BIOLOGIA PLANTARUM 52 (4): 668-673, 2008

Characterization of a rice metallothionein type 3 gene with different expression profiles under various nitrogen forms

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Abstract

A cDNA sequence *OsMT3* was initially isolated from the subtractive cDNA library of ammonium-fed rice (*Oryza sativa* L.) leaves, which was further confirmed by Northern blot to be highly ammonium-up-regulated as compared to nitrate. Its full-length cDNA was cloned by RT-PCR, and *in silico* analysis reveals that the cDNA includes an open reading frame of 186 bp and encodes a rice metallothionein type 3 peptide. Northern blotting analysis showed that *OsMT3* gene predominantly expressed in rice leaves, weakly in stems, and barely in buds and roots. The gene transcripts in leaves were significantly induced by polyethylene glycol (PEG), low temperature, NaCl and Cu^{2+}, but not by Pb^{2+}. Activities of three anti-oxidative enzymes (superoxide dismutase, catalase and peroxidases) and two non-enzymic antioxidants (reduced ascorbate and reduced glutathione) little differed in ammonium- and nitrate-fed rice leaves, indicating that the induced *OsMT3* expression was not mediated by ammonium-elicited oxidative signals.

Additional key words: ammonium, nitrate, *Oryza sativa, OsMT3* gene, oxidative stress.

Introduction

Metallothioneins (MTs) are defined as a class of proteins with low molecular mass, high cysteine content and metal-binding ability. Since MTs were firstly discovered from horse kidney as Cd-binding proteins in 1956, they have been widely identified in diverse organisms including animals, fungi, cyanobacteria and plants (Hamer 1986, Zenk 1996, Cobbett and Goldsbrough 2002). Considering their metal-binding ability, the roles of MTs have been proposed as detoxification of heavy metals and being involved in the homoeostasis of essential metal ions. Moreover, a large number of cysteines in MTs can also scavenge the reactive oxygen species (ROS) and protect cells against oxidative stress (Andrews 1990, Robinson *et al.* 1993, Van *et al.* 1999, Zhou *et al.* 2005). According to the classification proposed by Cobbett and Goldsbrough (2002), plant MTs are divided into four types. Type 1, 2 and 3 MTs contain two Cys-rich domains which are separated by a central cysteine free spacer. Type 1 MTs have six Cys-Xaa-Cys motifs exclusively (where Xaa is an amino acid other than Cys), while type 2 MTs have Cys-Xaa-Cys、Cys-Cys and Cys-Xaa-Xaa-Cys within the terminal domain. Type 3 MTs contain only four Cys residues in the N-terminal domain and the consensus sequence for the first three is Cys-Gly-Asn-Cys-Asp-Cys. The six Cys residues in C-terminal domain are arranged in Cys-Xaa-Cys motifs. Type 4 metallothioneins differ from other MTs by having three cysteine-rich domains.

During the identification of the differentially expressed genes under different nitrogen forms, a cDNA sequence *OsMT3* encoding metallothionein-like protein was isolated from the subtractive cDNA library of rice leaves, which was highly up-regulated in ammonium-fed leaves as compared to the nitrate-fed ones. To understand the characteristics and function of *OsMT3* gene, the genomic sequences of *OsMT3* gene including the integrated ORF and its expression patterns in different tissues and its response to abiotic stress were investigated in this study.

Received 22 January 2007, *accepted* 8 August 2007.

Abbreviations: Asc - reduced ascorbate; CAT - catalase; GSH - reduced glutathione; MT - metallothionein; PEG - polyethyleneglycol; POD - peroxidases; RT-PCR - reverse transcription - polymerase chain reaction; SOD - superoxide dismutase.

Acknowledgement: This work was supported by the National Science Foundation of China (No. 30470152) and President Foundation of South China Agricultural University (4600-k05030).

