BIOLOGIA PLANTARUM 53 (3): 444-450, 2009

Identification of differentially expressed genes associated with 5-methyltryptophan resistance in rice mutants

D.S. KIM*[1], C.S. JANG**, J.-B. KIM*, G.-J. LEE*, S.-Y. KANG*, W. KIM** and Y.W. SEO**[2]

*Division of Food Irradiation and Radiation Breeding, Advanced Radiation Research Institute, Korea Atomic Energy Research Institute, 1266 Sinjeong, Jeongeup, Jeonbuk, 580-185, Republic of Korea**
*Institute of Life Science and Natural Resources and Division of Biotechnology and Genetic Engineering, Korea University, Anam, Seongbuk, Seoul, 136-701, Republic of Korea***

Abstract

5-Methyltryptophan (5MT), a tryptophan analog, resistant M_4 rice mutants with high free amino acid contents were obtained through *in vitro* mutagenesis. To evaluate the 5MT resistance mechanism, a cDNA library was constructed by using the leaves and roots of the 5MT resistant mutant plants. Expressed sequenced tags (ESTs) of 1 019 randomly selected clones were analyzed and then assembled 588 unigens. A total of 389 unigenes had significant homologies with known protein sequences at the NCBI database and the remaining 199 unigenes were designated unidentified genes. These unigens were grouped into 13 categories according to their putative functions. Of the 233 randomly selected clones, 25 were identified as differentially expressed genes between 5MT resistant and 5MT sensitive wild type plants. For further study of the differential expression of the genes, expression patterns of 12 genes related to various biological functions were evaluated in response to 5MT treatment in both the resistant plants and sensitive plants. All of the tested 12 genes exhibited higher expression levels in mutant plants than wild type plants under the 5MT inhibition. These expression patterns of the 12 genes suggested that the genes related to 5MT resistance in the rice mutants have a variety of functions, and yield remarkably diverse expression patterns upon 5MT treatment. Many genes that were identified tend to be related to defense and stress responses, suggesting "cross-talking" between biotic/abiotic stresses including the 5MT treatment. Therefore, 5MT resistant mutants might be of value for identifying genes related to plant defenses and stresses.

Additional key words: abiotic stress, expressed sequence tags, Northern blot, *Oryza sativa*.

Introduction

The discovery of novel genes, the determination of their expression patterns in response to abiotic stresses, and an increased understanding of their roles in stress adaptation will provide a basis for effective plant engineering strategies leading to a greater stress tolerance (Cushman and Bohnert 2000). In a recent report, we demonstrated that the 5MT may induce stresses *in vivo*, similar to other environmental stresses using two-dimensional gel electrophoresis and native-PAGE coupled with antioxidant enzymes (Kim *et al*. 2005a). An amino acid analog can induce production of reactive oxygen species (ROS), and it is hypothesized that the mechanism of resistance to an analog may be similar to that used to cope with the oxidative damage caused by ROS. The increasing of antioxidant enzyme activity, especially superoxide dismutase (SOD) and ascorbate peroxidase (APX), by 5MT treatment in the 5MT resistant mutant rice lines demonstrated that 5MT inhibition might induce increased accumulation of ROS similar to plant responses affected by environmental stresses such as drought, excessive radiation and extreme temperature. The 5MT resistant rice mutants might activate antioxidant systems for protecting a cell from protein and DNA damages as well as lipid peroxidation by ROS.

The simplest way to predict genes related to resistance to an abiotic stress in the functional genome is

Received 30 July 2007, *accepted* 10 May 2008.
Abbreviations: ESTs - expressed sequence tags; ROS - reactive oxygen species; 5MT - 5-methyltryptophan.
Acknowledgements: This work was supported by a grant (Code 20070501034005) from BioGreen 21 Program, RDA (Rural Development Administration), and a grant (code 308020051SB040) from Agricultural R&D Promotion Center, Korea Rural Economic Institute, Republic of Korea.
Equally contributed corresponding authors; [1]fax: (+82) 63 570 3319, e-mail: bioplant@kaeri.re.kr;
[2]fax: (+82) 2 3290 3005; e-mail: seoag@korea.ac.kr

to generate and analyze the expressed sequence tags (ESTs) from the stressed plants. In an unbiased cDNA library, the number of ESTs that match a particular gene should reflect the abundance of the corresponding cDNA in the library and the level of mRNA in the tissue from which the library was derived (Ewing *et al*. 1999). ESTs from amino acid resistant mutants will provide a genomic tool for identifying the genes associated with amino acid analog resistance. Genes induced by the treatment of an amino acid analog are thought to have a role in protecting cells from a stress. For mining the genes involved in a stress adaptation or resistance, many functional genomic tools are available, for example, differential screening of appropriate cDNA libraries, differential display, subtractive hybridization, *etc*.

