Chapter 4

Structural and sequence analysis of metallothionein proteins in *Oryza sativa*

4.1 Background

In Chapter 2 transcriptome comparison of two contrasting genotypes of *Oryza sativa* (Pokkali vs IR64) under salt stress conditions identified several members playing key role in salt stress response (Kumari *et al.* 2009). Among these genes, metallothioneins also appeared as one of the member of this stress response which were found to be induced under stress conditions. Therefore, in order to identify all the members of metallothionein gene family, genome-wide analysis have been performed in *Oryza sativa* sp. Nipponbare. Moreover structural analysis of metallothionein protein member from *Oryza sativa* sp. IR64 was also attempted to get an insight to conserved structural and sequence features of these metallothionein proteins.

Plant usually experience stress from a multiple environmental sources, and hence multi-stress interactions leads to the increased uptake of toxic boron and cadmium by the exacerbated plant in the condition of salt stress. In order to maintain the concentration of essential metals within the physiological limits and to minimize the detrimental effects of nonessential metals, plants therefore have developed a complex network of homeostatic mechanism that serves to control
the uptake, accumulation, trafficking and detoxification of metals. Some of these mechanisms include: transport, chelation, compartmentalization, exclusion and sequestration process (Clemens 2001). Chelation constitutes one of the main mechanism among all and it involves participation of high-affinity ligands such as phytochelatins (PCs) and metallothioneins (MTs). Both PCs and MTs are one of the best characterized heavy metal-binding ligands in the plant cell (Hall 2002).

MTs were discovered in 1957 by Margoshes and Vallee, as a Cd-binding or Zn-binding protein in the horse kidney (Margoshes & Vallee 1957), and are widely found in animals, plants and prokaryotes (Palmiter 1998). Metallothioneins (MTs) are ubiquitous low-molecular weight (4-14 KDa), Cys-rich cytoplasmic metal binding proteins encoded by a family of genes that are able chelate to heavy metal ions by means of mercaptide bonding between the Cys residues and the heavy metal ions (Rauser 1999; van Hoof et al. 2001). MTs have been widely reported in the species of fungi, plants and cyanobacteria (Cobbett & Goldsbrough 2002). The vast majority of plant MT genes have been identified in the angiosperms (Cobbett & Goldsbrough 2002). In S. cerevisiae, MTs play a primary role in detoxification of excess Cu (Ecker et al. 1989). Genes encoding MT and MT-like proteins have been identified in many plant species including wheat (Kawashima et al. 1992), barley (Okumura et al. 1991) and Arabidopsis (Zhou & Goldsbrough 1995).

MT proteins are classified based on the arrangement of Cys residues. MT proteins had been classified into four types (Cobbett & Goldsbrough 2002). Type 1 MTs possess a total of six Cys-X-Cys motifs (X represents another amino acid) that are distributed equally among two domains. The two domains are separated by a spacer domain which is a common feature in plant MTs. It is observed that the type I MTs from Brassicaceae posses a number of distinguishing features such as shorter spacer between the cystein-rich domains and an additional Cys residues (Buchanan-Wollaston 1994; Zhou & Goldsbrough 1994). Type 2 MTs possess two cysteine-rich domains separated by a spacer of approximately 40 amino acid residues. However, the first pair of cysteine is present as a Cys-Cys motif. It is observed that the spacer region in type 2 MTs is much more variable between species. Type 3 MTs possess only four Cys residues in the N-terminal domain. Type 4 MTs differs from other plant MTs by having three cysteine-rich domains.
each containing 5 or 6 conserved cysteine residues, which are separated by 10 to 15 residues. It is observed that the expression of four types of MTs follow some general trend in expression like, type 1 MTs expressed predominantly in roots, type 2 MTs are expressed in leaves, type 3 MTs are expressed in fruits and type 4 are mainly expressed in seeds (Zhou & Goldsbrough 1994; Heish et al. 1995; Zhou & Goldsbrough 1995; Heish et al. 1996; Guo et al. 2003). The presence of diversity in MT gene family suggests that these may not only differ in sequence but also in their function. Arabidopsis MT gene expression follows the patterns found in other plant species but type 3 MTs had shown expression in the leaf mesophyll cells (Guo et al. 2003).

