Metallothionein Genes in Oryza Genus

Subjects: Plant Sciences
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Metallothionein (MT) proteins are low molecular mass, cysteine-rich, and metal-binding proteins that play an important role in maintaining metal homeostasis and stress response. However, the evolutionary relationships and functional differentiation of MT in the Oryza genus remain unclear.

Keywords: Oryza sativa ; metallothionein (MT) ; phylogenetic analysis ; promoter activity ; expression analysis

1. Introduction

Metallothioneins (MTs) are a family of low molecular mass (4–8 kD), cysteine (Cys)-rich proteins that bind metals via thiol groups of cysteine (Cys) residues [1]. *MT* genes are widespread in prokaryotes, plants, and animals [2]. MTs have a strong affinity for both essential (zinc, copper, selenium) and xenobiotic (cadmium, lead, mercury) metals, binding them through specific Cys-Cys and Cys-Xxx-Cys motifs [3][4][5]. As a result, MTs have various biological functions, including protective effects, resisting metal toxicity, controlling oxidative stress, and regulating physiological homeostasis [6][7][8].

So far, there is increasing evidence that various abiotic stresses can regulate *MT* gene expression; for example, drought, abscisic acid, salt, environment temperature, and reactive oxygen species [9][10][11][12]. This shows the importance of plant *MTs* genes in response to abiotic stress. For example, an *MT-2* gene (*MT2*) was upregulated in boron-stressed tomato plants, implying that it may protect against boron stress [13]. In *Chloris virgata* Swartz (*C. virgata*), *ChlMT1* expression was induced by several abiotic stresses, such as salts (NaCl and $NaHCO_3$), ROS inducer (paraquat), and metals ($CuSO_4$, $ZnSO_4$, and $CdCl_2$). Interestingly, alien *ChlMT1* overexpression could significantly improve the tolerance of yeasts to reactive oxygen species and salinity [14]. Similarly, the ectopic expression of *OsMT1e-P* can increase tolerance toward multiple abiotic stresses in transgenic tobacco; also, transgenic plants could survive and produce viable seeds under salt stress [15].

In many higher plant species, *MT* genes have been reported to be expressed specifically in different tissues. For example, the expression level of *OsMT* genes in mature rice plants is extremely high in stems relative to leaf blades, leaf sheaths, endosperm, and roots [16]. *METALLOTHIONEIN2b* (*OsMT2b*) is preferentially expressed in immature rice panicles, the scutellum of germinating embryos, and the primordium of lateral roots [2]. In cucumbers, *CsMT* genes exhibit different tissue expression patterns [17].

Although some *MT* genes have been characterized in rice [18], *Arabidopsis* [19], cucumbers [17], tomatoes [20], and soybeans [21], systematic and thorough studies are lacking in plants, especially in the *Gramineae* species. Most of the *MT* family remains unclear to date, limiting the depth of understanding of the evolutionary patterns of MTs in *Gramineae*. Therefore, it would be of important to study their evolution systematically and the possible physiological role of the *MT* gene family in *Gramineae*.

2. Identification and Structural Characterization of the MT Genes in the Oryza Genus

The hidden Markov model searching for proteins containing a metallothionein domain (Pfam accession no. PF01439) was downloaded from Pfam (http://pfam.xfam.org/) [22]. There were 9, 9, 12, 7, 7, and 9 *MT* members identified in *O. sativa* ssp.*japonica*, *O. rufipogon*, *O. sativa* ssp. *indica*, *O. nivara*, *O. glumaepatula*, and *O. barthii*, respectively. These genes are mainly dispersed across nine chromosomes, with a bias to Chr 12, where more than half of the genes are located on this chromosome. The 53 *MT* genes can be classified into four subfamilies according to the sequence identity. In detail, Chr1, Chr3, and Chr11 had six, six, and five genes, respectively, whereas two genes were found on Chr2, Chr5, and Chr8, and three genes on Chr10. Interestingly, the genes on Chr2, Chr8, and Chr12 were grouped into group 1; the Chr1, Chr5, and Chr10 *MT* genes belonged to group 2; the *MT* genes on Chr11 belonged to group 3; and the *MT* genes on Chr3 belonged

to group 4 (**Figure 1**). This characteristic is consistent with the classification of *MT* genes, reflecting the strict conservation of *MT* genes among the six *Oryza* species/subspecies.