In our previous study, we selected 5MT-resistant rice cell lines through an embryo culture system followed by γ-ray irradiation and subsequently developed mutant plants with 5MT resistance (Kim *et al*. 2004). Four 5MT-resistant homozygous M_4 lines (MRI-40, MRI-110, MRII-8 and MRII-12) were obtained from self-pollination of the regenerated 5MT resistant plants. These mutant rice lines were characterized by elevated free amino acid content and altered anthranilate synthase insensitivity to feedback inhibition by tryptophan suggesting that these properties might contribute to 5MT resistance (Kim *et al*. 2005b).

In this study, we constructed a cDNA library from the 4 5MT resistant rice mutants, which were subjected to a severe treatment of 0.5 mM 5MT for 3 d and subsequently selected random 1 048 clones for ESTs (Accession No. CD670586-CD671070, CF075623-CF075628 and CO435570-CO436077). Differential expression of interest genes was analyzed upon 5MT treatment between 5MT resistant mutant and wild type plants. Differential expressed genes (or interest ESTs) may provide useful insights for evaluating molecular mechanisms of amino acid analog resistance in mutants as well as wild types.

Materials and methods

Plants: Regenerated rice (*Oryza sativa* L.) plants from the 5MT resistant calli were obtained from a rice cv. Donganbyeo embryo culture which were irradiated with a γ-ray of 50 Gy, self-pollinated and the homozygous M_4 progeny was obtained as reported elsewhere (Kim *et al*. 2004). A cDNA library was constructed from the bulked leaves and roots of four 5MT resistant homozygous MR lines, MRI-40, MRI-110, MRII-8 and MRII-12, which were grown for 4 weeks in a growth chamber controlled at 27 °C with a 16-h photoperiod (irradiance of 27 μmol m^{-2} s^{-1}) and subsequently grown in a nutrient solution with 0.5 mM 5MT for 3 d under the same condition. The leaves and roots were harvested and immediately frozen in liquid nitrogen and stored at -70 °C for a cDNA library construction. For Northern blotting, both of the 5MT resistant mutant (MRI-40) and the wild type plants were grown for 4 weeks at day/night temperature of 25/18 °C and a 16-h photoperiod in a growth chamber and then grown on germination paper moistened with a nutrient solution containing 0.5 mM 5MT for 3 d. Also for a detection of the temporal expression pattern, treated plant tissues were harvested at 3, 6, 12, 24 and 48 h after the 5MT treatment and immediately frozen in liquid nitrogen.

ESTs analysis: Total RNA from the bulked leaf and root tissues of the four 5MT resistant homozygous MR lines was extracted by using a *Trizol* reagent (*Invitrogen*, Carlsbad, CA, USA). Poly(A)$^+$ mRNA was separated from the total RNA by using a *PolyATract* mRNA isolation system (*Promega*, Madison, WI, USA). Following the manufacturer's instructions, a cDNA library was directionally constructed from 5 μg of Poly(A)$^+$ mRNA by using a *ZAP-cDNA Gigapack III* cloning kit with a *Xho* I-oligo (dT) linker-primer (*Stratagene*, La Jolla, CA, USA).

An aliquot of the excised, amplified library was used for infecting *E. coli* SOLR cells of A_{600} 1.0 and subsequently plated on a Luria Bertani (LB) agar containing 100 μg cm^{-3} ampicilin. Individual colonies were selected randomly for plasmid DNA purification and sequencing. Template purification and sequencing of the plasmid cDNAs were performed by a modified alkaline lysis DNA preparation method from a 5 cm^3 liquid bacterial culture grown overnight in a LB medium containing 100 μg cm^{-3} ampicilin. Sequencing from the 5' end of the cDNA clones was performed by using T3 promoter primer according to the thermal cycling protocol of the *BigDye* terminator cycle sequencing ready reaction kit (*Perkin Elmer*, Boston, MA, USA). ESTs were clustered and assembled using with the *Paracel Transcript Assembly* program (version *2.7*; *Paracel*, Pasadena, CA). ESTs were assembled into high quality contigs if they had a 94 % similarity, a minimum overlap of 40 bp, and gaps less than 9 bps long. Unigenes were searched by using *BLASTX* against the NCBI non-redundant database. Putative function was assigned to a sequence by using an *E*-value cut-off of $< 1.0\ e^{-10}$. Sequences were grouped into 13 functional categories; primary metabolism, cell wall structure or metabolism, defense and stress related, cytoskeleton, cell division cycle, chromatin and DNA metabolism, gene expression and RNA metabolism, membrane transport and intracellular trafficking, secondary metabolism and hormone metabolism, signal transduction, function unknown or ambiguous and miscellaneous.

