20705, USA

Introduction

The food and feed value of most cereals could be improved by increasing their levels of proteinaceous lysine. While germplasm for enhanced lysine does exist for cereals, the number of accessions is limited in rice and wild germplasm is sometimes associated with soft, chalky endosperm, as is the case with the maize opaque2 mutant (Mertz et al. 1964). Improvements in the maize mutants have been in process for decades, and enhanced lysine phenotypes are still of great interest for research and development. A rice mutant with enhanced lysine was recovered from inhibitor selections from anther-derived callus at Beltsville, Maryland (Schaeffer and Sharpe 1987). The nutritional mutants of corn, barley and sorghum (Mertz 1986; Gengenbach et al. 1978) and rice (Schaeffer and Sharpe 1987) have been partially characterized. However, the specific biochemical and molecular mechanisms involved in the phenotypic expression of enriched lysine in cereal grains require further study. In addition to past research on seed storage proteins, recent efforts in maize (Habben et al. 1993) and earlier work with barley (Balasaraswathi 1984; Rasmussen et al. 1988) have targeted increases in non-storage proteins as contributors to the increased lysine phenotypes. Some of our earlier reports provided amino acid analyses of rice seed storage proteins and profiles of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)-separated proteins (Schaeffer and Sharpe 1990). This previous work was done with selfed mutants originally recovered from inhibitor selections in vitro.

The purpose of this report is to provide additional information on seed proteins of inhibitor-selected rice lines improved by successive backcrosses and grain selections. New information from refined multidimensional electrophoretic separations and analytical characterizations of endosperm proteins is presented. Endosperm proteins fractionated into solubility classes

and Coomassie-visualized after SDS-PAGE provide insights into quantitative shifts within families of proteins separated two dimensionally by charge and size. Analytical data from advanced mutants and controls are integrated with earlier data. These data are reinterpreted in light of information from two-dimensional (2-D) separation of proteins and N-terminal protein sequencing of several separated proteins. The data focuses on interpretations that the increased lysine content of the improved rice lines is induced by the constitutive stress biochemistry in the mutant, the reduction of lysine-poor proteins in the mutant and the incomplete processing of mutant proteins which change solubilities so that mutant glutelins or their precursors are extracted as salt-soluble globulins. Mutant precursor/product relationships are examined and compared with wild-type materials to identify targets for genetic modifications.

Materials and methods

Genetic sources

Selections in vitro, plant regeneration and development and progeny characterizations were as previously described (Schaeffer and Sharpe 1990). The original in vitro-derived mutant, 4C, was a product of 'Calrose 76' (Rutger et al. 1977), a California cultivar, subspecies japonica. The improved line, 2K41, was derived by successive backcrosses (BC) with 'Calrose 76' ($4C_{female} \times$ 'Calrose 76'$_{male}$) (Schaeffer et al. 1994). Selected progeny with enhanced lysine were selfed four times, checked for lysine at each selfing and then crossed to 'Calrose 76' ($2K41_{female} \times$ Calrose 76'$_{male}$). Selfed lines from BC_2 and BC_3 are described in this work. BC_3, produced a population of plants with enhanced protein and segregating for: (1) chalky endosperm linked with enhanced lysine; (2) partially chalky endosperm similar to white belly, a vitreous grain with a single stripe of chalkiness along one edge; and (3) vitreous (clear) endosperm with normal lysine but enhanced protein. All plants were greenhouse-grown. Our designations as enhanced or high protein are considered putative until tested in replicated field trials.

Protein fractionation and electrophoresis

Dehulled whole seed was defatted and fractionated by solubilities into water-soluble albumins, rsealb globulins (water-soluble after salt extraction), salt-soluble globulins, 70% ethanol-soluble prolamins and detergent-soluble glutelins as described earlier (Schaeffer and Sharpe 1990). One-dimensional (1-D) SDS-PAGE was as described earlier (Schaeffer and Sharpe 1987) and 2-D separations consisting of isoelectrofocusing followed by SDS-PAGE were similar to that described by O'Farrell et al. (1977).