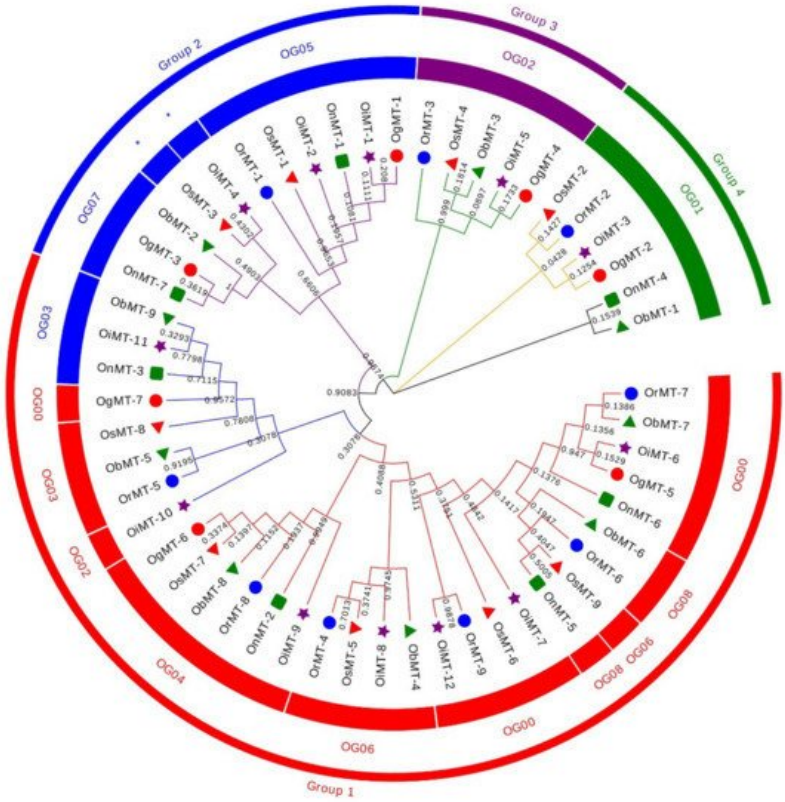

Figure 1. Phylogenetic tree of *MT* genes based on protein sequences from six *Oryza* species/subspecies. ClustalW is used for multiple sequence alignment. MEGA v.7.0 is adopted for phylogenetic reconstruction using the neighbor-joining (NJ) clustering method. Bootstrap numbers (1000 replicates) are shown. Different color of circles represents different subfamilies. Different-shaped markers indicate the different species. The numbers inside the red circles represent the different orthogroups (OGs). * means unassigned proteins.

The phylogenetic tree of *MT* genes based on protein sequences from six *Oryza* species/subspecies is shown in **Figure 2**A. Furthermore, the *MT* gene's structure was characterized in order to acquire more viewpoints into the structural diversity of *MT* genes in the *Oryza* species (**Figure 2**C). Results indicated that the intron number of *MT* genes in six *Oryza* species/subspecies ranged from 1 to 8, and the exon number ranged from 2 to 6. A total of 31 *MT* genes (58.5%) had three exons, followed by the 16 (30.2%), 4 (7.5%), 1 (1.9%), and 1 (1.9%) gene, possessing two, four, five, and six exons, respectively. Further, 20 conserved motifs were identified from the 53 *MT* proteins using the MEME [23], and all 53 *MT* proteins showed a similar motif arrangement (**Figure 2**B). Notably, we found that MTs from the same group showed variations in the number and length of exons/introns, suggesting the functional diversification of the *MT* genes in the same group.