Isolation of differential expressed clones and Northern blotting: The differential hybridization was performed by

using probes labeled with the digoxigenin (DIG) and the signal detected by using alkali phosphatase and chemi-luminescent substrate CSPD (*Boehringer Mannheim*, Indianapolis, IN, USA) as described by Lee *et al*. (2006) with some modification. Each DIG-labeled probe was synthesized from each of mRNAs of the 5MT treated wild type plants (cv. Donganbyeo), the 5MT treated resistant plants or non-treated resistant plants.

The differentially expressed clones were amplified by using a T3 promoter primer (*Promega*) with the *BigDye* terminator cycle sequencing ready reaction kit (*Perkin Elmer*) for sequencing. The sequences were assembled and processed according to the procedures described above.

Total RNAs (20 μg) were fractionated on a 1 % formaldehyde agarose gel and transferred to a nylon membrane (*Amersham Intermational*, Little Chalfont, Bucks, UK). cDNAs to be used as probes were labeled by using the random rrimed DNA labeling kit (*Boehringer Mannheim*). The blots were prehybridized for 1 h and then hybridized for 18 h at 65 °C in a hybridization solution [1 mM EDTA, 7 % SDS, 0.25 M disodium phosphate (pH 7.2), and 5 % dextran sulfate]. The membrane was washed with a washing buffer followed by incubation with *Avidx-AP* conjugation, and then incubated with a *CDP-star* solution (*Applied Biosystems*, Foster, CA, USA). The membrane was covered with X-ray film, and exposed for 20 min at room temperature.

Results and discussion

ESTs sequencing and general characteristics: A rice cDNA library was constructed from the high-amino acid accumulating 5MT resistant homozygous MR lines (M_4), MRI-40, MRI-110, MRII-8, and MRII-12. Since the 5' untranslated region (UTRs) of many plant genes are relatively short (Joshi 1987), sequencing was carried out from the 5' end of the transcripts in anticipation of a higher chance of sequencing the coding regions, which are helpful to identifying ESTs. The ESTs from randomly selected cDNA clones of 1 048 were generated by single-run partial sequencing. Of the 1 048 sequencing reactions attempted, 1 019 produced readable sequences. A total of 676 non-overlapping sequences comprised of 98 contigs and 578 singletons were assembled through the assembly program as described above. To enable assigning putative functions to the unigenes from the 5MT resistant mutant rice, the *BLASTx* analysis was performed against a non-redundant protein database of the NCBI ($E < 10^{-10}$). A total of 389 unigenes showed significant similarity to known amino acid sequences while 199 unigenes did not match any genes reported. A total of 88 unigenes matched genes that might be from *E. coli,* human, mouse, pig or *Drosophila*, and thus could be excluded from further analyses. As a result, a total of 588 unigenes were used for further study.

Most of the abundant ESTs are involved in defense and stress response such as methallothionein-like protein (33 clones), fructose-bisphosphate aldolase, chloroplast precursor (ALDP, 14 clones) and phenylalanineammonia lyase (PAL, 5 clones) (data not shown). The relative high frequencies of these clones were consistent with the responses of the plants by the treatment of 5MT as if plants had been exposed to other environmental stresses. The methallothioneins are small proteins with a high affinity for binding metal ions (Andrews 1990) and they were up-regulated under both drought and salt stresses as established by monitoring with microarrary (Oztur *et al*. 2002). The ALDP which catalyzes the cleavage of fructose-1,6-bisphosphate into *D*-glyceraldehyde-3-phosphate and dihydroxylacetone phosphate exists, two forms in higher plants: cytoplasmic and plastidic. The cytoplasmic ALDP gene is inducible under anaerobic conditions and is considered to have an important role in producing ATP by stimulating the glycolysis under such conditions (Russell *et al*. 1990, Mujer *et al*. 1993). In plants, the PAL is the entry point enzyme into phenyl-propanoid metabolism that regulates the biosynthesis of a wide range of secondary compounds, including lignin, flavonoids, furanocoumarin and isoflavonoid phyto-alexins and wound protectant hydroxylcinnamic acid esters (Jones 1984). PAL activity can be induced by a pathogen attack, and by environmental stresses such as a wounding and light (Hahlbrock and Schroder 1975).