The function of MTs in plants is still not very well understood, despite of their widespread occurrence and their relatively high level of RNA expression (Matsumura et al. 1999; Bausher et al. 2003; Moyle et al. 2005). In animals, MTs protect against cadmium toxicity (Klaassen et al. 1999), while in plants protection against cadmium toxicity is provided by Phytochelatins (PCs) (Cobbett & Goldsbrough 2002). In Arabidopsis and Rice, the MT gene expression is strongly induced by Cu treatment and to lesser extent, by Cd and Zn (Zhou & Goldsbrough 1994; Heish et al. 1995). In Arabidopsis ecotypes, expression of MT genes is correlated with the Cu tolerance (Murphy & Taiz 1995). MT gene were also induced by ethylene in Sambucus nigra (Coupe et al. 1995) and increased during senescence in Arabidopsis (Garcia-Hernandez et al. 1998; Navabpour et al. 2003). In kiwifruit and pineapple, the expression of MT genes is confined to specific stages of fruit development (Moyle et al. 2005). In some plants, MT gene has also been proposed to have functions during development (Lanc et al. 1987; Ledger & Gardner 1994; Zhou et al. 2005), and in protection against oxidative stress (Akashi et al. 2004; Gisela et al. 2004; Wong et al. 2004). All the Arabidopsis MTs can functions as metal chelators in vivo (Guo et al. 2008). In rice, MT2b has been demonstrated to have important role in initiation of lateral root and embryo germination (Yuan et al. 2008).

With the availability of whole-genome sequences of Oryza sativa and Arabidopsis thaliana, the tools of bioinformatics were used to examine the key sequence and structural features of MT proteins. The analysis is based on the high-quality, finished (TIGR version 6.1) O. sativa genome sequence (IRGSP, 2005).
A genome wide analysis of Rice MT proteins with a comparison to their counterparts in *Arabidopsis* gives an insight into evolution of MT family members in these plants. Previously similar genome wide analysis for MTs had been performed in rice (Zhou et al. 2006) along with their expression analysis using Northern blots. With the further advancement in the rice and *Arabidopsis* genome annotation level and to identify any possible new members of MT family genome wide analysis has been carried out. These analyses mainly focus on the genome localization and evolutionary relationship of each member in Rice and *Arabidopsis* MT proteins. Additionally, we have carried out comprehensive analysis of the OsMT1 in IR64 salt tolerant rice variety. We have also carried out structural analysis of the MT protein in *Oryza sativa* var. IR64 variety using homology modeling in order to analyse its key structural features.

4.2 Methods

4.2.1 Sequence Analysis of Rice Metallothionein Genes

The homologous protein members of metallothionein protein family were searched from NCBI non-redundant protein sequence database (Pruitt et al. 2003) using PSI-BLAST (Altschul et al. 1997) with cut-off for expected (E) values of $10^{-10}$. All the members were then aligned using MUSCLE-multiple sequence alignment software (Edgar 2004). The alignment was then used to make profile using hmmbuild program of HMMER package (version 3.3.2; http://hmmr.wustl.edu/). This profile was further used for searching the MT proteins members in rice (TIGR version 6.1) and *Arabidopsis* (TAIR version 8) using hmmsearch program of HMMER package. The sequences thus obtained were aligned using MUSCLE (Edgar 2004). Using the sequence alignment, bootstrap analyses were performed with 2000 replicas. The parsimonious tree was calculated using protpars program from Phylip package (version 3.6) (Felsenstein 1989). A consensus tree was obtained, and unrooted tree was plotted using drawtree program from the phylip package. Pairwise alignment of the metallothiomein genomic sequences from *Oryza sativa* were carried out using Needle program of EMBOSS package (6.0.1) (Rice et al. 2000). Mummer software package (v 3.20) (Kurtz et al. 2004)
and dottup program of EMBOSS package (6.0.1) (Rice et al. 2000) with word size 15 was used for analysing the duplication of gene present on chromosome XII. The final alignments were prepared using Jalview (Waterhouse et al. 2009).

