Biochemical Characterization of 5-Methyltryptophan Resistant Rice Mutants through SDS-PAGE and 2-Dimentional Gel Electrophoresis

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Abstract − To increase the contents of specific amino acids in the rice (*Oriza sativa* L.) cv. Dongan, original variety (control), we developed 4 homologous 5-methyltryptophan (5MT) resistant rice M_5 mutant lines via *in vitro* mutagenesis with gamma-rays. These mutant lines exhibited elevated amino acid content, in addition to an increased tolerance to 5MT inhibition. SDS-PAGE analysis was conducted to identify changes in total proteins and purified protein fractions based on their solubility properties between the control and 4 mutant lines. Significant differences in total protein fractions were detected at the molecular weight of about 26 kDa and 18 kDa between the control and 2 mutant lines. In the patterns of 4 solubility classes, enhanced polypeptides with high signal intensity were observed in mutant lines compared with that of the control. It was also interesting that the significant differences in prolamin band patterns between MRI and MRII were found at about 18 kDa and 16 kDa. Proteins produced in elevated amounts or *de novo* in response to 5MT were studied by comparing silver-stained two-dimensional gels of leaf proteins between the control and 4 5MT resistant lines. Five N-terminal sequences were obtained and the database was searched to tentatively identify them. Sequencing data of *de novo* or enhanced proteins in mutant lines demonstrate that the resistance for 5MT may be similar to mechanism to cope with the oxidative damage caused by reactive oxygen species (ROS).

Key words : 2-Dimentional gel electrophoresis, Mutants, Rice, SDS-PAGE

INTRODUCTION

Rice is an important source for human dietary protein especially in Asian countries. However, similar to those of other cereals, rice seed proteins are deficient in some essential amino acids. It will greatly benefit our efforts to improve the nutritional quality of rice if suitable mutants associated with the metabolism or catabolism of essential amino acids are identified and characterized. In vitro techniques provide some advantages for the application of biochemical selection pressure and the recovery of specific metabolic mutants (Green and Philips 1974; Schaeffer and Sharpe 1981).

A wide range of mutants in endosperm storage proteins have been isolated and characterized (Ogawa *et al.* 1989; Schaffer and Sharpe 1997; Krishnan 1999). Seed storage proteins can be divided into four classes; albumins, globulins, prolamines, and glutelins, based on their solubility properties. Glutelins, the major storage proteins in rice, account for 80% or more of the total seed proteins, and the remaining 20% is consisted with globulins (4～15%) prolamines (2～8%), and albumins (1～5%) (Houston *et al.* 1968). Schaeffer

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and Sharpe (1987) selected callus cultures resistant to inhibition of lysine plus threonine and S-(2-aminoethyl) cysteine. Plants regenerated from these cultures and their progenies had improved seed protein lysine as well as improved protein levels. Recently, Schaeffer and Sharpe (1990) reported that the most obvious amino acid composition changes in the mutants was a higher lysine level in all protein solubility fractions and a decrease in tyrosine through sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. The endosperm proteins of the high lysine mutants revealed reduced amounts of the 30-kDa low-lysine globulin and an increase in several high-molecular weight globulin components (Schaeffer and Sharpe 1990). Seed storage proteins from these mutants improved by several backcrosses to 'Calrose 76' were characterized for changes in five different solubility classes by SDS-PAGE and some two-dimensional gel electrophoresis (2-DE).

The development of these polyacrylamide gel electrophoresis techniques has been extremely useful as an analytical tool for the separation and quantification of protein species from complex mixtures containing higher plants. Wilkins *et al.* (1996) invented the new term "proteome" to refer to the total set of proteins encoded by the genome of an organism. "Proteomics" is the global study of the proteins comprising the proteome, including the changes in structure and environmental cues. Recently, the proteomics has also been applied to all the proteins expressed in a particular organelle or tissue or in response to a particular stress (Salekdeh *et al.* 2002). The principal technique for the global analysis of proteome is 2-DE. The two-dimensional separation makes use of two independent protein characteristics: one is the charge, which is reflected by the isoelectric point (p*I*), and the other is the molecular weight, which determines the mobility of the SDS-protein complexes in polyacrylamide gels (Weber and Osborn 1969). Direct micro-sequencing analysis of the protein spots on the gel is also applicable to confirm the identification of the proteins. Since O'Farrell (1975) first demonstrated the great potential of 2-DE technique using isotopically labeled protein, this method has been extensively applied in resolving proteins from both prokaryotic and eukaryotic organisms and can be used for the separation of many types of cellular proteins (Bravo 1984). As a result of these improvements, this technique has been employed in studying alterations in protein expression during development and differentiation or in response to environmental changes. Plants have highly developed defense mechanisms against abiotic environmental stresses. *De novo* protein synthesis is one of the major physiological responses to the environmental stresses such as wounding, drought, pathogens, and salinity.