Protein sequencing

Proteins separated two-dimensionally were electroblotted onto polyvinylidene difloride (PVDF) membranes in 25 m*M* TRIS, 192 m*M* glycine and 0.1% sodium dodecylsulfate for 16 h at 25 V. Membranes were washed in 3% acetic acid, 40% methanol, stained in 0.1% imido black and rinsed to remove excessive stain. Protein spots were excised, rinsed extensively in water and stored at $-20^{\circ}C$ in water. Protein sequencing was performed on a Hewlett Packard model 1005G gas-phase sequencer using the supplied PVDF program.

Salt-soluble protein fractionation

Salt-soluble proteins were electrophoretically separated by 1-D SDS-PAGE. Individual bands were identified by Coomassie blue staining of external lanes as well as of lanes with molecular-weight markers. The gels which were neither fixed nor stained were cut into 14 bands, eluted with NaCl and detergent and processed as described earlier (Sharpe and Schaeffer 1993). Amino acid analyses were performed as previously reported (Schaeffer and Sharpe 1987), and protein was quantified using the Pierce BCA protein assay kit.

Nitrogen analyses

Percentage total nitrogen in whole seed was determined at the Ohio Research and Development Center, Ohio State University. Protein was estimated by multiplying the percentage nitrogen by 5.95 (Jones 1931).

Results

Initial protein characterizations of enhanced lysine mutants before backcross improvements showed major protein differences between mutant and controls (Schaeffer and Sharpe 1987, 1990). These lines were phenotypically suboptimal as demonstrated by infertility and small grains with soft, crumbly endosperm from plants that were shorter with uneven culm height and culm number and darker green than the controls. Seed size and fertility were restored to normal by successive backcrosses to 'Calrose 76'. Progeny from BC_3 segregated into chalky, partially chalky (similar to white belly) and non-chalky (vitreous/clear) grain classes with varying levels of lysine and protein.

Lysine and protein relationships

The analyses of BC_3S1 progeny confirmed observations that line improvements for enhanced lysine and protein and are genetically transmitted. Table 1 demonstrates variability/segregation for enhanced protein in BC_2S4 and for percentage lysine in BC_3S1. Line 2K41Ep had 19.7% higher grain protein than the control. Enhanced protein lines had a near-normal percentage lysine in protein but increased total lysine per grain by virtue of an increased protein content per seed. Backcrossed segregants identified as chalky or vitreous, had 89 μg and 79 μg lysine per grain, respectively; whereas the controls had 67 μg. Soft endosperm and chalkiness remained linked to enhanced lysine. Enhanced protein levels were not linked to chalkiness but occurred in chalky, partially chalky and vitreous grains. We consider the high protein clear-grain

Table 1 Mean rice grain protein from individual plants grown in the greenhouse, Beltsville, Md. Control line is 'Calrose 76', and 2K41*Ep* and 2K41*Np* represent selfed elevated and normal segregants, respectively, from progeny of third backcross with 'Calrose 76', the source parent of the in vitro-derived mutant ($2K41_{female} \times$ 'Calrose $76'_{male}$)

	n	Protein (%) SE	Protein (μg/grain)	Lysine[a] (% of total)	Lysine (μg/grain)	Grain weight (mg) ± SE
Chalky, selfed, BC_2 S4, *Ep*	4	10.62 ± 0.49	2246	3.52	79	22.1 ± 0.19
Chalky, selfed, BC_2 S4, *Np*	5	9.31 ± 0.30	2004	3.92	79	21.5 ± 0.17
Chalky, segregant, BC_3 S1[b]	4	11.34 ± 0.43	2355	3.79	89	20.8 ± 0.23
Vitreous, segregant, BC_3S1	4	11.41 ± 0.34	2413	3.27	79	21.2 ± 0.14
Calrose 76, control	6	8.87 ± 0.31	1885	3.54	67	21.3 ± 0.27

[a] Lysine, % of total, is from a single amino acid analysis
[b] BC_3S1 segregants are progeny from a BC_2 (Chalky $S4_{female} \times$ 'Calrose $76'_{male}$)

phenotype significant because it has a greater value in breeding programs than chalky phenotypes. Whether these lines will express grain characteristics the same way under field conditions as they did in the glasshouse needs to be determined.