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Materials and methods

Plant materials and treatments: Rice (*Oryza sativa* L. cv. Xiangzhongxian 2) was grown in a greenhouse at 14-h photoperiod (irradiance of 600 - 1000 μmol m^{-2} s^{-1}), day/night temperature of 29/24 °C and relative humidity 60 - 80 %. At four-leaf stage, the seedlings were treated with different nitrogen forms by replacing the normal culture solution (Yoshida *et al.* 1976) with those containing sole nitrate or ammonium (3 mM in the form of calcium nitrate and ammonium sulfate, respectively). At 1, 2 and 3 d after the treatments leaves were harvested for physiological determination and the 3-d-treated leaves were also used for suppression subtractive hybridization (SSH) analysis. For various stress treatments, roots of four-leaf-stage seedlings were grown separately in culture solution containing 15 % polyethyleneglycol-6000, 150 mM NaCl, 0.2 mM $CuSO_4$, 0.2 mM lead acetate, or the seedlings were treated with low temperature (4 °C). Leaves were harvested at 12 and 24 h after treatments. All the harvested materials were immediately frozen in liquid nitrogen and stored at -75 °C until use.

Assay of enzyme activities and glutathione and ascorbate quantitation: For estimation of total protein content and enzyme activity, 0.2 g of each fresh sample was homogenized in 5 cm^3 of 50 mM chilled phosphate buffer (pH 7.0), The homogenate was centrifuged at 12 000 *g* for 20 min at 4 °C and the supernatant was used for enzyme assays and protein determination. Protein concentration was measured by the method of Bradford (1976). The superoxide dismutase (SOD) activity was estimated according to the method of Beauchamp and Fridovich (1971) with modifications. The 4 cm^3 reaction mixture containing 50 mM phosphate buffer (pH 7.8), 0.1 M EDTA, 13 mM methionine, 63 μM nitroblue tetrazolium chloride (NBT), 13 μM riboflavin and 0.02 cm^3 enzyme extract. Reaction was started by exposing the mixture to white fluorescent light for 15 min. Absorbance was recorded at 560 nm (*Hitachi UV 2000*, Tokyo, Japan). Catalase (CAT) activity was assayed from the rate of H_2O_2 decomposition as measured by decrease of absorbance at 240 nm, following the procedure of Aebi (1984). The reaction mixture contained 3.0 cm^3 50 mM phosphate buffer (pH 7.0), 0.2 cm^3 H_2O_2 (3 %), and 0.01 cm^3 enzyme extract. Peroxidase (POD) activity was assayed in a reaction mixture containing 50 mM phosphate buffer (pH 7.0), 10 mM H_2O_2 solution, 20 mM guaiacol and 0.01 cm^3 of crude extract (Chance and Maehly 1955). The activity was determined by monitoring the increase of absorbance at 470 nm.

For estimation of reduced ascorbate (Asc) and reduced glutathione (GSH) content, 0.2 g each fresh seedling was homogenized in 5 % chilled trichloroacetic acid. The homogenate was centrifuged at 12 000 *g* for 20 min and the supernatant was then used. Ascorbate was estimated as described by Kampfenkel *et al*. (1995). The 4 cm^3 reaction mixture contained 0.6 cm^3 0.2 M phosphate buffer (pH 7.4), 1 cm^3 10 % TCA, 0.8 cm^3 42 % H_3PO_4, 0.8 cm^3 4 % 2,2'-dipyridyl, 0.4 cm^3 3 % $FeCl_3$ and 0.2 cm^3 extract solution. The reaction was carried out in 42 °C for 40 min and then be determined at 525 nm. The glutathione was assayed in a 3 cm^3 reaction mixture containing 100 mM phosphate buffer (pH 7.7), 0.2 cm^3 12.5 mM 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) and 0.5 cm^3 crude extract (Ellman 1955). The mixture was determined at 412 nm after 5 min reaction.

Construction and identification of subtractive library: Total RNA was extracted with the *Trizol Reagent* (*Qiagen*, Hilden, Germany) and mRNA was purified with the PolyAT tract mRNA isolation systems Ⅲ kit (*Promega*, Madison, WI, USA). SSH was carried out using a *Clontech* PCR-select cDNA subtraction kit (*Clontech,* Palo Alto, CA, USA) according to the manufacturer's instruction. The cDNA of ammonium-treated rice leaves was used as tester and nitrate one was used as driver. Products from the secondary PCR were inserted into pGEM-T easy vector (*Promega*) and then transformed into DH10B cells. Positive clones were picked and transferred into 384-well plates, which formed the subtractive cDNA library.