In this study, different endosperm protein fractions of the control and high amino acid accumulating mutant lines were characterized by SDS-PAGE. For the investigation of the changes of gene expression according to 5MT inhibition, total leaf proteins of the control and mutant lines were separated by 2-DE, and *de novo* or enhanced proteins were identified by N-terminal amino acid sequencing.

MATERIALS AND METHODS

1. Plant materials

Mature seeds of rice (*Oryza sativa* L.) *japonica* cv. Dongan and the high-amino acid accumulating M_5 mutant lines, MRI-40, MRI-110, MRII-21 and MRII-63, derived from cv. Dongan by *in vitro* mutagenesis with gamma rays (Kim *et al.* 2004a) were harvested at the end of the growing season and stored at 4°C until used.

The original variety Dongan and homozygous 5MT resistant 4 MR lines were sown separately in pots filled with vermiculite and grown in a growth chamber set at 27°C with a 18 h light photoperiod for 3 weeks. Three-week-old seedlings were transferred to fresh water or 500 μM 5MT solution, and grown separately for 4 days. For the 2-DE analysis, the aerial parts (approximately 5 g F.W.) of plants grown in fresh water or 500 μM 5MT solution were collected, and immediately ground in the liquid nitrogen.

2. Amino acid contents within seed storage protein

The total amino acid contents of the seeds were measured using the Pico-Tag method (Waters) as described in previous reports (Kim *et al.* 2004a, b). General amino acids and cysteine were hydrolyzed using constant boiling HCl containing 0.5% phenol in a reaction vial for 24 h at 110°C. The hydrolysis of tryptophan was conducted in 4 M methanesulfonic acid for 24 h at 110°C and neutralized by adding 4 M KOH to the sample for 5 min.

3. SDS-PAGE of the seed storage proteins

Seed storage proteins were obtained by extracting 0.2 g

of dehulled rice meal with 1 ml of an extraction buffer (62.76 mM Tris-HCl pH 6.8, 1% 2-mercaptoethanol, 1% SDS, 10% glycerol and 0.01% bromophenol blue). All the bulk protein fractions were brown rice with aleurone and embryo intact. The extracted proteins were dissolved in a sample buffer (Sigma S3401) at a 1 : 5 ratio, boiled for 5 min and microfuged for 5 min. For the determination of the protein contents, the resulting supernatant containing the total rice seed protein was measured by the Bradford method (Bradford 1976), and then an equal amount of the proteins was subjected to SDS-PAGE.

Seed storage proteins were fractionated into solubility classes using the procedures described by Luthe (1983), with minor changes. Briefly, one-gram of seed meal was defatted by stirring in 5 volumes of cyclohexane for 2 h. The storage protein fractions were sequentially extracted in the order given below by stirring the defatted powder for 2 h at room temperature in the following solvents: water for albumin extractions; 1 M NaCl for globulin extractions; 70% ethanol for prolamine extractions and 0.5% SDS and 1% 2-mercaptoethanol for the glutelin extractions. All the solvents used for the fractionation contained 1 mM phenylmethyl-sulfonylfloride (PMSF) and were buffered with 10 mM Tris-HCl (pH 7.5). Protein fractions were precipitated with 2 volumes of acetone at −20°C overnight and centrifuged at 27,000 g for 20 min at 4°C. To remove the residual salt and solvents, the globulin and glutelin fractions were dialyzed overnight with water containing 1 mM PMSF, and reprecipitated with acetone as described above. Fraction protein contents were determined using the Bradford method (Bradford 1976). Protein subunits were fractionated using the Laemmli's method (Laemmli 1970) and silver stained.