Protein electrophoretic profiles

Proteins separated according to solvent solubilities were additionally purified to characterize and distinguish mutant and wild-type rice. The 2-D separations showed quantitative differences between mutant and control proteins. Figure 1A–D illustrates the compositional shifts in 2K41 and 'Calrose 76' endosperm proteins. Major differences (note arrows) were particularly obvious in the distribution of the salt-soluble globulins and water-soluble allergens. The profiles included the water-soluble albumins (A, B), the water-soluble rsealb fraction from the globulins (C, D) and the true salt-soluble globulins (E, F). Mutant albumins were enhanced at 50 kDa and 33 kDa, whereas proteins at 25 kDa and 14–16 kDa were greatly diminished. Mutant rsealb (C, D) proteins are slightly higher at 22 kDa but diminished at 14–18 kDa. Most of the salt-soluble (E, F) mutant proteins accumulated high levels of protein at 50 kDa, 32–33 kDa and 26–27 kDa but were significantly diminished at 23–24 kDa and 16 kDa. In general, the major globulins at 24–25 kDa were diminished in the mutant as were those proteins below 25 kDa.

Protein profiles in different phenotypes and solubility fractions

Some prominent shifts in banding patterns of the proteins occurred at different protein levels. Figure 2 illustrates SDS-PAGE patterns in responses to different protein levels. Represented are BC_2 segregants with normal protein levels (Fig. 2A), BC_2S4 segregants with high protein (Fig. 2B) and a selfed line, 4C (Fig. 2C).

Normal protein lines

One-dimensional patterns for the mutant and control albumins were similar. Minor pattern changes in the globulin and glutelin fractions were visible (50–58, 33, 22, 14–15 kDa). The rsealb fraction showed increases at 10 kDa and 22 kDa and a reduction in the 16-kDa band. Essentially all mutant globulins ranging from 27 to 58 kDa were increased, whereas the major 25-kDa globulin and bands at 14–16 kDa were reduced. The mutant prolamins and the glutelines appeared somewhat reduced in this normal protein line (Fig. 2A).

High-protein variants

The selfed high-protein line showed little change from the control in the 25-kDa globulins (see Fig. 2B), and all major globulins, prolamins and glutelin proteins were increased. Most prominent were the increases in the 58-kDa globulins, the prolamins and glutelins at 33 kDa and 25 kDa.

Selfed unimproved line

Changes in the Coomassie protein profiles in the mutant occurred in the globulin fraction with an elevated expression at 27 kDa and 50–58 kDa. There was an obvious reduction in the 22- to 25-kDa fraction. This globulin fraction is quite similar to the globulin fraction of the normal protein line (Fig. 2A). The prolamins were much reduced in the mutant and the glutelins appeared to be nominal in this selfed line. Globulin pattern changes were documented in normal, high-protein and selfed lines. The decrease in some of the 25-kDa subunits of the globulins and the increases at 50 kDa were among the most salient features of these rice mutants. Lysine, as percentage of total amino acids was affected by elevated protein levels. This is probably due to the large increase in prolamins and glutelins (Fig. 2), which comprise 60–80% of the total seed