Classification of the ESTs according to biological processes: As indicated by their putative identifications, a total of 588 unigenes were grouped into the following 13 functional categories. The relative lack of information about the mechanism of feedback inhibition by a 5MT is reflected in the highest abundance of the unknown protein category (21.4 %). A major portion (21.4 %) of the unigenes represented transcripts involved in primary metabolism including photosynthesis followed by defense- and stress-related (13.7 %) and protein synthesis and processing (13.1 %). The ESTs related to cell wall structure and metabolism account for an additional 4.2 % of the identified clones.

Interest of EST clones: We classified the genes that may be related to the 5MT resistance mechanism by comparisons with the stress-inducible genes previously identified in other reports. A total of 126 unigenes were considered to be related to 5MT resistance. These genes are involved in many different functions including reactive oxygen scavengers, stress proteins, membrane transport and ion homeostasis, cell wall fortification, signaling components, transcriptional regulators and other metabolic processes. In our previous report, we demonstrated that the cytotoxic effect of 5MT may induce stresses *in vivo* similar to other environmental stresses (Kim *et al*. 2005a). The genes that may be inducible by the treatment of 5MT would be good

candidates for characterizing the key mechanism of amino acid analog resistances although further works are needed.

We had previously reported that the amino acid contents of four mutant lines used for a construction of the cDNA library were 2.4 - 3.5 times greater than in the control seeds (Kim *et al.* 2005a). As well as the increase of aromatic amino acids, tryptophan, phenylalanine and tyrosine, which coincide with the selection scheme, the content of the other amino acids was increased in the mutant lines. These observations were considered to be metabolic shifts in the pathways, which were not directly related to tryptophan synthesis which would satisfy the cell charge or the hydrophobic/hydrophilic balance in seed storage proteins. As was our expectation, the genes related to amino acid biosynthesis were identified in the EST analysis, which included the glutamine synthase root isozyme, glutamine-dependant asparagine synthetase 1, plastidic cysteine synthase 1 and S-adenosylmethionine synthetase 1 (data not shown).

Genes encoding anthranilate synthase α2 subunit (*OASA2*) and tryptophan synthase β-subunit directly related to a tryptophan biosynthesis were also isolated in the ESTs. Previously, we reported on an accumulation of the *OASA2* transcripts in the mutant lines by an altered anthranilate synthase form after 5MT treatment (Kim *et al*. 2005b). Changes in both the level and sensitivity of an anthranilate synthase can contribute to resistance and hence be responsible for the overproduction of tryptophan.

Identification of differentially expressed cDNA in 5MT resistant mutant plants: In other to compare the relative transcript abundance between 5MT resistant and sensitive plants, 223 randomly selected clones were analyzed by the differential hybridization method.

Twenty-five clones expressed higher more than 2-folds in 5MT resistant plants compared to sensitive plants under 5MT treatment were selected (Table 1). Six of the 25 clones showed higher expression more than 2-folds in the 5MT resistant plants treated with 5MT than those non-treated (Fig. 1, Table 1)

To evaluate that differential expression of genes were caused by the difference of genotype, we randomly selected 12 out of 25 clones and performed Northern blot analysis using the wild type plant and a 5MT resistant M_4 line (MRI-40) under the stress condition of 5MT. Except for the RE01 clone encoding unknown protein, expression levels of the remaining 11 cDNA clones in the mutant plants were 1.34-fold (RD07) to 3.82-fold (RJ05) higher than in the wild type for 5MT non-treatment. Whereas, under the treatment of 5MT between both