4. Two-dimentional gel electrophoresis

The powder was homogenized in an aqueous extraction buffer containing 0.5 M Tris-HCl (pH 8.3), 20 mM $MgCl_2 \cdot 6H_2O$, 2% Nonidet P-40, and 2% 2-mercaptoethanol. Soluble proteins were extracted from homogenate according to the acetone precipitation method. The final protein pellet was redissolved in 9 M Urea, 10% Nonidet P-40, 40% ampholine (pH 3-10), and 2 M dithiothreitol. Two-dimensional gel electrophoresis was conducted essentially according to the procedure of O'Farrell (1975). The isoelectric focusing (IEF) gel electrophoresis was done using a gel containing 9.5 M Urea, 10% Nonidet P-40, 30% acrylamide, 1% bis-acrylamide, 2.5%, 1%, 2.5%, and 1% ampholine pH 4-6, pH 6-8, pH 5-7, and pH 3-10, respectively, to which the extract containing 500 μg protein determined by the Bradford method (1976) was applied. The first gel electrophoresis was carried out at 250 V for 0.5 h, 300 V for 0.5 h, 400 V for 0.5 h, and 600 V for 17.5 h. The upper (anode) buffer was 0.1% 2 N NaOH and the lower (cathode) buffer was 38% phosphoric acid. After the first gel electrophoresis, the gel was removed and equilibrated for 30 min using equilibration buffer containing 1 M Tris-HCl (pH 6.8), 10% SDS, 50% grycerol, 1% 2-mercaptoethanol, and 5% bromophenol blue. The second dimension SDS gels were 0.75 mm thick and consisted of a 14 cm separation gel containing 30 : 1 acrylamide-bisacrylamide, 1 M Tris-HCl (pH 6.8), and 10% SDS. Electrophoresis was done at 140 V constant current until the tracking dye had reached the bottom of the gel. Two-dimensional gels were fixed and silver stained for detection.

The two dimensional gels were electroblotted to polyvinylidene difluoride (PVDF) membranes at 200 mA for 12 h and the membranes were stained with 0.3% Ponceaus in acetic acid. The protein spots were excised from the membranes and placed into the reaction chamber of the gas-phase protein sequencer. Protein sequencing was conducted using Procise491 and Procise cLC492 Automatic Protein Sequencer (Applies Biosystems).

RESULTS AND DISCUSSION

1. Amino acid contents in 5MT resistant M_5 rice mutants

Four homologous 5MT resistant M_5 lines, MRI-40, MRI-110, MRII-21 and MRII-63, were analyzed for the measuring of amino acid incorporated into the protein synthesis through hydrolysis of the storage protein in the endosperm. The amino acid profiles of the 4 mutant lines revealed a similar pattern. In these mutant lines. The total amino acid contents were 35%, 28%, 97%, and 89%, respectively, which was greater than those in the original variety, Dongan. Increasing rates of essential amino acid relative to the control seed were 88 (MRII-21) ~ 20% (MRI-110) in the mutant lines (Table 1). As expected, significant increases in the levels of tryptophan which was a target amino acid corresponding to 5MT

Table 1. Essential amino acid profiles in brown rice seeds of four homozygous 5MT resistant mutant lines compared to those of the original cv. Dongan

Amino acid	Dongan	MRI-40	R/D†	MRI-110	R/D	MRII-21	R/D	MRII-63	R/D
His	4.04	5.29	1.31	4.54	1.12	8.68	2.15	7.66	1.89
Thr	5.75	7.54	1.31	7.00	1.22	10.98	1.91	10.82	1.88
Val	8.76	11.41	1.30	9.59	1.10	16.51	1.89	15.27	1.74
Met	0.76	0.92	1.20	1.26	1.65	1.52	2.00	1.30	1.70
Ile	4.54	6.00	1.32	5.79	1.28	8.41	1.85	7.46	1.64
Leu	8.54	11.31	1.32	9.80	1.15	16.56	1.94	14.75	1.73
Phe	3.91	4.75	1.21	4.39	1.12	6.75	1.73	6.69	1.71
Trp	0.01	0.02	2.20	0.03	3.03	0.03	3.28	0.03	2.91
Lys	2.36	2.91	1.23	3.96	1.68	3.37	1.43	3.43	1.46
Sum‡	38.67	50.14	1.30	46.37	1.20	72.82	1.88	67.39	1.74
Total a.a.	118.79	160.20	1.35	152.26	1.28	233.94	1.97	225.01	1.89

Numbers given are μmole g^{-1} fresh weight.
†Ratio of the mean value of mutants to the original variety Dongan, ‡Total contents of essential amino acid.

were observed in the mutant lines. The increase of tryptophan was 2.2, 3.0, 3.3 and 2.9 times in MRI-40, MRI-110, MRII-21 and MRII-63, respectively. However, the increasing rates of the phenylalanine and tyrosine levels in the shikimate pathway were lower than the total increase rates of the amino acids in the mutant lines. Increase of the lysine level, one of the least abundant amino acids in rice, was 1.2, 1.7, 1.4 and 1.5 times higher than that of the control seed. In addition, the amino acid profiles from the seed hydrolysates of MRII showed significantly increased levels of histidine, threonine, and methionine compared to the other amino acids in the mutant lines. The MRII-21 to control ratio for these amino acids was 2.2, 1.9 and 2.0, respectively. These might be metabolic shifts in the pathways not directly related to the tryptophan synthesis in order to satisfy the cell charge or the hydrophobic-hydrophilic balance in the seed storage proteins. Remarkable increases of the isoleucine and leusine levels in the MRI lines were also observed.