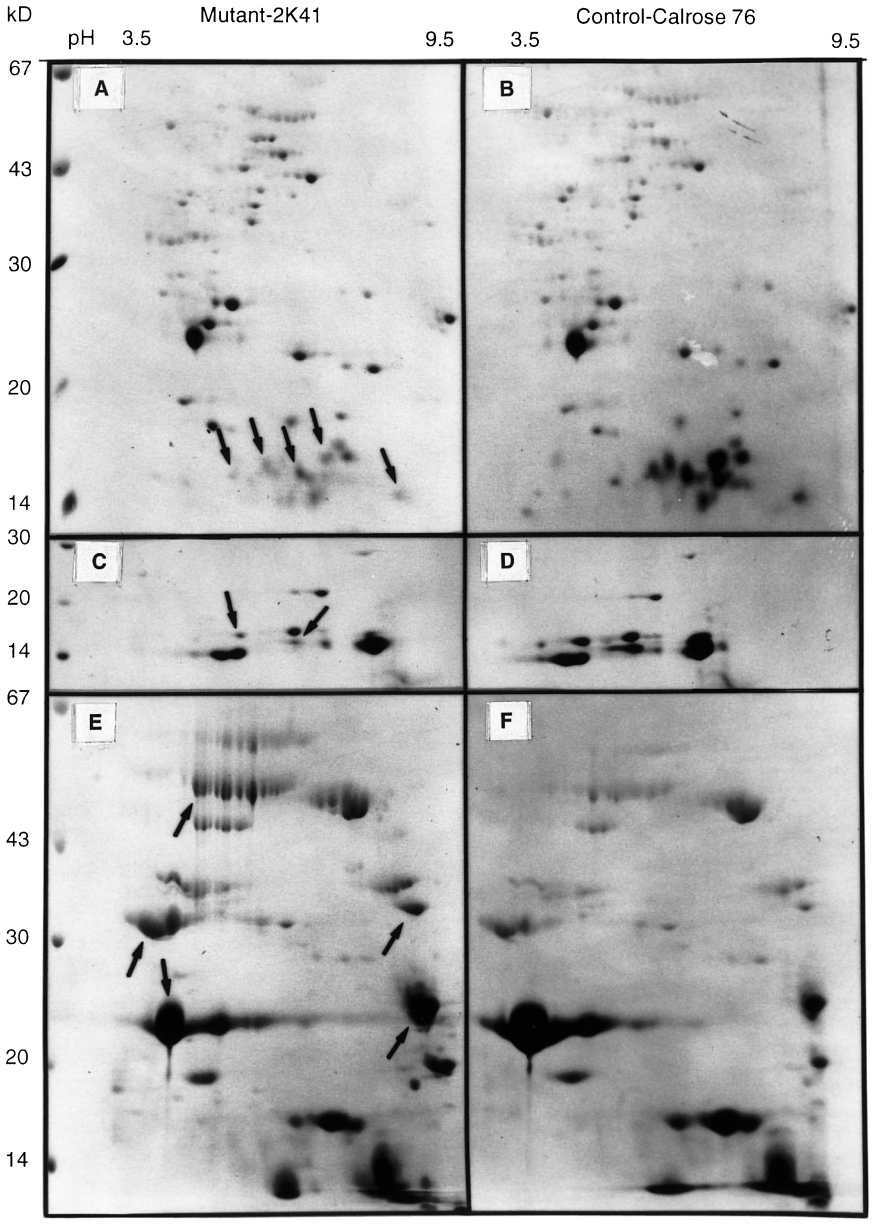

Fig. 1A–F Two-dimensional separation of mutant and control proteins from germplasm backcrossed three times (BC_3S1) to the source control cultivar, 'Calrose 76.' In Vitro-derived mutant BC_2S4 was selfed and then crossed, $2K41_{female} \times$ 'Calrose 76'$_{male}$. Mutants are displayed on *left half*, controls on the *right half*. **A**, **B** Water-soluble albumins, **C**, **D** rsealb water-soluble globulins **E**, **F** salt-soluble globulins. *Arrows* highlight differences between mutants and controls: *up arrow* indicates higher levels in mutant and *down arrow* indicates lower levels

protein (Schaeffer and Sharpe 1990), and high levels would normalize the percentage lysine.

Differences in protein banding patterns across segregants were also obvious in the SDS-PAGE profile of whole grains (Fig. 3). These lines varied widely in protein content; however, substantial increases compared to 'Calrose 76' in the 21-kDa, 33-kDa and 50-kDa fractions were common to all mutant lines. The protein differences at 14–15 kDa were difficult to interpret because this region of the gel had not only proteins from the prolamins but from all fractions, with only a small contribution from the glutelins, which are predominant in the endosperm portion of the whole grains.

Protein distribution in acrylamide gels

The protein content of individual mutant and control gel bands from isolated globulins is illustrated in Fig. 4 as is the mutant and control ratio for percentage lysine, based on total amino acids in the acid digest of proteins, in different gel bands. The mutant had enhanced protein in band 2. This was a section of the gel that did not stain well with Coomassie blue. Gel band 9 showed the reduction of the major globulin in the mutant; gel bands 8 and 9 had the highest mutant/control ratio due in part to the low lysine level in the controls in the major globulin fraction and the tendency for proteins in the lower half of the gel to have more basic amino

