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FRUCTOSE 1,6-BISPHOSPHATE ALDOLASE ACTIVITY IN LEAVES OF A RICE MUTANT SELECTED FOR ENHANCED LYSINE

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Key Word Index—*Oryza sativa*; Gramineae; aldolase; mutant; lysine.

Abstract—Unknown proteins isolated from mutant tissues of rice (*Oryza sativa* L.) recovered from inhibitor selections were subsequently peptide microsequenced. Database searches putatively identified one peptide as fructose 1,6-bisphosphate aldolase (EC 4.1.2.13). Tissues of mutant rice, PI564784, and wild type (cv Calrose 76) tissues were evaluated for aldolase activity. Total enzyme activities were slightly lower in the mutant than the control but the differences were not significant. Although the mutant phenotype is for enhanced lysine and protein, we ascribe the small aldolase differences to physiological adjustments, rather than to DNA modifications of the aldolase gene(s). Homologies of rice peptides with aldolases from a range of species, as well as rice cell culture expressed sequence tags (ESTs) are presented. Some amino acids sequences are highly conserved. The mutant phenotype expressing stress proteins is not likely to be defined by a change in rice aldolases.

INTRODUCTION

Fructose-bisphosphate aldolase is a widely distributed enzyme, and functions in both the chloroplasts and cytoplasm of plants and in numerous animal tissue, including the brain, muscle and liver. This enzyme, which has at least four loci in the rice genome [1] plays a vital role in carbohydrate metabolism and in the production of triose phosphates and derivatives important in signal transduction.

Mutants of rice, with enhanced lysine in endosperm proteins [2] and some lines with enhanced seed proteins have a range of attributes which have been partially characterized. We concluded from past experiments that the mutant described here, PI564784, is constitutive for specific stress-related and possibly membrane proteins significant in protein transport/export, including β1,3-glucanases and chitinases [3]. Alterations in proteins, particularly membrane and processing proteins, might be reflected in the activity of a ubiquitous enzyme such as aldolase. This work was based, in part, on the observations that electrophoretic profiles developed with Coomassie blue showed subtle quantitative differences in the banding patterns of mutants and controls separated on both denaturing and non-denaturing acrylamide gels. Proteins were eluted from the native gels of the mutant and subsequently separated on SDS-PAGE gels, blotted and sequenced. Microsequencing data led to the identification of several proteins, including the aldolase reported here.

RESULTS AND DISCUSSION

Mutant and control seedling proteins separated under non-denaturing conditions showed similar banding patterns with subtle quantitative differences. However, 1-dimensional SDS-PAGE profiles of proteins eluted from native gels showed the presence of substantially greater Coomassie positive material at about 35 kD in the mutant than in the wild type. Amino acid sequencing of the purified 35 kD band (Fig. 1) provides evidence that the peptide is an aldolase. Figure 2 illustrates complete homology for 21 amino acids with rice cDNA sequences derived from an expression library of rice seedlings. In addition to the complete homology of proteins isolated from rice there are many highly conserved regions which occur across a wide range of species and tissue types including animal brain and liver extracts. Amino acids that are unique for plants and highly conserved include Y (tyrosine) at position 3, K (lysine) at position 9, and T (threonine) at position 30 and G (glycine) at position 32. The N terminal end seems variable in plant systems but more constant in animal systems. Amino acids that are highly conserved across plant and animal systems include E (glutamic acid) and L (leucine) at positions 6 and 7. Amino acids A (alanine) at 11, G

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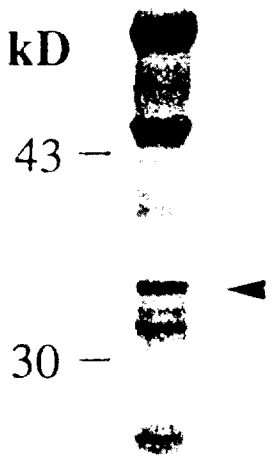

Fig 1. SDS-PAGE electrophoretic profile of Coomassie blue stained proteins eluted from non-denaturing gels of the mutant PI564784. Arrow marks the 35 kD band which was microsequenced.

at positions 18 and 20, and the triplet I (isoleucine), L (leucine) and A at positions 21, 22, 23, respectively, are highly conserved across Kingdoms as are L at 35, G at 39 and E and N (asparagine) at positions 41 and 42 respectively. Finally, E at position 44 appears highly conserved. These data suggest coding regions for sequence modifications which were likely to be tolerated and regions in which changes would probably alter enzyme functions.