The positive clones were further identified by reverse Northern blot according to the methods of Zhang *et al*. (1996) and Mou (1994). PCR products of selected clones were transferred to *Hybond*-N^+ nylon membrane (*Amersham*, Piscataway, NJ, USA) after electrophoresis. 5 μg RNA of ammonium- treated leaves and nitrate-treated ones were reverse transcripted and used as probes respectively. Probes were labeled with random primer DNA labeling kit (*Takara*, Tokyo, Japan) and then hybridized with the above membrane.

Cloning of *OsMT3* gene by RT-PCR: The integral *OsMT3* gene was cloned by reverse transcription - polymerase chain reaction (RT-PCR). The two specific primers were: 5'-GGGATTAACTCATCA CTCAC-3' (sense) and 5'-ATAGCACGGGTACGTCACAT-3' (antisense). The amplification products were ligated into the vector pGEM-T easy (*Promega*) and then transformed to competent DH10B cells. Positive recombinant clones were selected for further sequencing confirmation.

Northern blotting: Total RNA extracted from various rice tissues were subjected to electrophoresis in 1.2 % formaldehyde agarose gels and blotted to a nylon membrane (*Hybond* N^+, *Amersham*) according to the methods of Sambrook *et al*. (1989). Hybridizations were performed overnight at 42 °C and a hybridization buffer containing 50 % formamide, 5× SSC, 1× Denhardt's solution, 1 % SDS and 100 μg cm^{-3} heat-denatured salmon sperm. After hybridization the blot was washed in 2× SSC, 0.5 % SDS solution at 42 and 65 °C,

respectively for 15 min, and once in 0.2× SSC, 0.2 % SDS solution at 65 ℃ for 15 min, and then were exposed to an imaging plate and the radioimage of the plate was analyzed using the *Molecular Imager FX-Plus* (*Bio-Rad*, Ivry sur Seine, France).

Results

Screening of the subtractive cDNA library: PCR products of about 1 000 clones were transferred onto the membrane and hybridized with ammonium-treated cDNA and nitrate-treated cDNA, respectively. The clones with stronger hybridization signal with ammonium-treated cDNA probe than with nitrate-treated cDNA probe were selected as positive clones. Ultimately, 68 clones were identified to be uniquely expressed in ammonium-treated leaves (Fig. 1), the cDNAs were sequenced and then analyzed at NCBI using the *Blastn* and *Blastx* algorithm (Table 1). Interestingly, the gene of metallothionein-like protein was detected 5 times among these differential expressed clones. Moreover, the result was further confirmed by Northern blot (Fig. 2).

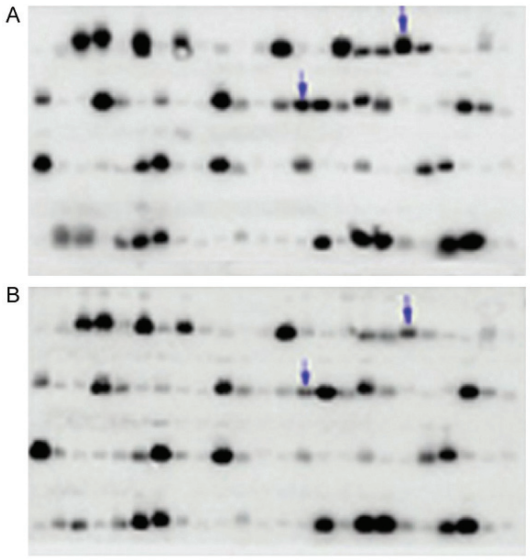

Fig. 1. Differential screening for the subtractive cDNA library (partial). *A* - the subtractive cDNA library hybridized with ammonium-treated cDNA probe; *B* - the subtractive cDNA library hybridized with nitrate-treated cDNA probe. *Arrows* indicate positive clones.

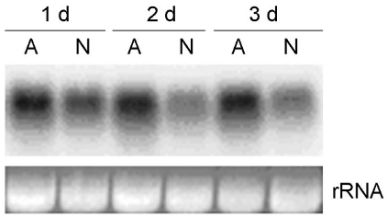

Fig. 2. Northern blot of metallothionein-like protein. Rice seedlings were treated with 3 mM ammonium nitrogen (A) and nitrate nitrogen (N), respectively. RNA was extracted from leaves after treatment for 1, 2 and 3 d.