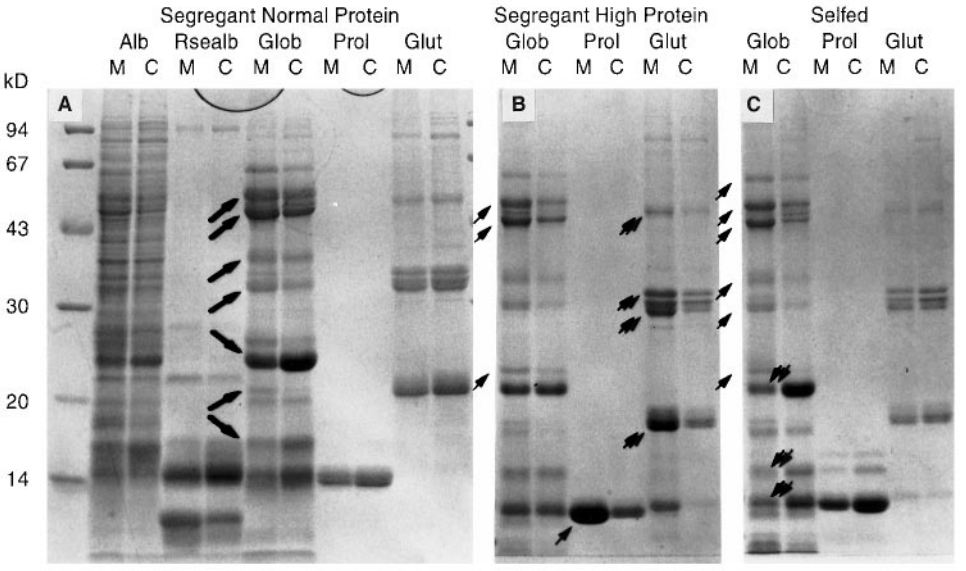

Fig. 2A–C Mutant (*M*) and control (*C*) SDS-PAGE profiles of endosperm proteins from **A** BC_2S4 grains with normal proteins, **B** proteins from high-protein grains, **C** proteins from unimproved selfed mutant. Wells were loaded with extracts from equal amounts of rice grain. *Alb* Albumins, water soluble; *Rsealb* water-soluble globulins; *Glob* globulins, salt soluble; *Prol* prolamins, ethanol soluble; *Glut* glutelins, detergent soluble. *Arrows* highlight differences between mutants and controls: *up arrow* indicates higher levels in the mutant and *down arrow* indicates a lower level

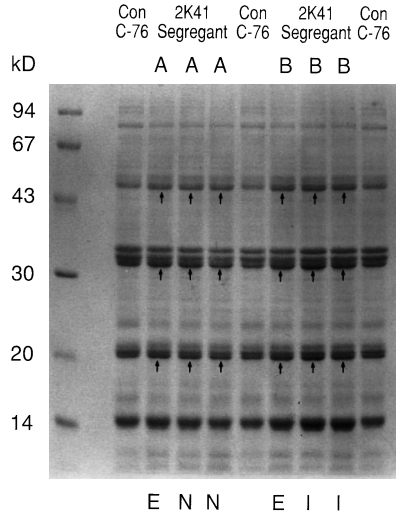

Fig. 3 SDS-PAGE profiles of whole-grain extracts, without solubility fractionations, from individual plants of controls and three mutant plants (2k41 group A and 2K41 group B). *Con* is the control and is the same as the cultivar 'Calrose 76' (*C-76*). Extracts from equal seed weights were loaded for electrophoresis. *E* Enhanced protein, *N* normal protein, *I* intermediate protein level. *Arrows* indicate greater protein in the mutants than the corresponding controls