4.2.2 Molecular Modelling of OsMT1-IR64

The secondary structures of the OsMT1-IR64 protein were predicted using JNET secondary structure prediction server (http://barton.ebi.ac.uk/jpred2.html) (Cuff & Barton 1999; Cole et al. 2008), PREDATOR (Protein secondary structure prediction from sequences) (Kabsch & Sander 1983; Frishman & Argos 1995; Frishman & Argos 1996; Frishman & Argos 1997), STRIDE (STRuctural IDENTification) (Frishman & Argos 1995), PSIPRED (Protein Structure Prediction server) (http://bioinf.cs.ucl.ac.uk) (Bryson et al. 2005).

The fold recognition analysis of OsMT1-IR64 protein sequence was performed using FUGUE (Sequence structure homology recognition) (Shi et al. 2001), GenTHREADER (McGuffin & Jones 2003), 3DPSSM (Kelley et al. 2000) and Phyre server (http://www.sbg.bio.ic.ac.uk). The architectural motifs and the topology of proteins with known three-dimensional structure were analysed according to SCOP (Structure Classification Of Protein) (Murzin et al. 1995) and CATH (Orengo et al. 1995; Cuff et al. 2009) classifications.

The three-dimensional structure of OsMT1-IR64 was modeled in a stepwise procedure, starting with the identification of template structures with PDB id: 1CLD.pdb, 2F2J.pdb, 1M1X.pdb 1ZME.pdb, 1HWT.pdb and 1J5L.pdb. The identified templates were obtained from protein structure database, PDB and were aligned using structure alignment software STAMP (Russell & Barton 1992). The aligned structures were used as a profile for aligning the target sequence. Comparative protein modelling program MODELLER9v2 (Sali & Blundell 1993; Eswar et al. 2000; Fisher et al. 2000; Marti-Renom et al. 2000) was then used to generate a 100 all-atom model by alignment of the target sequence with the selected template sequence in an alignment file. The best model was chosen on the basis of the stereochemistry quality report generated using PROCHECK (Morris et al. 1992; Laskowski et al. 1993) and side chains were optimized using SCWRL 3.0 (Camtepec et al. 2003). The bond distances and dihedral angel...
restraints on the target sequences were derived from its alignment with the template three-dimensional structures. The spatial restraints and the energy minimization steps were performed within Modeller using the CHARMM22 force field for proper stereochemistry of proteins. Further evaluation of the modeled OsMT1-IR64 structure was done using the PROSA web server (Sippl 1993; Weiderstaein & Sippl 2007). Molecular visualization and analysis of the final model were carried out with VMD (Humphrey et al. 1996).

4.3 Results and Discussion

4.3.1 Analysis of OsMT1-IR64 Gene Family of Rice

To gain an insight into the member proteins of metallothionein family in *Oryza sativa*, whole genome protein sequences were searched using profile of metallothionein protein members (see Methods). One of the library clones obtained using subtractive hybridization approach (Chapter 1) showed strong upregulation under the salt stress condition in *Oryza sativa* sp. IR64. Sequence of OsMT1-IR64 gene was compared with other members of the metallothionein family. The whole genome analysis revealed the presence of 13 genes and 16 protein products in rice, while in *Arabidopsis* 9 members of the metallothionein family have been identified (Ziemer et al. 2005). However, only eight out of these nine genes of metallothionein could only be established as for AtMt1b, EST was not previously found. New members were not observed in genome wide analysis of MT members in *Arabidopsis* using TAIR ver 8. The sequence alignment of the deduced amino acid sequence in rice MT along with the MT protein isolated from the library of Rice IR-64 and *Arabidopsis* MTs shows the presence of conserved cysteine residues in the sequence (Figure 4.1).