Table 1 illustrates the aldolase activity in mutant and wild type extracts of rice seedlings. The aldolase activities in this experiment were generally lower in the mutant than the controls when evaluated on the basis of crude extracts, leaf area, and fresh weight. However, when expressed on a protein basis the specific enzyme activities (μmol mg^{-1} protein) of the mutant and control were similar or higher in the mutant. An initial experiment designed to optimize conditions produced similar enzyme patterns. The protein contents in crude extracts were also lower in the mutant than in the control.

Our conclusions from this work are that aldolases isolated from mutant rice seedlings appear normal in N-terminal amino acid sequences and have similar enzymatic properties to the control enzymes. A further implication of this work is that the mutant phenotype which expresses altered stress enzyme activities compared with the control is probably not conditioned by a major change in rice aldolases. However, enzyme activities do not provide information on protein aggregation and post translational modifications such as phosphorylation and glycosylation which could produce modified electrophoretic profiles.

EXPERIMENTAL

Rice, (*Oryza sativa* L.) seeds were germinated in 1 cm deep vermiculite/potting soil mix in 10 cm clay pots in chambers with 14 hr photoperiod from cool white fluorescent lamps at 90–100 μE m^{-2} s^{-1}. Plants were watered daily, cut at the soil level 10–14 days after sowing and frozen in liquid N_2. About 0.2 g frozen powder was transferred to a ground-glass tissue homogenizer and extracted at 0° with 4 to 4.5 ml of extraction buffer consisting of 50 mM Bicine–NaOH, pH 8.1, 5 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 1% soluble PVP-40, 0.01% Triton × 100 and 5 mM DTT. Homogenates were centrifuged at 27000 *g* for 15 min at 2–4°. Supernatants were quickly transferred to 1.5 ml micro centrifuge tubes and frozen in liquid N_2. The spectrophotometric assay for FBP-aldolase (EC 4.1.2.13) activity was performed essentially as described in ref [4]. Assay soln contained 50 mM Tris–HCl, ph 7.5, 1 mM EDTA, 1.6 mM fructose-1,6-bisphosphate, 0.2 mM NADH, 10 units triose-phosphate isomerase, 1 unit alpha-glycerophosphate dehydrogenase and 20 μl of thawed leaf extract in a total vol. of 1 ml. Assays were performed at 25° and *A* changes measured at 340 nm. Soluble protein was measured according to ref [5] using BSA as standard and following vendor's specifications.

Preps of proteins for microsequencing: Seedlings ground in liquid N_2 were extracted × 2 with 50 mM Tris pH 6.8, 5% HOC_2H_5SH, 1 mM phenylmethylsulphonylfluoride (PMSF) and insoluble PVP at 2–4°. The proteins were pptd with 4 vol. of cold Me_2CO and pelleted by centrifugation at 15000 *g* for 10 min. The pellets were solubilized with 20 mM NaOAc, pH 5.2, and 5% glycerol and layered onto non-denaturing Tris/borate, pH 8.3, 5–16% polyacrylamide gels. Margins of the gels were stained with Coomassie blue and 1–2 cm sections of the non stained

Table 1. Fructose-1,6 bisphosphate aldolase activity in crude extracts of seedlings and expanded (Epd) leaves of an *in vitro*-derived mutant (PI564784) and Calrose 76 (Cal-76), the source cultivar

Sample	Protein mg (Fr. wt.)$^{-1}$	Protein mg (cm^{-2})	Protein mg sample^{-1}	Leaf Area (LA) cm^2	Fr. wt. g	Rate μmol min^{-1} mg^{-1} protein	Rate μmol min^{-1} g^{-1} fr. wt.	Rate μmol min^{-1} cm^{-2} LA
Cal-76 Epd Leaf	12.7	0.213	2.86	13.4	0.225	0.256	3.26	0.055
Mut Epd Leaf	10.6	0.157	1.8	11.4	0.17	0.292	3.10	0.046
Cal-76 Seedling	32.3	0.447	8.73	18.3	0.27	0.389	12.54	0.185
Mut Seedling	26.1	0.383	5.4	15.0	0.22	0.394	10.28	0.151