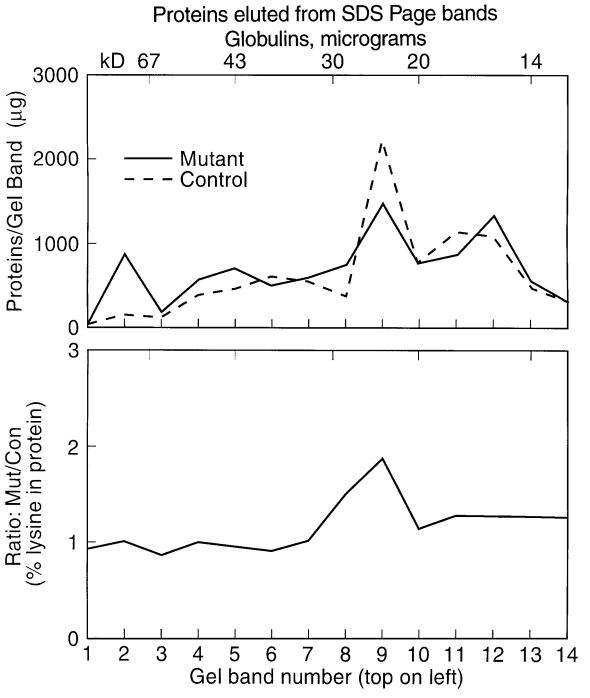

Fig. 4 Micrograms protein recovered and mutant/control (*Mut/Con*) ratio or percentage lysine in eluted proteins from bands across the SDS PAGE gels. Equal weights of mutant and control globulins were loaded onto gels for separation and analysis of eluate in a single experiment

acids than the upper portion. The compositions of the starting materials used for SDS-PAGE are presented in Table 2.

Table 2 illustrates the protein levels and the lysine content of the globulin fraction for line 4C described earlier (Schaeffer and Sharpe 1990), an early selection improved by selfing only. Proteins were eluted for quantitation with Pierce reagents. The mutant/control ratio was 1.1 for the total protein eluted from 14 gel bands. The percentage of lysine in the total extract produced a mutant/control ratio of 1.30, and total lysine, reflecting increased protein, produced a ratio of 1.45.

Discussion

Database homologies of several microsequenced water-/salt-soluble proteins demonstrated mutant

Table 2 Total protein and lysine in protein, eluted from globulin (salt-soluble) fraction separated into 14 gel bands by SDS-PAGE, and percentage lysine in total non-electrophoresed fraction

	Total protein (μg)	Ratio M/C	Lysine (% of total extract)	Ratio M/C	Total lysine	Ratio M/C
Mutant, 4C	9478	1.11	3.13	1.30	297	1.45
Control, C-76	8553		2.40		205	

down-regulation of a family of genes identified in the control as allergens (Fig. 1B). These allergens show specific homologies to both the salt-soluble alpha amylase/trypsin inhibitors (Adachi et al. 1993) and proteins identified as in vitro protein-synthesis inhibitors (Limas et al. 1990). Also, we found several salt-soluble proteins, at higher levels in the mutant than in the control, which show homologies to a number of storage proteins, including legumins, glutelins, globulins and vicilins. The reduced levels of allergens in the mutant and the increased levels in some globulins leads to some intriguing questions. For example, do endogenous allergens, also defined as protease and translation inhibitors, promote the precise orientation of proteins within protein bodies to produce a clear endosperm or perhaps function in protein body aggregation? Two-dimensional electrophoresis clearly indicated that the decreases in this class of proteins occurred in the enhanced-lysine/chalky line. Both whole seed extracts from controls with normal protein and vitreous mutants with high protein had no reduction in these proteins. Probably modifiers or proteases and peptidases function in the expression of stress biochemistry, and the allergens could then play a role in the modulation of the constitutive stress biochemistry assigned to this rice mutant.

The enhanced accumulation of storage-type proteins in the mutant suggests important differences in protein processing between the mutant and the control. The loss of translation inhibitors or other regulators, for example, could promote the synthesis and processing of the legumin-/glutelin-type precursor or primitive proteins at the expense of other types of endosperm proteins. For example, the 25-kDa globulins which are expressed at a higher level in the control are down-regulated in the mutant. Note that the increases in the 50- to 58-kDa precursor-type proteins, which are known to be salt soluble (Yamagata 1982) are correlated with the increases in the 21-kDa and 33-kDa glutelins (Fig. 3). The loss or the acquisition of translational modifiers could be important in driving specific evolutionary biochemistries associated with the processing of seed storage proteins.

Acknowledgements The authors thank J. N. Rutger for his help on sample analyses and technical information and R. Dilday and C. Johnson for seed increases in the field. We thank Dorothy Roach and Audry Lemucchi for clerical support.

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