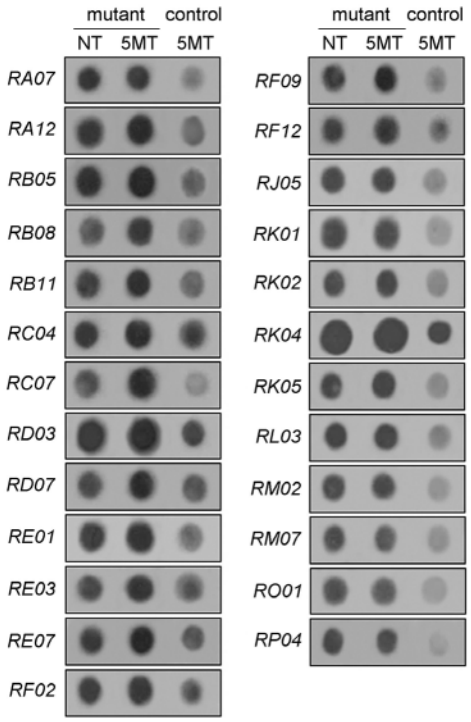

Fig. 1. Differential hybridization of the cDNA library from the leaves and roots of the 5MT resistant rice mutants. A positive nylon membrane containing randomly selected clones was hybridized with digoxigenin-labeled cDNA from 5MT resistant plants without or with a 5MT treatment, and 5MT sensitive plants with a 5MT treatment.

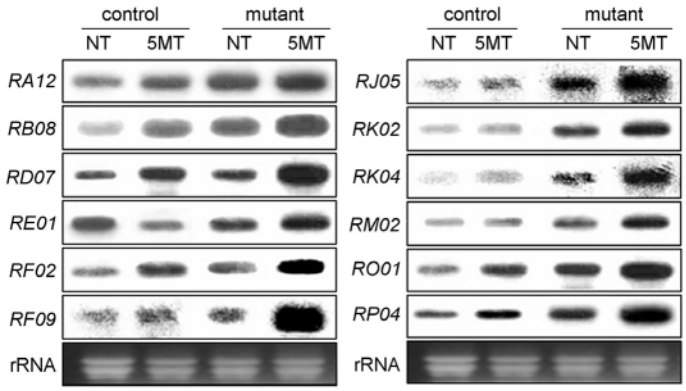

Fig. 2. Northern blot analysis of the transcription of the differentially expressed genes after 0.5 mM 5MT treatment. Total RNAs (20 μg per lane) from 4-week-old leaves in the control (the original cv. Donganbyeo) and 5MT resistant M_4 mutant (MRI-40) were fractionated on 1 % denaturing agarose gel.

Table 1. Identification of the highly expressed clones in the 5MT resistant rice mutants. Relative expression levels of cDNA clones were described between the 5MT resistant plants with and without 5MT treatment [RT/RTN = 5MT resistant plants with a 5MT treatment/5MT resistant plants without a 5MT treatment] and between the 5MT resistant plants and 5MT sensitive (control) plants with a 5MT treatment [RT/ST = RT/5MT sensitive plants with a 5MT treatment].

Clone	Inserted cDNA size [kb]	Identification	Accession	A.A. homology score [bits]	RT/RNT	RT/ST
RA07	0.5	heat shock protein 90	BAD04054	239	1.40	7.06
RA12	1.3	putative chorismate mutase/prephenate dehydratase	AAC73018	307	1.45	2.90
RB05	0.5	acyl carrier protein	P02902	103	1.67	3.83
RB08	0.35	ADP-ribosylation factor	BAA04607	324	2.89	6.08
RB11	1.6	putative glucosyltransferase	AAP53973	233	1.78	3.23
RC04	1.5	Zn-induced protein	AAA87049	166	1.73	2.02
RC07	0.5	putative lipase	BAB94238	103	2.82	7.09
RD03	1.4	putative tetratricopeptide repeat (TDR)-containing protein	BAD08177	106	1.70	2.87
RD07	1.3	putative phophatidylinositol/phosphatidylcholine transfer protein	BAD07999	278	2.46	2.72
RE01	0.7	unknown	-	-	1.66	5.73
RE03	1.3	enolase (2-phosphoglycerate dehydratase) (2-phospho-glycerate hydrolyase) (OSE1)	Q42971	315	1.99	5.06
RE07	1.2	glycine dehydrogenase P protein	AAS16361	57.8	1.97	4.55
RF02	1.1	glycine-rich cell wall structural protein 2 precursor	P29834	52.0	1.70	5.21
RF09	2.3	polyubiquitin	CAA53665	411	2.11	6.27
RF12	1.5	fructose-bishosphate aldolase, chloroplast precursor (ALDP)	Q40677	138	2.14	3.06
RJ05	0.8	putative hydroxyproline-rich glycoprotein	AAK92705	57.8	1.61	3.78
RK01	1.1	Harpin-induced protein 1 family (HIN1)-like	BAD03295	94.7	1.49	7.55
RK02	1.6	heat shock protein 82	CAA77978	272	1.56	5.56
RK04	0.7	type 1 rice metallothionein-like gene	AAD10376	81.3	1.69	2.71
RK05	1.5	putative cytochrom c1 precursor	BAB64199	111	2.02	3.77
RL03	1.5	GB protein	AAL68853	58	1.58	3.28
RM02	1.9	enolase (2-phosphoglycerate dehydratase) (2-phospho-glycerate hydrolyase) (OSE1)	Q42971	365	1.69	8.33
RM07	0.8	*D*-ribulose-5-phosphate 3-epimerase	AAF01048	100	1.63	7.16
RO01	1.5	unknown protein	BAB64786	133	1.56	7.84
RP04	0.9	probable peroxidase	AAQ56548	91.7	1.59	8.64