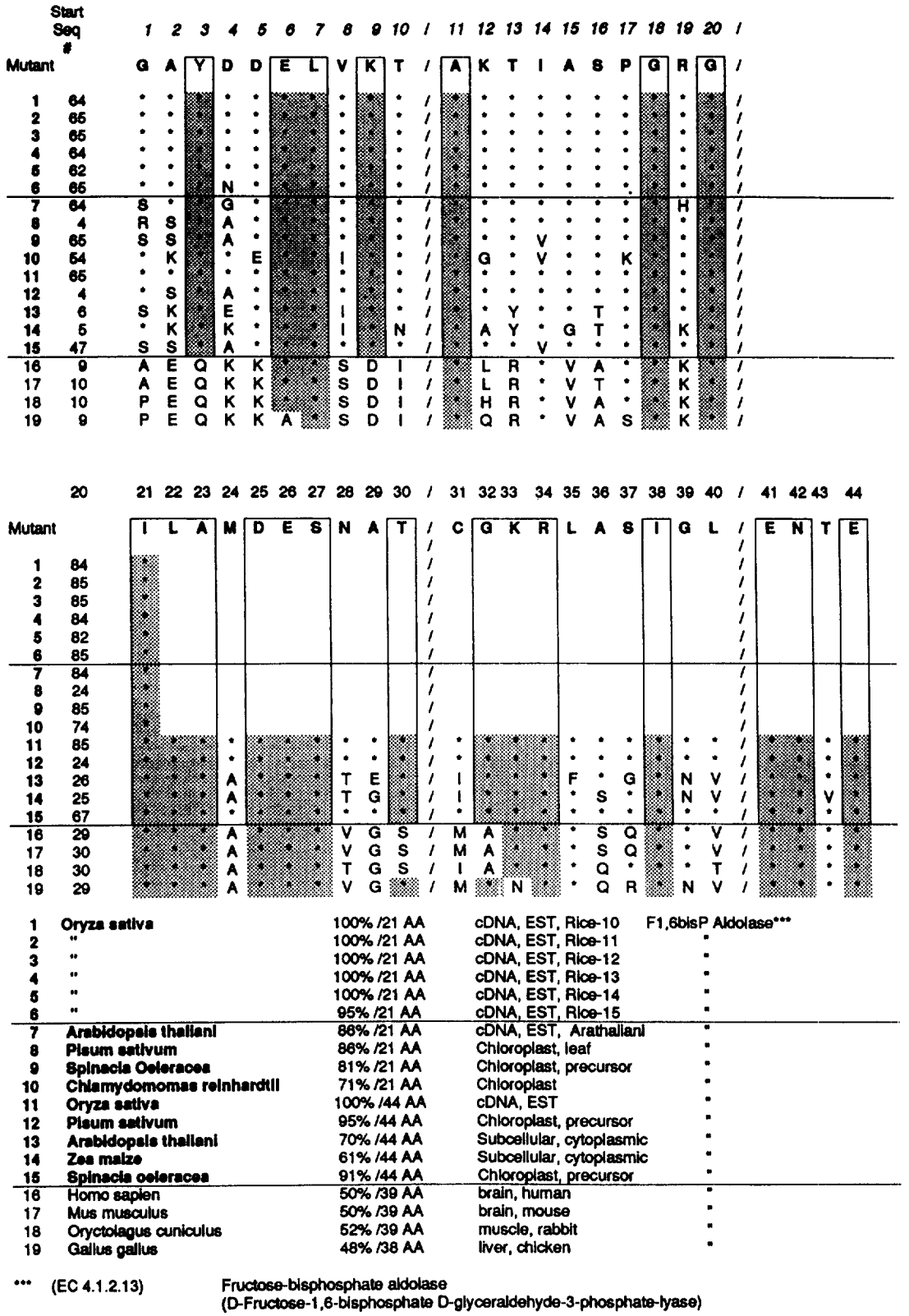

Fig 2. Homology of isolated microsequenced proteins from mutant rice seedlings with Fructose-1,6-bisphosphate aldolase from plant and animal sources retrieved from Genbank databases. Amino acids from the database corresponding with the rice amino acids are designated by *. The highly conserved regions are indicated by * and shading.

portions representing the major protein bands were eluted with H_2O by shaking overnight. Eluted proteins were re-precipitated with Me_2CO and centrifuged. Pellets were re-solubilized in SDS and sepd electrophoretically in 12% SDS PAGE gels. Sepd proteins were blotted into PVDF membranes, visualized with amido black in 0.5 M HOAc and subjected to amino acid microsequencing.

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