The classification of MT genes in *Oryza sativa* sub pokkali was performed on the basis of existing criteria which was based on conserved cysteine residue positions (Cobbett & Goldsbrough 2002). Previously reported OsMT-I-2a was found here to be hypothetical protein. Similarly earlier repoted OsMT-I-3b was also not found in the analysis. The members of metallothionein protein family members have been named according to scheme of nomenclature as per TIGR rice
Figure 4.1: Alignment of the amino acid sequences of OsMT proteins in *Oryza sativa*. The metallothionein sequence from the *Oryza sativa* sp. IR-64 were named as OsMT1-IR64, rest of the other amino acid sequences were from *Oryza sativa* sp. japonica. The * above the sequences shows the conserved amino acid residues present on the OsMT1 proteins. The bar below the sequences shows the level of conservation of motifs in the amino acid sequences.

genome nomenclature guidelines such as OsMT, where the first two initials would represent the name of the organism followed by Metallothionein abbreviated as 'MT'. The numeral followed would represent the type of Metallothionein protein member and alphabet followed represents the member number identified. Earlier OsMT members in rice have been searched by Zhou et al. (2006). With more finished genome level information on TIGR, searches have been made in the whole genome of *Oryza sativa* sp. Nipponbare for metallothionein protein members. Table 4.1 shows the identified member of the metallothionein family with respect to the earlier proposed report (Zimeri *et al.* 2005). The sequences of MT show the identity ranging 55% to 98%, in their respective types. OsMT1-IR64 shows 100% identity with that of OsMT1e member of *Oryza sativa* sp. Nipponbare
metallothionein family at protein level.

Among all the MT genes, most of the type 1 MT genes (van Hoof et al. 2001) were located on chromosome XII in rice except OsMT1c located on chromosome III, and OsMT1e on chromosome XI (Figure 4.2). Analysis of gene duplication records from TIGR revealed (http://rice.plantbiology.msu.edu), OsMT1e to be the result of duplicated OsMT1a which is present on chromosome XII. Two members of the type 2 MT proteins were located on chromosome I, while the third, OsMT2c, was located on chromosome V. The single type 3 gene identified in *Oryza sativa* sp. Nipponbare (OsMT3a) was located on chromosome I. As shown in Figure 4.2, all the type 1 genes present on chromosome XII shows identical gene structure i.e. two introns per gene while only the alternatively spliced OsMT1a2 shows the presence of single intron. Analysis of all OsMT1 genes present on chromosome XII showed similar intron arrangement.

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4.3 Results and Discussion

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Table 4.1: Metallothionein members in *Oryza sativa*. The members were identified using HMMER search; nomenclature compared to earlier identified members in *Oryza sativa* (Zimeri *et al.* 2005).

Two genes of type 1 member of the metallothionein family namely OsMT1c and OsMT1f were found to be in the antisense position while other three OsMT1a, OsMT1g and OsMT1d were found to be on sense strand. With the view to find if there is any repeat of the gene segment, analysis using Mummer (*v* 3.20) (Kurtz *et al.* 2004) and dot plot using dottup program of EMBOSS software package (Rice *et al.* 2000) have been performed (Figure 4.3).

Analysis of the plots obtained using dottup and Mummer program (Figure 4.3) does not reflect duplication events in this region due to absence of repeats. With these results, it can be hypothesized that certain regions of the segment might have got duplicated and diverged further during the course of evolution or genes present in the segment might have co-evolved during the course of evolution. Further analysis using pairwise alignment using Needle program of EMBOSS package (*v* 6.0.1) of the genomic sequences shows all the type 1 members of the metallothionein family located on chromosome XII shows identity ranging from 49% to 72% among themselves. While OsMT1e shows low genomic sequence level identity (39%-42%) with the OsMT1 members present on chromosome XII. OsMT1b present on chromosome III also shows similar pattern with members of OsMT1 present on the chromosome XII. Analysis show that OsMT1b and OsMT1e genes might have evolved differentially after duplication. Sequence analysis further shows that in OsMT1c, OsMT1d, OsMT1f and OsMT1g one N-terminal cysteine is absent while in OsMT1a1, OsMT1a2, OsMT1-IR64, OsMT1b it was found to be present.