Cloning and sequencing of *OsMT3*: To get the integral gene sequence of the type 3 metallothionein-like protein, primers were designed according to the homologous sequence in GenBank, and then the gene was cloned by RT-PCR (GenBank accession EF136378). Sequencing data revealed that the coding region of this gene is 186 bp; when translated it is demonstrated to contain 9 cysteine residues and the first three is CGNCDC and the other six Cys residues in the C-terminal cysteine are arranged in Cys-Xaa-Cys motifs. The abundance (14.5 %) and the distribution of cysteines in this gene were shown to have structural features characteristic of the type 3 MT-like proteins according to the classified method of Cobbett and Goldsbrough (2002), so we named the gene *OsMT3*. It has high homology with the other plant type 3 MT-like proteins (Fig. 3), with the overall sequence similarity varying from 60.7 to 51.6 %.

Table 1. Features of some sequenced clones which were ammonium-up-regulated and results of *BLAST* search (partial).

Clone No.	Length [bp]	GenBank hit	Result
A1c12	291	M82426	*Oryza sativa* 18S ribosomal RNA
A1d8	311	X68807	*Oryza sativa* light-induced mRNA
A1e4	470	ABI79452	DNA-binding protein DSP1
A1h6	340	ZP_00231457	conserved hypothetical protein
A2a11	614	AP006728	*Oryza* chloroplast DNA
A3b4	460	ABA97179	Ribulose bisphosphate carboxylase small chain A
A3b7	284	NP_917982	putative 29 kDa ribonucleoprotein A
A4a1	290	ABA97179	Ribulose bisphosphate carboxylase small chain C
A4d11	358	BAD52245	*Oryza sativa* metallothionein-like protein
A5e1	337	BAB33421	putative senescence-associated protein
A8b8	615	AF1828061	*Oryza sativa* carbonic anhydrase mRNA

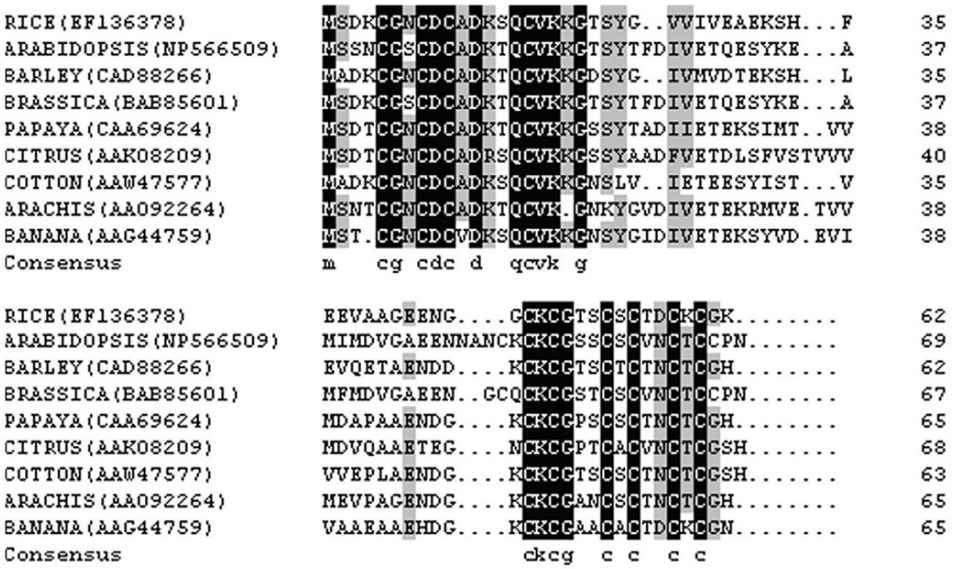

Fig. 3. Comparison of the deduced amino acid sequence of *OsMT3* from various organisms.

Through database searches, A phylogenetic tree of all known and predicted rice MT-like proteins was constructed, which showed four distinct groups corresponding to their predicted amino acid sequences (Fig. 4), the four groups also follow the MT classification of Cobbett and Goldsbrough (2002). The predicted amino acid sequences of rice metallothionein genes aligned and analyzed using DNAMAN6.0, and the phylogeny is constructed by Mega 2.0.