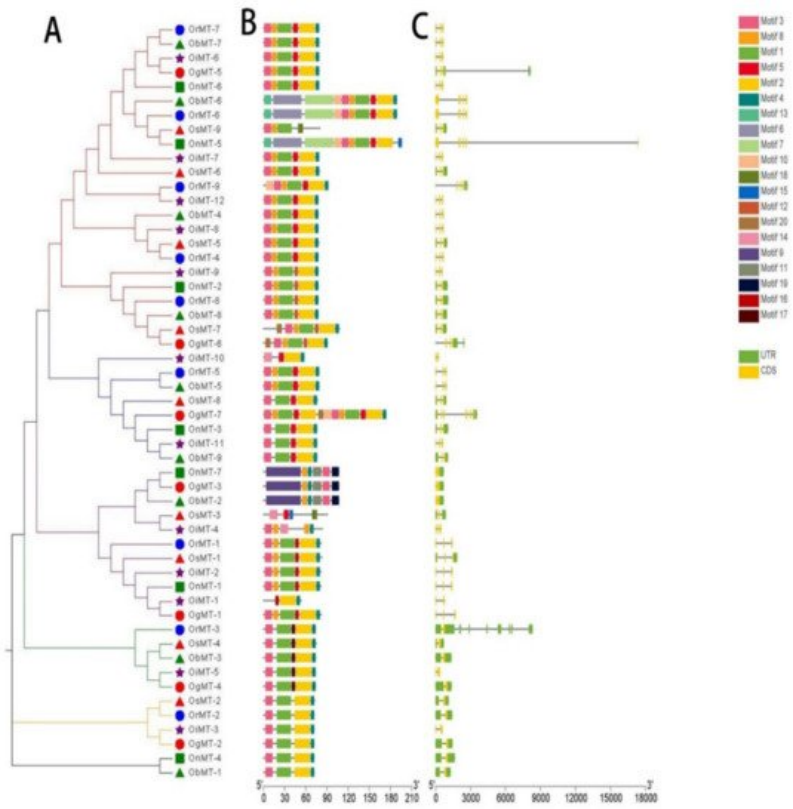

Figure 2. The phylogenetic tree (**A**), motif composition (**B**), and exon/intron structure (**C**) of the *MT* genes in six *Oryza* species/subspecies. (**A**) Sequence alignments and the NJ-phylogenetic trees were made using ClustalW and MEGA v.7.0, respectively. A bootstrap number (1000 replicates) is adopted; (**B**,**C**) the widths of the gray bars represent the relative lengths of genes and proteins. The green boxes and gray lines display exons and introns, respectively.

3. Chromosomal Distribution, and Evolutionary Characters

The chromosome location results showed that *MT* genes show an unbalanced distribution pattern, where no *MT* genes were mapped on Chr4, Chr6, Chr7, and Chr9 (**Figure 3**). Furthermore, we discovered five pairs of segmental duplication events and ten pairs of tandem duplication events in the six *Oryza* species/subspecies (**Table 1** and **Figure 3**). Interestingly, segmental duplication events were detected in all species/subspecies except *Oryza nivara* (**Figure 3**); moreover, nine pairs of tandem duplication clusters were observed on chromosome 12, reflecting that gene duplication may be the major cause for the expansion of the *MT* family in the *Oryza* species. The segmental duplication events of these six gene pairs were estimated to occur between 7.65 and 10.32 Mya (**Table 1**).

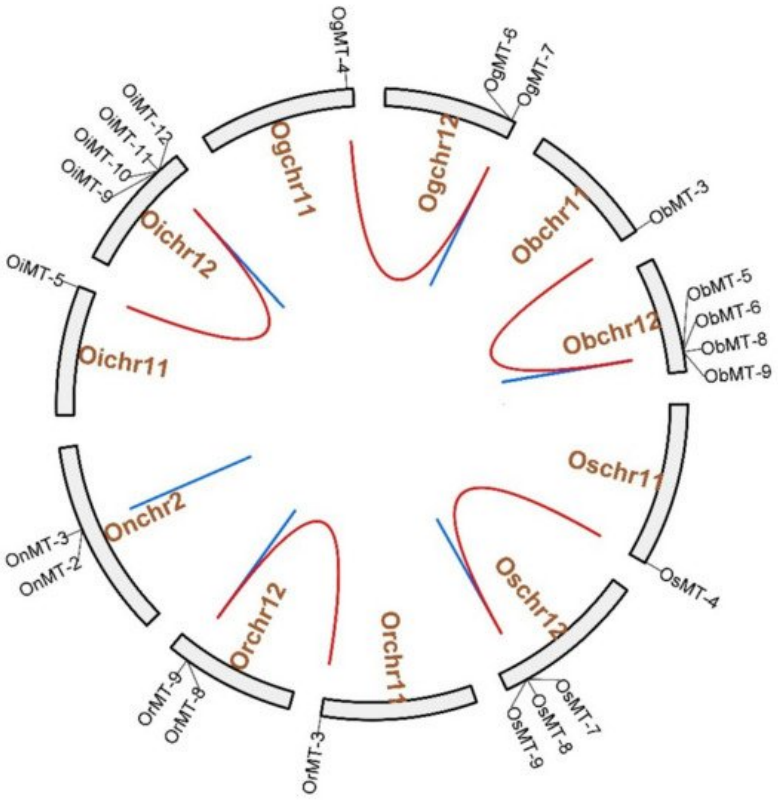

Figure 3. The chromosome location and duplication events of *MT* genes in six species/subspecies. Os represents *Oryza sativa* ssp. *japonica*. Or represents *Oryza rufipogon*. On represents *Oryza nivara*. Oi represents *Oryza sativa* ssp. *indica*. Og represents *Oryza glumaepatula*. Ob represents *Oryza barthii*. The location of each *MT* gene is marked with a gray line using Circos software. The whole-genome duplication (WGD) or segmental duplication/tandem duplication gene pairs are linked by red/blue lines.