2. SDS-PAGE analysis of endosperm storage proteins

SDS-PAGE analysis was conducted to identify changes in total proteins and purified protein fractions based on their solubility properties of mutant lines. Total proteins extracted as resolved by SDS-PAGE revealed a little different protein profiles between the control and mutant lines (Fig. 1). More than 40 bands were visualized by silver staining. Interestingly, about 50 and 38 kDa protein was present at higher concentration in the mutant lines than in the control. Significant differences between the control and the mutant lines were detected at the molecular weights of about 26 kDa and 18 kDa. MRII-21 and MRII-63 had 26 and 18 kDa proteins absent in the control (Fig. 1). These differences between MRI and MRII groups might be due to two groups derived from each different cultures.

In order to assign these polypeptides to a solubility class, they were compared with the electrophoretic patterns, sequentially extracted from the seed powder. Minor protein changes between the albumins of the control and the mutants were observed at the molecular weights of ca. 42 and 62 kDa (Fig. 1). In an early work, Iwasaki *et al.* (1982) reported the separation of about 20 albumin components using starch gel electrophoresis. In rice, albumins of three types showed many similarity and some clear differences. They observed significant differences in band patterns and the intensities of several bands among varieties and resulted that albumins might be used in electrophoretic variety identification. However, in this study, significant differences in albumins were not observed between the control and the mutant lines.

In the rice glutelins, the most abundant storage protein of rice endosperm, the predominant species exhibit sizes of 34 ~ 39 kDa (α-subunit) and 21 ~ 23 kDa (β-subunit) (Krishinan and Okita 1986). Luthe (1983) suggested the α- and β-subunits may be proteolytic products of a larger precursor, 57 kDa protein, as results from *in vivo* pulse-chase labeling studies. Different intensities between the control and mutant lines were found at about 57 kDa and 39 kDa located at a larger precursor and within the range of α-subunit, respectively (Fig. 1).

Prolamines, typified by their solubility in alcohol solutions, are the major seed storage proteins in most of the cereals

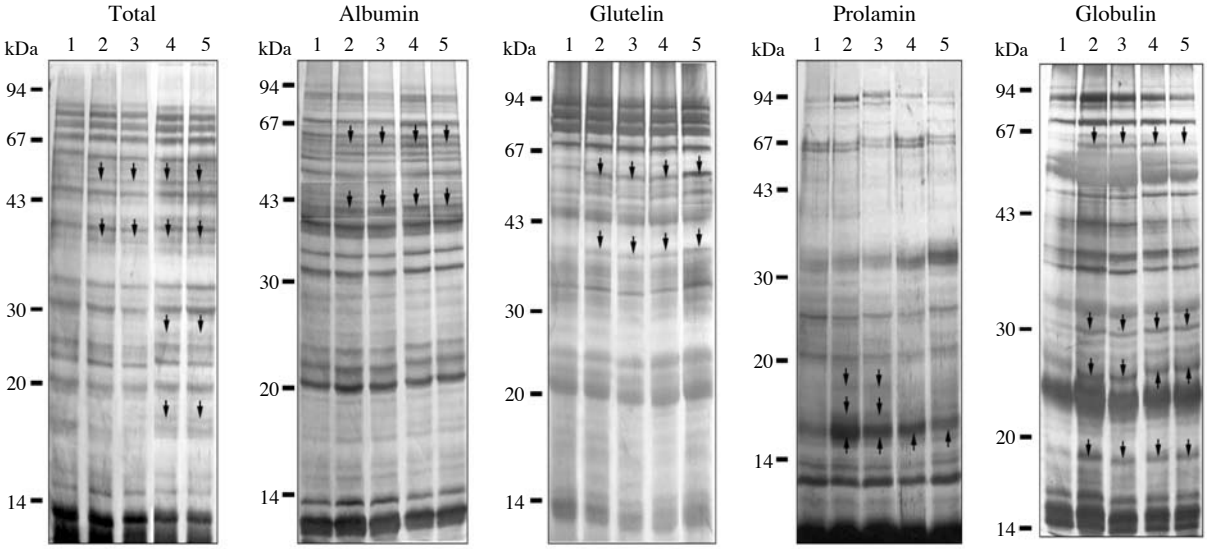

Fig. 1. SDS-PAGE analysis of the total seed proteins, albumin, glutelin, prolamin, and globulin in the 5MT resistant mutant lines. The proteins were resolved on a 12.5% SDS polyacrylamide gel and stained by silver staining method. Lane 1, control; lane 2, MRI-40; lane 3, MRI-110; lane 4, MRII-21; lane 5, MRII-63. Molecular weights are described in the left of each panel. The arrowheads point to the enhanced or specific polypeptides in mutant lines.