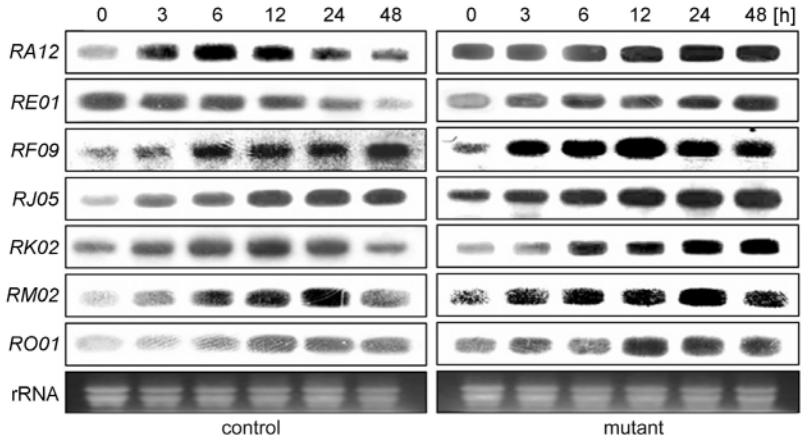

Fig. 3. Northern blot analysis of the transcription of the differentially expressed genes after 0.5 mM 5MT treatment for the indicated time periods. Total RNAs (20 µg per lane) from the 4-week-old leaves in the control and the 5MT resistant mutant (MRI-40) were fractionated on a 1 % denaturing agarose gel.

genotypes, all of genes tested showed higher transcription levels of in 5MT resistant plants than in wild types (Fig. 2).

We performed Northern blot analyses to identify the temporal expression patterns of seven out of twelve cDNA clones tested as above (Fig. 3). All of seven genes in both genotypes showed a peak of expression after the 5MT treatment, while RE01 showed the decline of transcripts after the treatment in wild type plants. In the

mutant plants, four genes such as RA12, RE01, RJ05, and RK02 exhibited a peak of expression at 48 h after the treatment but others showed the increasing of their transcripts until 12 or 24 h. However, the wild type plants showed rather different patterns of transcripts compared to the mutants. Only two genes, RF09 and RJ05, showed the highest expression at 48 h after the 5MT treatment. The transcription of four genes, RA12, RK02, RM02, and RO01 were increased by the treatment and then gradually decreased until 48 h.

It is believed that the genes induced by 5MT treatment in the resistant plants would be good candidates for characterizing the resistance mechanism(s). The expression patterns of RA12 clone encoding putative chorismate mutase would be in a good example of characterization of the 5MT resistant mechanism. We have already reported on an increase of the phenylalanine and tyrosine contents in mutant rice cell lines (Kim *et al*. 2005b). These observations were consistent, to a degree, with coordination of the production of these amino acids as well as tryptophan in the shikimate pathway. A likely cause of the phenylalanine and tyrosine biosynthetic branch may be via the elevated tryptophan level. It is known that tryptophan both activates and reverses the phenylalanine and tyrosine feedback inhibition in chorismate mutase isoforms.

To date little of the 5MT resistant mechanism have been known, therefore another case of worthy of further study is uncharacterized genes with highly expression through the 5MT treatment. We identified three unknown genes, *i.e*. RE01, RJ05, and RO01 *via* Northern blotting. Expression patterns of the transcripts of RJ05 and RO01 clones were similar to each other between both genotypes (Fig. 3), suggesting that both genes might be related to the response of 5MT in rice. However, the different responses of the RE01 clone between the resistant plants and wild types pointed to the hypothesis that this gene might be related to the resistance mechanism in the mutant plants.