Table 1. Ka, Ks, and Ka/Ks values for duplicated gene pairs in rice.

Seq1	Seq2	Ks	Ka	Ka/Ks Ratio	Date (MY)	Duplication Type
OsMT-4	OsMT-7	0.1392	0.9012	0.1544	7.65	WGD or segmental duplications
OsMT-7	OsMT-8	0.1217	0.7707	0.1579	6.69	tandem duplication
OsMT-8	OsMT-9	0.2080	0.6090	0.3416	11.43	tandem duplication
OrMT-3	OrMT-8	0.1392	0.9745	0.1428	7.65	WGD or segmental duplications
OrMT-8	OrMT-9	0.0882	0.4947	0.1784	4.85	tandem duplication
OnMT-2	OnMT-3	0.1117	0.7852	0.1422	6.14	tandem duplication
OiMT-5	OiMT-9	0.1392	0.9745	0.1428	7.65	WGD or segmental duplications
OiMT-9	OiMT-10	0.2444	0.6186	0.3951	13.43	tandem duplication
OiMT-10	OiMT-11	0.2195	0.2856	0.7686	12.06	tandem duplication
OiMT-11	OiMT-12	0.1101	0.4226	0.2606	6.05	tandem duplication
OgMT-4	OgMT-6	0.1878	0.9203	0.2041	10.32	WGD or segmental duplications
OgMT-6	OgMT-7	0.1861	0.5980	0.3112	10.22	tandem duplication
ObMT-3	ObMT-8	0.1392	1.0556	0.1318	7.65	WGD or segmental duplications
ObMT-5	ObMT-6	0.1212	0.4932	0.2458	6.66	tandem duplication
ObMT-8	ObMT-9	0.1117	0.7852	0.1422	6.14	tandem duplication

Synonymous (Ks) and nonsynonymous (Ka) substitution rates of duplicate gene pairs (Ka/Ks ratios).

Next, Ka/Ks values of *MT* duplicate gene pairs were calculated to evaluate the driving force underlying *MT* gene evolution. The results showed that Ka/Ks values of the 15 duplicated *MT* genes ranged from 0.1318 to 0.7686, and all Ka/Ks values

were less than 1 (**Table 1**), indicating that the duplicated *MT* genes were under a strong negative selection during evolution [24].

To better understand the evolutionary relationship of the *MT* genes in the *Oryza* genus, the orthogroup clustering was analyzed and a phylogenetic tree was constructed, and showed nine orthogroups in six *Oryza* genera; namely, eight in *O. sativa* ssp. *japonica*, eight in *O. rufipogon*, seven in *O. sativa* ssp. *indica*, eleven in *O. nivara*, eight in *O. glumaepatula*, and nine in *O. barthii*. However, *OsMT-3* and *OiMT-4* were assigned to the *MT* family. Furthermore, the gene numbers in each orthogroup were different, ranging from 3 to 10. Orthogroup 0 was the largest, and single-copy orthogroups were found in orthogroups 1 and 4. Besides, we found that the number of orthologs was also different among these species (**Table 1**). These results showed that the unequal loss and expansion of most orthogroups might have occurred during the domestication process.

4. Collinearity Relations of O. sativa ssp. japonica with Other Tested Species

To evaluate the evolutionary relationship of the *MT* genes within *Gramineae*, the molecular phylogeny of the *MT* family was analyzed using the MCScanX toolkit. Referenced to the genome of *japonica* rice Nipponbare, we found seven, five, nine, six, and seven collinear gene pairs between *O. sativa* ssp. *japonica* and *O. rufipogon*, *Oryza nivara*, *Oryza sativa* ssp. *indica*, *Oryza glumaepatula*, and *Oryza barthii*, respectively. The *MT* genes showed a strongly conserved collinearity among the six *Oryza* species and subspecies, and the collinearity of *MT* genes between *japonica* and *indica* was closer than the other species, supporting their close evolutionary distance.