and serve as a source of nitrogen, carbon, and sulfur for the young developing seedling. The rice prolamines have molecular sizes of about 12～17 kDa (Mandac and Juliano 1978). The elevated expression in all the mutant lines was found at ca. 15 kDa. Interestingly, the 16 and 17 kDa proteins were present at a higher concentration in the two mutant lines, MRI-40 and MRI-110, than in the control (Fig. 1). SDS-PAGE analysis of *in vitro* translation products purified by immunoprecifitation using a rice prolamine antibody revealed the synthesis of a 16-kDa precursor form presumably containing a single peptide (Krishinan and Okita 1986). Complementary DNA encoding a putative rice prolamine precursor was also cloned based on cross-hybridization and restriction enzyme map analyses (Kim and Okita 1988). Like the glutelins, the prolamines are also packaged, albeit separately into protein bodies. Ogawa *et al.* (1987) isolated protein body type I (PB-I) from developing rice grain and concluded that PB-I is the accumulation site of rice prolamine.

In terms of globulin protein, in addition to many discrete minor proteins, six major bands were observed at about 50～53 kDa, 35～36 kDa, 24～25 kDa, 18 kDa, 16 kDa, and 14 kDa. These results were similar to that by Krishinan *et al.* (1992) except 35～36 kDa proteins. The SDS-PAGE profiles of mutant globulins revealed three distinct bands of different intensities at ca. 18, 31 and 65 kDa. It was also interesting that significant differences in the globulin band patterns between MRI and MRII were found at about 26 kDa. Schaeffer and Sharpe (1990) confirmed that lysine per unit weight of protein is higher in all solubility classes of the high lysine mutants, but the greatest change is in the globulin fraction.

The enhanced and *de novo* accumulation of storage-type proteins in the mutant lines suggests important differences in protein processing between the mutant lines and the control. The loss or the acquisition of translational modifiers could be important in driving specific evolutionary biochemistries associated with the processing of seed storage proteins. In addition, increases in the protein intensities of these four solubility classes, especially in the prolamins and globulins, might affect the elevated protein levels and amino acids containing lysine and tryptophan.

3. Two-dimensional electrophoresis analysis

In order to investigate the changes of gene expression in response to 5MT inhibition, 5MT was treated for 96 h, and total leaf proteins of the control and mutant lines were analyzed by 2-DE analysis. For simplicity, I have currently marked only the major proteins that are most affected under 5MT and show reproducible and prominent differences. Mutant specific proteins that were preferentially synthesized