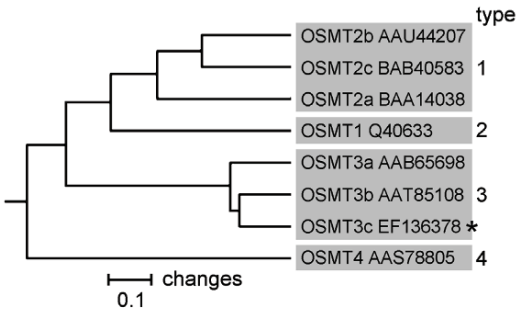

Fig. 4. A phylogenetic tree of rice metallothioneins. The numbers following rice metallohionein genes indicate the GenBank accession numbers of the corresponding genes. The number of 1, 2, 3 and 4 means the 4 type plant MTs. *Asterisk* indicates the gene we cloned.

***OsMT3* expression in different plant tissue and in response to stresses:** Total RNA from the tissues of buds, roots, stems, mature leaves and old leaves were subjected to Northern blot analysis. The results showed that the transcripts of *OsMT3* were specifically abundant in old leaves and a weak hybridization signal was detected in mature leaves and stems. The hybridization signal was hardly detected in buds and roots (Fig. 5), indicating the fact that expression of *OsMT3* is only restricted to a specific tissue such as in leaves.

To investigate the effects of stress factors on *OsMT3* gene expression in rice leaves, PEG, low temperature and several metal ions including Na^+, Cu^{2+} and Pb^{2+} were taken into account in the study. The expression of *OsMT3* was obviously up-regulated by the treatments of PEG and low temperature (Fig. 6). Metal ions, such as Na^+ and Cu^{2+}, also induced the expression of *OsMT3* gene, whereas the expression level was not affected by Pb^{2+} treatment.

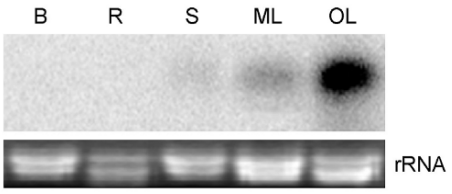

Fig. 5. Northern blot analysis of *OsMT3* expression in various tissues. B: buds; R: roots; S: stems; ML: mature leaves; OL: old leaves.

Difference in response of anti-oxidation reactions to ammonium or nitrate in leaves: Why ammonium-fed leaves had a much higher expression of *OsMT3* gene than nitrate-fed ones? Many studies have proved that the expression of plant *MT* gene was frequently up-regulated by oxidative stress (Wong *et al*. 2004, Akashi *et al*. 2004), so the *MT* response could be mediated by redox state. The activities of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POD) and the contents of non-enzymic antioxidants such as reduced ascorbate (Asc) and reduced glutathione (GSH) were analyzed in both ammonium and nitrate-fed leaves. POD activity was a little higher in ammonium-treated leaves than in nitrate-treated ones, whereas SOD activity displayed the opposite trends and CAT activity

showed no obviously difference between the two nitrogen forms (Table 2). Contents of two non-enzymic antioxidants, Asc and GSH, presented the similar results. Both of them were relatively higher in nitrate-treated leaves. The above overall results suggested that rice had no significant difference in internal redox state between ammonium and nitrate nitrogen forms. As a result, it can be inferred that the induced expression of *OsMT3* genes in ammonium-fed leaves could be mediated by ammonium itself or certain factors rather than oxidative signals.

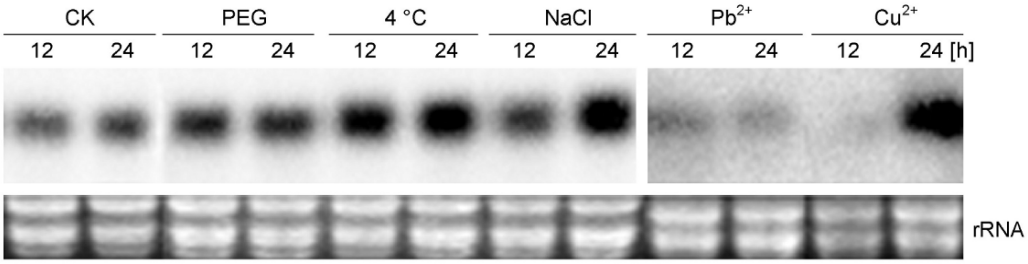

Fig. 6. Effects of stress factors on the expression of *OsMT3* in rice leaves. RNA was extracted from leaves after treatment for 12 h and 24 h. Exogenous stress factors are 15 % polyethyleneglycol-6000 (PEG), 4 °C, 150 mM NaCl, 0.2 mM $CuSO_4$ (Cu^{2+}), 0.2 mM lead acetate (Pb^{2+}); CK: control.