5. Promoter Activity and Cis-Elements Identification of OsMT Genes in O. sativa ssp. japonica

Cis-elements in the promoter usually play a vital role in responding to different environments and determining the tissue-specificity of genes [25][26]. Thus, potential cis-regulatory elements in the promoter regions of *japonica* rice *OsMT* genes were identified by searching the PlantCARE database [27]. In total, 31 types of cis-regulatory elements were identified; they can be primarily classified into three categories based on their functionality: phytohormone response, growth and development, and stress response [28]. The cis-regulatory elements in the growth and development category had a higher percentage than the other two categories. In the growth and development category, the light-responsive/responsiveness subcategory had a total of 74 motifs that belonged to 15 types of cis-elements, which indicated that light-responsive/responsiveness is widely present in the promoter region of *MT* genes. In the phytohormone response category, the MeJA-responsiveness element was the largest subcategory (40), which includes the TGACG- and CGTCA motif, followed by the abscisic acid responsiveness subcategory, including the ABRE cis-element. The top three subcategories in the stress response category were the anaerobic induction, drought-inducibility, and low-temperature responsiveness elements. The marked cis-element related to biological and abiotic stress in the promoters means that *OsMT* genes are widely involved in the environmental stress response.

Further analysis showed that cis-regulatory elements are unevenly distributed in the *OsMT* genes, and that some cis-regulatory elements were preferentially present on individual *OsMT* genes. For example, *OsMT-5* and *OsMT-8* had many MeJA-responsive cis-regulatory elements, and *OsMT-7* had the most auxin regulatory elements, which is the functional specificity for a few of the *MT* genes.

The functional specificities of plant genes are often reflected by the promoter activities [29]; thus, the promoter activity of the nine *OsMT* genes in rice was investigated in planta using pGreen-0800 as a control, where the vector constructs are used in the dual-luciferase assay (**Figure 4**A). Results showed that the *OsMT-9* promoter showed the highest LUC/RUC ratio in the protoplast of rice (**Figure 4**B), the promoter of *OsMT-5*, *OsMT-7*, and *OsMT-8* showed the weakest activity, and the promoter of *OsMT-1, OsMT-3, OsMT-4*, *OsMT-6*, and *OsMT-7* showed a higher fluorescence intensity than the control in *Nicotiana benthamiana* leaves (**Figure 4**C), reflecting a tissues-specific functional differentiation of the *OsMT* genes in rice.

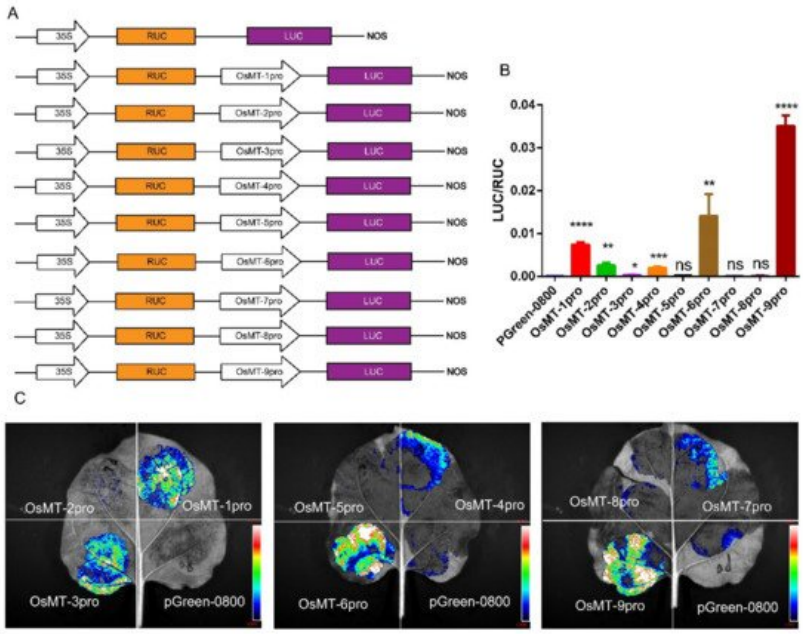

Figure 4. Analysis of the promoter function of *OsMT* genes in vivo: (**A**) the vector constructs are used in the dual-luciferase assay; (**B**) dual-luciferase assay in the protoplast of rice; (**C**) dual-luciferase assay in *Nicotiana benthamiana* leaves. The error bars show the standard deviations of the three independent biological replicates. Significance analysis was performed using *t*-test; *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$.

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