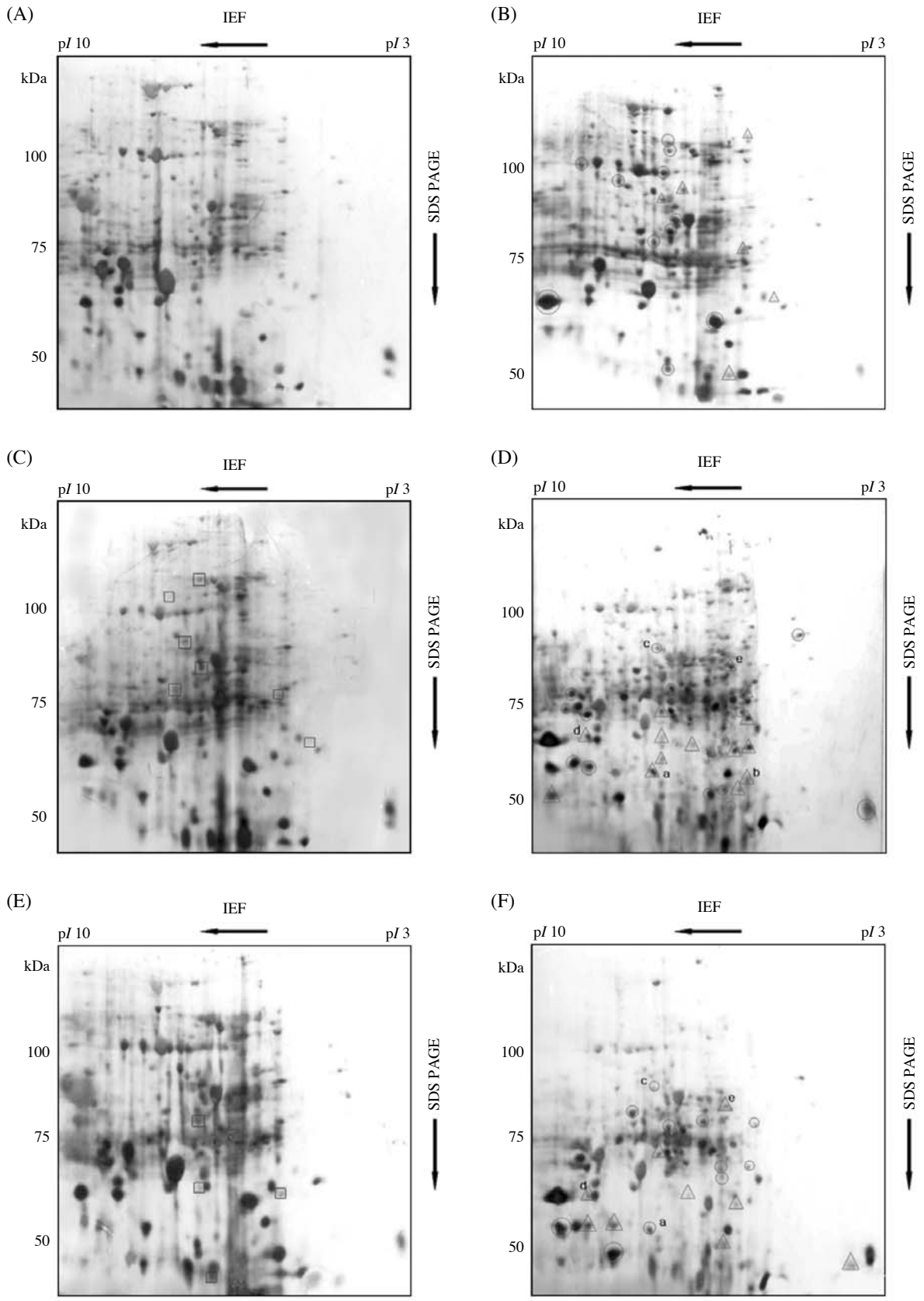

Fig. 2. Continued.