The rooted tree based on the amino acid sequence showed the close relationship between the various MT types present in *Arabidopsis* (Zimeri *et al.* 2005)
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Figure 4.2: Graphical (scaled) representation of location of putative genes for MT family on *Oryza sativa* chromosomes. The centromeres on chromosomes have been marked in ovals. The position of first exon of genes (in Mb) has been marked in the parentheses along with their names at same location on chromosomes. Arrow marks the direction of the ORF specific to the OsMT genes. The region corresponding to the clustered MT members located on chromosome XII has been enlarged to depict the striking similarities in gene structure of various members.

and rice (Figure 4.4). The rooted tree showed that classification of various MT types present in *Arabidopsis* and rice. Interestingly it was observed that type 2 MT in rice (OsMT2d and OsMT2a) belong to larger subgroup having both type 1 and type 2 MT proteins, which shows that these sequences have not differentiated much but have preserved type 2 signature in their respective sequences. Similarly, type 1 MT (AtMT1a and AtMT1c) were observed more closer to the
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Figure 4.3: (a) Mummer plot to of 23.3 Mbp to 23.6 Mbp region of chromosome 12. (b) Dotplot analysis of 23.3 Mbp to 23.6 Mbp region of chromosome 12.

root showing that these sequence might have also preserved their type 1 signature but have not differentiated much during the course of evolution.

4.3.2 MPSS analysis for MT family genes

Massively parallel signature sequencing (MPSS) provides a quantitative measure of gene expression for nearly all genes in the genome. To comment on the expression of metallothionein genes in various tissues/organs under different conditions, analysis of MPSS signature data available for both 17 base and 20 base libraries representing 6 different parts of the plant *Oryza* MPSS project (http://mpss.udel.edu/rice/) (see Appendix C.1 to C.8) has been performed. On an average, transcript abundance of OsMT1a, OsMT2c and OsMT3a was found to be very high under all the conditions tested as per the MPSS database. Expression of OsMT1e and OsMT1f was reported exclusively in young and mature roots of rice with latter showing slight accumulation in growing seedlings also. A member of type 2 metallothioneins, OsMT2b was found to be highly abundant in merismatic tissues at vegetative and reproductive stages suggesting
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Figure 4.4: Rooted phylogenetic tree of metallothionein protein members from *Oryza sativa* sp japonica, *Arabidopsis thaliana* and OsMT1 from *Oryza sativa* sp IR-64.

its developmental regulation. A type 2 metallothionein, OsMT2c shows higher expression in roots of stressed rice seedlings. Expression of metallothionein gene members OsMT2c and OsMT3a (located on chromosome I) and OsMT1a was documented under biotic stressors (*X. oryzae* and *M. grisea*) which may be an indication of their involvement in stress regulated expression. Analysis of OsMT1c and OsMT1d showed absence of transcripts under various conditions tested in MPSS database. Type 4 metallothionein signatures were not available in the MPSS database. In summary, the expression of various MT family members
appears to be strictly tissue and developmental stage specific.

4.3.3 Comparative modeling of OsMT1-IR64

The template X-ray structures were obtained from Protein Data Bank (PDB) (see Methods). The model of OsMT1-IR64 was obtained using comparative modeling strategy (Tramontano 1998). The multiple sequence alignment obtained from the known sequences can provide a reasonable approach to comparative structure modelling. In order to verify the quality of the sequence alignment and optimise the position of gaps, corresponding positions from secondary structures were used. Experimentally determined structures were aligned and used as a profile for aligning the target sequence and to obtain an alignment, which was then used for modelling OsMT1-IR64 protein. To generate a 3D model of OsMT-IR64 protein, 100 structure models were generated using MODELLER9v2. Ramachandran plots were generated for all the modeled structures to determine deviations from normal bond lengths, dihedrals and nonbonded atom-atom distances. The plot shows that the modeled OsMT-IR64 protein has 94% residues in favourable regions with the remaining 5% of residues occurring in allowed regions, only 1% of the residue was found in the disallowed region (Figure 4.5).