Recently, another our report suggested that the cytotoxic effect of 5MT in plants was similar to, *in vivo*, other environmental stresses, *e.g*., temperature stresses, water stresses, and salt stress (Kim *et al*. 2005a). The finding that stresses-inducible genes such as heat shock protein (HSP) genes (RA07 and RK02), metallothionein-like gene (RK04) and peroxidase (RP04) were highly expressed by the 5MT treatment in the resistance plants was consistent with our previous data. HSPs, whose function is as molecular chaperones to guide an initial folding of proteins and refolding of the unfolded proteins (Nelson *et al*. 1992) may include an enzyme associated with thermal tolerance, such as membrane lipid saturation for protein stability (Van Breusegem *et al*. 1994). The type 1 methallothionein-like gene of rice was highly expressed in response to heavy metals, high temperatures, sucrose starvation and aging. Peroxidase (POD) is of great interest because of its significant role in the plant response to such stresses as air pollution, low temperature, ozone, heavy metals, wounding, pathogens, salts, drought and a UV radiation (Castillo 1992). Most of higher plants possess a number of POD isozymes which are involved in many physiological processes, including organogenesis, cross-linking of cell wall polysaccharides, lignification, suberization and auxin metabolism (Huh *et al*. 1997). These discoveries have raised the possibility that plants once exposed to one stress attain cross-protection against other stresses.

The transcript level of the RM02 clone encoding enolase revealed similar temporal patterns between the mutant and the control plants. The expression of this gene, however, was strongly induced in the mutant plant. The enolase activity was highly increased by salt stress (Forsthoefel *et al*. 1995) and desiccation stress (Minhas and Grover 1999). Increases of the enolase transcript levels might be due to the increased glycolysis rates to compensate for the reduced energy levels brought about by 5MT exposure. According to Plaxton (1996), the cytosolic network of glycolytic enzymes may provide an essential metabolic flexibility that facilitates plant development and acclimation to environmental stresses. In addition, an increase of the enolase level promotes synthesis of the phosphoenolpyruvate (PEP) by catalyzing the conversion of 2-phosphoglycerate into PEP. PEP synthesizes shikimic acid, a precursor of chorismate in the shikimate pathway, by a combination with erythrose-4-phosphate. Successively, the shikimic acid synthesizes the aromatic amino acids such as tryptophan, phenylalanine, and tyrosine.

The steady-state level of the RF09 (polyubiquitin) mRNA transcript increased markedly in the mutants after an exposure to 5MT; maximal levels were obtained after 12 h treatment. In the wild type plants, only a minor transient increase in the RF09 mRNA transcript level was apparent at 6 h, followed by a small yet stable increase in the later stages of 5MT treatment. The increase of the RF09 transcript level indicates conjugation to target proteins as a recognition signal for protein degradation. Fraser *et al*. (1991) reported that polyubiquitin genes are transiently induced as an adaptation of the cell to environmental changes so as to ensure a re-programming of cellular metabolism and rapid turnover of the regulatory or enzymatically active proteins, which are no longer necessary under the new conditions.

The other genes that were induced by the 5 MT treatment in the resistant plants, such as ADP-ribosylation factor (RB08), phosphatidylinositol transfer-like protein III (RD07) and glycine cell wall structural protein (RF02) would also be good candidates for characterizing the 5MT resistant pathway in further study.

Further work will focus on an analysis of clones from the libraries of other stages and tissues of 5MT or other amino acid analog resistant mutant plants and on the identification of the physiological response and resistance mechanisms of plants in response to amino acid analogs as related to other stresses. Transgenic experiments by using sense and antisense clones of useful genes are also planned for identifying the gene expression or gene silencing that occur *in vivo*.

References

Andrews, G.K.: Regulation of methallothionein gene expression. - Prog. Food Nutr. Sci. **14**: 193-258, 1990.

Castillo, F.J.: Peroxidase and stress. - In: Penel, C, Gaspar, T, Greppin, H (ed.): Plant Peroxidase 1980-1990, Topics and Detailed Literature on Molecular, Biochemical and Physiological Aspects. Pp. 187-203. University of Geneva, Geneva 1992.

Cushman, J.C., Bohnert, H.J.: Genomic approaches to plant stress tolerance. - Curr. Opin. Plant Biol. **3**: 117-124, 2000.