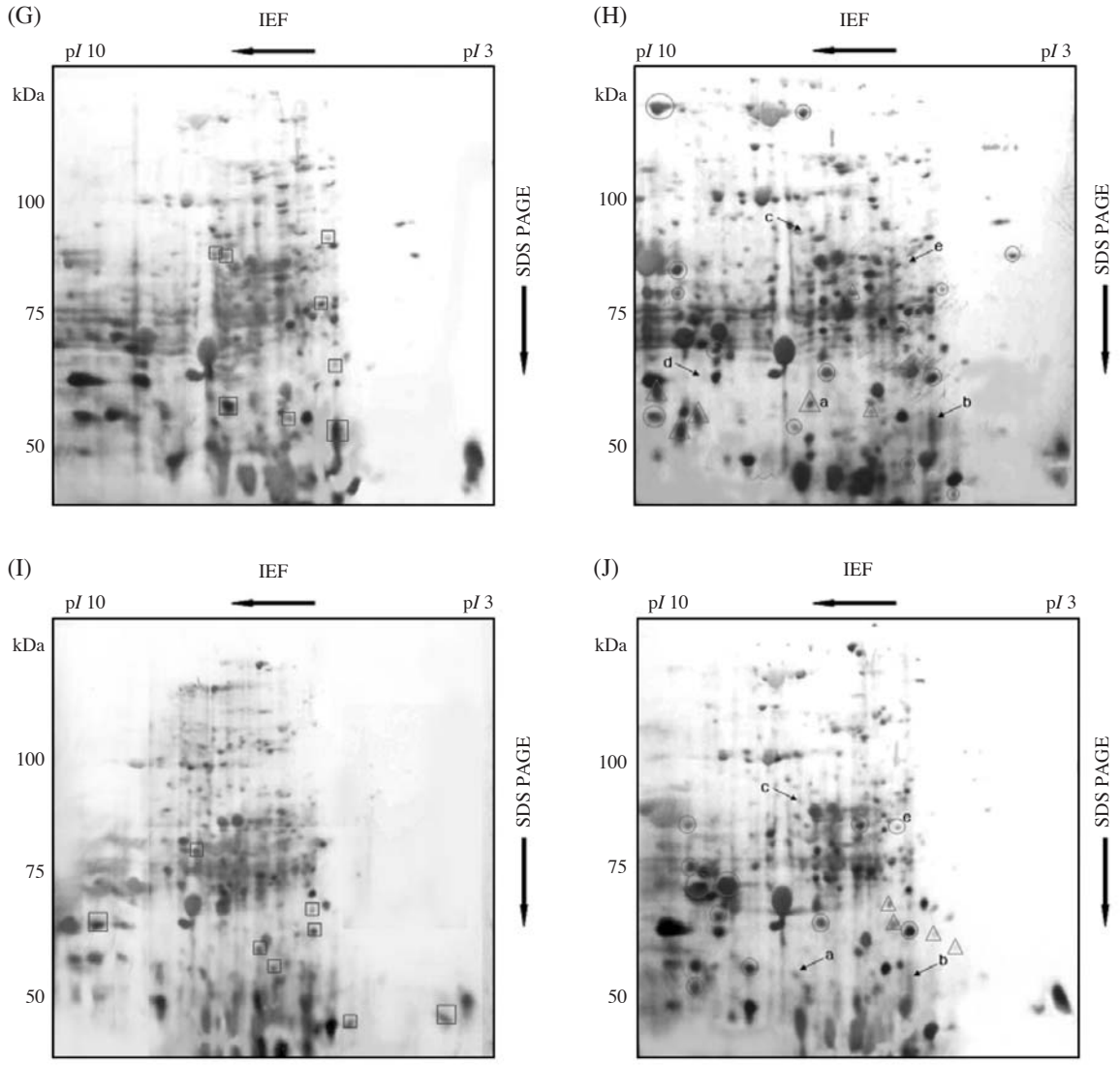

Fig. 2. Silver stained 2-DE image changes of the control (panel A and B), MRI-40 (panel C and D), MRI-110 (panel E and F), MRII-21 (panel G and H), and MRII-63 (panel I and J) leaf proteins obtained with a linear IEF from pH 3 to 10 for the first dimension in response to 5MT treatment. Panel A, C, E, G and I, 5MT non-treatment; and panel B, D, F, H and J, 96 h treatment. The squares indicate mutant specific proteins. The circles in the gels show the proteins that are increased in amount on addition of 5MT. The triangles indicate *de novo* proteins in response to 5MT. The values on the left indicate the molecular mass of the reference proteins.

in only the mutant lines are shown by squares; triangles show the proteins that were *de novo* synthesized in response to 5MT; proteins shown increased intensity by 5MT are noted by circles. Out of >500 leaf proteins detected by 2-DE, about 320 proteins were reliably quantified (Fig. 2). After silver staining in the control, at least 11 protein species were consistently more abundant in 5MT treated tissues, 6 proteins were *de novo* synthesized, and 9 proteins consistently less abundant, as shown in Fig. 2A and B. Mutant specific proteins in MRI-40, MRI-110, MRII-21, and MRII-63 were 7, 4, 8, and 8 spots, respectively (Fig. 2C, E, G, and I). *De novo* synthesized and enhanced proteins in these mutant lines against 5MT were 14, 9, 7, and 4, and 13, 11, 15, and 11 spots, respectively (Fig. 2D, F, H, and J). Polypeptides of the control and the mutant lines were distributed from 10～50 kDa and had p*Is* ranging from 4～10. Distribution of important proteins in the mutant lines were revealed from molecular weights of 40～80 kDa and ranged from 5～9 p*Is*.

The protein spots were electroblotted onto a PVDF membrane and stained with Ponceaus. Stained protein spots were sequenced using automatic protein sequencer. Five protein spots that revealed from 55～80 kDa and ranged from 6～

Table 2. Partial amino acid sequence analysis of 5MT-induced proteins from rice leaf

Spot name	Mr/p*I*†	Matching identity	Accession No.	E-value/%identity	Mr/p*I* of matched protein‡
Ma	55/6.8	Enolase (OSE 1)	Q42971	$6.2*e^{-2}$/100	48.0/5.42
Mb	52/4.8	Rubisco activase	AAK31173	$4*e^{-3}$/100	21.7/4.78
Mc	85/6.8	Rubisco large subunit	BAA00147	$6*e^{-3}$/100	52.9/6.22
Md	60/7.5	Cu-Zn SOD	P28756	8.2/94	15.1/5.72
Me	80/5.4	GS shoot isozyme	P14655	$6*e^{-3}$/100	46.6/5.96

†Experimental molecular weight and p*I*. ‡Theoretical molecular weight and p*I*. Mr and p*I* were calculated from the protein sequence using the Mr/p*I* calculator available at ExPASy (http://expasy.proteome.org.au/tools/pi_tool.html).