The PROCHECK result summary showed 9 out of 72 residues labeled while the torsion angles of the side chain designated by $\chi_1$-$\chi_2$ plots showed only no labeled residues. All main-chain and side-chain parameters were found to be in the ‘better’ region. G-factor is essentially a log odds score based on the observed distribution of stereochemical parameters such as main chain bond angles, bond length and phi-psi torsion angles. The score for G-factors should be above -0.50 for a reliable model. The observed G-factor scores of the present model were -0.36 for dihedral bonds, -0.40 for covalent bonds and -0.37 overall. The distribution of the main chain bond lengths and bond angles were 97.4% and 90.6% within limits. This distribution in the bond angles is attributed to many residues that are not conserved in OsMT1-IR64 in comparison to the template structures. The PROSA-Web analysis plots show z-score of -3.59 for the modeled OsMT1-IR64. The PROSA energy plots and z-score further confirm the quality of modeled structure (Figure 4.6).
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4.3.4 Structural Analysis of OsMT1-IR64

The prediction of secondary structure using Jnet and PREDATOR (see Method) confirms the presence of two beta sheets in OsMT1-IR64. The modeled structure was observed to have the presence of two beta sheets in the protein (Figure 4.7 and 4.8). The sequence analysis has revealed the presence of Cys-Xaa-Cys signature motif. Earlier many attempts were made to solve structure of metallothioneins in apo form, with Cu and Ag using NMR. The sequence and structure of the OsMt1-IR64 shows the presence of conserved cystein as observed the type 1 members of the metallothionein protein family which has been highlighted in the cartoon structure representation.

The metal cluster fragment of the enzyme contains six Cys-Xaa-Cys groups, in groups of three separated by the 42 amino acid spacer. The first group of metal cluster is extended from 3-5, 9-11 and 15-17. The other group of metal cluster ranges from 61-63, 67-69 and 72-74. It is earlier assumed that each Cys-Xaa-Cys
OsMT1-IR64 was observed to have an upregulated expression under the salt stress condition (chapter 1). This gene showed high homology with other known type I metallothionein genes in plants. The significant level of identity (70%-85%) with their respective MT family members shows that the motifs of the proteins have remained conserved throughout the evolutionary process. The residues which are needed for the chelation of the heavy metals were found to be conserved among the metallothionein proteins in Oryza sativa. The chromosomal localization of the metallothionein genes shows that the OsMT1 genes were clustered on chromosome XII of the Oryza sativa which may be due to the result of duplication event that
Figure 4.7: Cartoon representation of the OsMT1-IR64 protein from *Oryza sativa* sp. IR64 showing presence of two $\beta$-sheets in the secondary structure. The conserved residues of OsMT1-IR64 are shown as CPK representation. The figure was prepared using VMD.

Figure 4.8: Electrostatic stereo-view of the modeled OsMT1-IR64 protein showing presence of the conserved cystein.
had taken place on the segment. A structural study also reveals the conservation of the structural domains required for the essential functions of the protein.

The role of metallothionein protein family members in salt stress conditions is not clearly understood while earlier research have proven their role in heavy metal detoxification, ion homeostasis maintenance, and storage of essential metal is well established in plants (Cobbett & Goldsbrough 2002). Few reports earlier have suggested that the expression of metallothioneins is altered when plant is exposed to abiotic stresses (Hsieh et al. 1995; Jin et al. 2006), implicating their role in abiotic stress tolerance. Hence detailed experimental analysis of metallothionein protein family member can gives an insight to abiotic stress tolerance in plants.