Ewing, R.M., Kahla, A.B., Poirot, O., Lopez, F., Audic, S., Claverie, J.M.: Large-scale statistics analyses of rice ESTs reveal correlated patterns of gene expression. - Genome Res. **9**: 950-959, 1999.

Forsthoefel, N.R., Cushman, M.A.F., Cushman, J.C.: Posttranscriptional and posttranslational control of enolase expression in the facultative Crassulacean acid metabolism plant *Mesembryanthemum crystallinum* L. - Plant Physiol. **108**: 1185-1195, 1995.

Fraser, J., Luu, H.A., Neculcea, J., Thomas, D.Y., Storms, R.K.: Ubiquitin gene expression: response to environmental changes. - Curr. Genet. **20**: 17-23, 1991.

Hahlbrock, K., Schroder, J.: Light-induced changes of enzyme activities in parsley cell suspension cultures. Increased rate of synthesis of phenylalanine ammonia-lyase. - Arch. Biochem. Biophys. **166**: 47-53, 1975.

Huh, G.-H., Lee, S.-J., Bae, Y.-S., Liu, J.R., Kwak, S.S.: Molecular cloning and characterization of cDNAs for anionic and neutral peroxidase from suspensioncultured-cells of sweet potato and their differential expression in response to stress. - Mol. gen. Genet. **255**: 382-391, 1997.

Jones, D.H.: Phenylalanine ammonia-lyase: regulation of its induction and its role in plant development. - Phytochemistry **23**: 1349-1359, 1984.

Joshi, C.D.: An inspection of the domain between putative TATA box and translation start site in 79 plants. - Nucl. Acids Res. **15**: 6643-6653, 1987.

Kim, D.S., Lee, I.S., Jang, C.S., Hyun, D.Y., Seo, Y.W., Lee, Y.I.: Selection of 5-methyltryptophan resistant rice mutants from irradiated calli derived from embryos. - Euphytica **135**: 9-19, 2004.

Kim, D.S., Lee, I.S., Jang, C.S., Kang, S.-Y., Park, I.S., Song, H.S., Seo, Y.W.: High amino acid accumulating 5-methyl-tryptophan resistant rice mutants may include an increased antioxidative response system. - Physiol. Plant. **123**: 302-313, 2005a.

Kim, D.S., Lee, I.S., Jang, C.S., Kang, S.-Y., Song, H.S., Seo, Y.W.: Altered anthranilate synthase in 5-methyltryptophan resistant rice mutants. - Plant Cell. Rep. **24**: 357-365, 2005b

Lee, M.S., Jang, C.S., Lee, S.S., Kim, J.Y., Lee, B.M., Seong, R.C., Seo, Y.W.: Hordoindolines are predominantly expressed in the aleurone layer in late kernel development in barley. - Breed. Sci. **56**: 63-68, 2006.

Minhas, D., Grover, A.: Transcript levels of genes encoding various glycolytic and fermentation enzymes change in response to abiotic stresses. - Plant Sci. **146**: 41-51, 1999.

Mujer, C.V., Rumpho, M.E., Lin, J.-J., Kennedy, R.A.: Constitutive and inducible aerobic and anaerobic stress proteins in the Echinochloa complex and rice. - Plant Physiol. **101**: 217-226, 1993.

Nelson, R.J., Ziegelhoffer, T., Nicolet, C., Werner-Washburne, M., Craing, E.A.: The translation a machinery and 70 kd heat shock protein cooperate in protein synthesis. - Cell **71**: 97-105, 1992.

Oztur, Z.N., Talam, V., Deyholos, M., Michalowski, C.B., Galbraith, D.W., Gozukirmizi, N., Tuberosa, R., Bohnert, H.J.: Monitoring large-scale changes in transcript abundance in drought- and salt-stressed barley. - Plant mol. Biol. **48**: 551-573, 2002.

Plaxton, W.C.: The organization and regulation of plant glycolysis. - Annu. Rev. Plant Physiol. Plant mol. Biol. **47**: 185-214, 1996.

Russell, D.A., Wong, D.M.-L., Sachs, M.M.: The anaerobic response of soybean. - Plant Physiol. **92**: 401-407, 1990.

Van Breusegem, F., Dekeyser, R., Garcia, A.B., Claes, B., Gielen, J., Van, M, Caplan, A.B.: Heat-inducible rice hsp82 and hsp70 are not always co-regulated. - Planta **193**: 57-66, 1994.