9 p*Is* were subjected to sequencing, designated from Ma to Me (Fig. 2, Table 2). Five sequenced proteins were identified by comparing with the database at National Center for Biotechnology Information (NCBI) using BLAST program, and compared against the SWISS-PROT and TREMBI databases using the PeptIdent (http://www.expasy.ch./tools/peptident.html). Molecular weights and p*Is* were calculated from the protein sequence using the Mr/p*I* calculator available at ExPASy (http://expasy.proteome.org.au/tools/pi_tool.html).

The amino acid sequence of the deduced protein of an open reading frame identified based on the N-terminus sequence of Ma showed strong homology to an *Oryza sativa* polypeptide, enolase (2-phosphoglycerate dehydrogenase), OSE1 with 100% identity. Ma revealed the molecular weight of 55 kDa and p*I* of 6.8. Enolase catalyzes the conversion from 2-phosphoglycerate (PG) into phosphoenolpyruvate (PEP) in glycolytic metabolism. Minhas and Grover (1999) reported that the level of transcripts of rice enolase gene (*eno*) increased in response to O_2 deprivation, desiccation, and salt in both shoot and root tissue. The database search identified Mb and Mc as ribulose-1,5-bisphosphate carboxylase (Rubisco) activase and Rubisco large subunit, respectively. Rubisco is a key enzyme in plant photosynthesis. *De novo* synthesized Rubisco activase (Mb) in response to 5MT had 52 kDa and 4.8 p*I*. The function of Rubisco activase protect chloroplast protein synthesis from drought stress or any associated temperature stress as a chaperon in associated with tylakoid-bound ribosomes (Salekdeh *et al*. 2002). The enhancements of Rubisco activity were also induced by salt (Sivakumar *et al*. 2000) and drought stresses (Bose and Ghosh 1994). These reports support this experiments that the *in vivo* mechanism by 5MT inhibition is similar to that by other stresses. The 18 amino acids sequenced from the N-terminus of Md protein showed high homology to Cu/Zn superoxide dismutase (Cu/Zn SOD) of *Oryza sativa*. Oxidative overproduction of reactive oxygen species (ROS) may be induced by a wide spectrum of abiotic and biotic stress factors. One of the primary enzymatic components of the antioxidative response systems (ARS) is SOD, which converts superoxide to H_2O_2 and oxygen (Slesak *et al*. 2002). Increasement in activities of cytosolic and chloroplastic Cu/Zn SOD by abiotic stresses such as water deficits, slat, and heat were extensively investigated in many species. Me protein was identified as glutamine synthetase (GS) by the database search. This protein is a key enzyme for assimilating ammonium into organic compounds. The multiple isoforms of GS have been identified to be localized to different subcellular compartments (chloroplast/plastid and cytosol) and differentially present in various organs (Hirel *et al*. 1980; Zhang *et al*. 1997). Recently, Hoshida *et al*. (2000) explained chloroplast GS was overexpressed about 1.5-fold in response to salt with rice transformed with a chloroplastic GS2 gene.

Biomass production is strongly affected by environmental stresses. Many of these adverse situations such as drought, excess light and extreme temperatures have been claimed to produce at least part of their deleterious effect through increased accumulation of reactive oxygen species (ROS) resulting in protein and DNA damage and lipid peroxidation. In chloroplasts, ROS are known to cause extensive modifications in several stromal and thylakoid proteins, including inactivation and degradation of Rubisco, fragmentation of GS, aggregation and breakdown of thylakoid proteins, and membrane solubilization of ferredoxin-$NADP^+$ reductase (FNR) (Palatnik *et al*. 2002). Most plants respond to oxidative stimuli by over-expressing antioxidant enzymes and metabolites. In this study, 2-DE analysis of mutant plants in response to 5MT, sequencing data of *de novo* or enhanced proteins in mutant lines demonstrate that the resistance for 5MT may be similar to mechanism to cope with the oxidative damage caused by ROS. Further study will be required in order to characterize the relationship between 5MT or other amino acid analog inhibition, and ROS-mediated oxi-

dative stresses.

ABBREVIATIONS

2-DE: 2-dimentional gel electrophoresis, 5MT: 5-Methyltryptophan, ROS: reactive oxygen species, ARS: antioxidative response systems, SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis

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