Table 2. Superoxide dismutase (SOD), catalase (CAT) and peroxidases (POD) activities and the contents of reduced ascorbate (Asc) and reduced glutathione (GSH) in rice leaves after 1, 2 and 3 d of exposure to different nitrogen forms (ammonium and nitrate). SOD activity was expressed as [U mg^{-1}(protein)], where U means extract volume able to induce 50 % of NBT reduction, POD activity [ΔA_{470} mg^{-1}(protein) min^{-1}], CAT activity [ΔA_{240} mg^{-1}(protein) min^{-1}], AsA content [mg g^{-1}(f.m.)] and GSH content [μg g^{-1}(f.m.)]. Values are means ± SE of 3 determinations.

	NH_4-N			NO_3-N		
	1 d	2 d	3 d	1 d	2 d	3 d
SOD	19.82 ± 1.78	21.72 ± 2.00	20.54 ± 1.31	21.70 ± 1.06	24.90 ± 1.87	25.11 ± 2.31
CAT	5.20 ± 0.23	5.04 ± 0.50	4.47 ± 0.18	5.14 ± 0.92	4.88 ± 0.41	4.22 ± 0.13
POD	0.66 ± 0.10	0.66 ± 0.14	0.70 ± 0.12	0.49 ± 0.10	0.63 ± 0.16	0.54 ± 0.07
AsA	2.14 ± 0.11	2.05 ± 0.07	2.12 ± 0.11	2.19 ± 0.20	2.16 ± 0.21	2.30 ± 0.06
GSH	281.50 ± 8.10	257.20 ± 25.4	270.70 ± 2.40	275.20 ± 34.6	309.50 ± 23.0	309.50 ± 12.5

Discussion

Differential expression of MT gene in various tissues has been widely reported in plants (Zhou and Goldsbrough 1995, Cobbett and Goldsbrough 2002, Zhou *et al*. 2006). Our results showed that *OsMT3* gene, which belongs to type 3 MTs, predominantly expressed in rice leaves, weakly in stems, and barely in buds and roots (Fig. 5). A number of investigations have demonstrated that the expression of plant MTs are markedly regulated by exogenous factors, such as salt stress, heat shock, wounding, H_2O_2 and so on (Hsieh *et al*. 1995, Wong *et al*. 2004, Akashi *et al*. 2004, Zhou *et al.* 2005). Our data also showed that expression of *OsMT3* in rice leaves was obviously increased by osmotic stress (15 % PEG) and low temperature (4 °C) (Fig. 6), indicating that *OsMT3* may have taken part in scavenging the ROS which brought about by osmotic stress and low temperature. In some plants expression of MT genes can be enhanced by various metals (Kawashima *et al*. 1991, Foley and Singh 1994). The effects of Na^+, Cu^{2+} and Pb^{2+} were investigated in this experiment, the up-regulated expression of *OsMT3* in rice leaves treated with Na^+ and Cu^{2+} (Fig. 6) suggested that *OsMT3* may play an important role in maintaining the homeostasis of essential metals. Unexpectedly, Pb^{2+}, as a heavy metal ion, failed to induce the expression of *OsMT3* gene, it may be inferred that *OsMT3* played no functional roles in detoxification of Pb.

Since *OsMT3* expression can be immediately induced by abiotic stress, was the highly expression of *OsMT3* under ammonium-treated leaves directly caused by ammonium toxicity stress? The further physiological analysis showed that activities of three anti-oxidative enzymes (SOD, POD and CAT) and two non-enzymic antioxidants (Asc and GSH) little differed between the plants fed by two nitrogen forms (Table 2), which indicated that rice may have not suffered obvious oxidative stress under ammonium nitrogen compared with nitrate nitrogen, and this is not surprising since rice

is a well-known ammonium-preferring plant (Troelstra *et al*. 1995). So, the induced expression of *OsMT3* under ammonium-treated rice may not be mediated by stressful signals, and the detailed mechanism remains to be further investigated.

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