Chapter 4

Isolation and Cloning of Metallothionein Genes
4.1 Introduction:

Heavy metals pollution in water, soil and sediments has become a serious environmental problem due to anthropogenic activities. Excess metal ions can be toxic to plants, causing a wide range of detrimental effects, such as the inhibition of photosynthesis and impairment in pigment synthesis, plasma membrane damage, functional changes and other metabolic disturbances[1]. It has been reported that a limited number of wild plants are able to grow normally on metal-contaminated sites and accumulate high concentrations of metals in their tissues without suffering any damage[2]. Plants from different species have evolved several mechanisms for metal detoxification that include exclusion, compartmentalization, chelation and binding to organic ligands (organic acids, amino acids, PCs and MTs)[3], [4].

MTs are low-molecular weight cysteine (Cys)-rich proteins that can effectively bind metals via their Cys residues[3]. Based on the arrangement of Cys residues, all plant MTs (class II) were further distributed into four types, according to the distribution of cysteine residues in the amino- and carboxy-terminal regions. The type 2 MTs spacer region is more variable between species[3]. Although their precise cellular function is uncertain, by virtue of their high affinity of binding with heavy metal ions, they take part in protection against heavy metal toxicity via sequestration, play a role in maintaining the homeostasis of essential metal ions, detoxifying heavy metals, and scavenging reactive oxygen species (ROS)[3], [5]–[8]. In some plant species, metal tolerance is associated with high copy transcript levels of a type-2 MT constitutively[9]–[11]. Functional gene expression analyses in yeast have revealed a role for MTs in metal homeostasis and detoxification[12]–[15]. Besides binding to metal ions plant MTs are also involved in fruit ripening[16], root development, embryo germination[17], and suberin deposition[5].

Plant MT-like proteins have been isolated from different tissues and species. Gene expression has been well characterized in maize seeds[18], Brassica campestris shoot apex during floral transition[19], somatic and zygotic embryos of Douglas-fir[20], kiwifruit early development[21], foliar trichromes of Vicia faba[22], senescent leaves of Brassica napus[23]–[25], ethylene- promoted abscission of Sambucus nigra L. leaflets [26], tobacco mosaic virus infection of Nicotiana glutinosa leaflets[27] and wounding. These reports suggest that MT genes are different in structure and likely to
play diverse roles and functions in plants in order to cope with complex developmental and environmental cues. Gene expression of MT-like proteins is regulated by different endogenous and exogenous factors. The synthesis of MTs is induced through transcriptional activation systems, composed of MREs (metal response elements), AREs (antioxidant response elements) and GREs (glucocorticoid response elements) in the enhancer/promoter region of the MT gene and MRE/ARE/GRE-binding proteins which are, directly or indirectly, activated by a variety of heavy metals, glucocorticoid hormones, interferon, interleukin-I, bacterial endo-toxin, UV radiation, and oxidative stress[28], [29]. They respond differentially to inducers including ethylene, natural senescence, wounding, ABA, sucrose starvation, viral infection and CuCl₂[27], [30], [31].

Expressing plant MTs in microbial hosts provides important evidence of plant MTs functioning in metal stressed condition. For instance, *E. coli* transformed with type 1 MT (PsMTa) from pea plant showed the highest affinity for Cu metal[32]. MT-deficient strains of yeast expressed arabidopsis MTs have restored the yeast mutant’s heavy metal tolerance[33]. The effect of heavy metals on the expression of plant MTs and MT-like genes has been studied extensively, but no general conclusion is proposed. The levels of MT mRNA could be decreased[34], [35], increased[30], or unaffected[36], [37] when exposed to heavy metal treatment. The transcription of MT-like genes by plants grown in excess heavy metals is plant species-, tissue- and metal-specific. For example, the level of type 2 MT-like mRNA in *Brassica juncea* decreased in response to elevated external Cu²⁺, but increased when exposed to Zn²⁺[38]. In addition, the expression of plant MTs was also detected in response to various stress conditions. Heat shock and glucose starvation induce expression of MTs in rice[30], leaf senescence in *Brassica*[23], wounding and viral infection in *Nicotiana*[27], and fruit development in kiwi[27].

Therefore, an understanding of the molecular mechanisms and genetic basis is an important aspect of developing plants as agents for the phytoremediation of contaminated sites[39], [40]. Using molecular techniques, some fast growing and high biomass producing nonaccumulators can be engineered to achieve some of the properties of the hyperaccumulators. Accessing the molecular mechanism of metal accumulation will be a key point in achieving this goal. Heavy metals effect on MT
(metallothionein) expression has disclosed wide variation from species to species. Molecular mechanisms underlying the transcript levels (enhancement, depression or stasis) in response to metal stresses, remain largely unknown in plants[41]. Functional properties of plant MTs were demonstrated by their over-expression in E.coli as fusion proteins[42], through complementation studies in yeast[43], and by RNAi knock down approach in Arabidopsis[44]. In view of disclosing the role of molecules that is critical for the hyperaccumulation of heavy metals by plant, molecular characterization of metallothionein gene was addressed in the present study. This chapter deals with the molecular characterization of metal uptake, with particular reference to Metallothionein genes.

4.2 Materials and methods:

4.2.1 Primer design:
Seven pairs of primers were designed manually and using online primer 3.0 algorithm tool from sequences available in NCBI database (http://www.ncbi.nlm.nih.gov/) for metallothionein genes of Araceae, Malvaceae, Euphorbiaceae and Poaceae family plants. Sequences of these primers are as follows.

1) CEMT-2
   
   FP = 5’- CTC GAG TAT GTC TTG CTG TGG TGG T -3’
   RP = 5’- AAG CTT TCA ACA GTT GCA AGG GTC G -3’

2) MAL GHL
   
   FP = 5’- GGA AAA TTC GGA TCC ATG TCT TGC TGT GGT GGA-3’
   RP = 5’- ATA TGC CGC CTC GAG TCA TTT ACA ATT GCA AGG-3’

3) MAL GHS
   
   FP = 5’- GGA AAA TTC GGA TCC ATG TCT GAC AGG TGC GGC-3’
   RP = 5’- ATA TGC CGC CTC GAG TTA GTG GCC ACAGGT GCA-3’

4) Croton
   
   FP = 5’ ATG TCT TGC TGC GGA GGA AAC TG-3’
   RP = 5’ TCA TTT GCA GTT GCA TGG ATC AC-3’

5) Grass1
   
   FP = 5’ CTC GAG TAT GTC TTG CTG CGG-3’
   RP = 5’ AAG CTT TCA GTT GCA GGA GCA-3’
6) \(JC\)

FP = 5’ AAA TTC GGA TCC ATG TCT TGC TGC GGA GGA 3’
RP = 5’ TGC CGC CTC GAG TTA GCA GTT GCA TGG GTC 3’

7) \(RC\)

FP = 5’ AAA TTC GGA TCC ATG TCT TGC TGT GGA GGA AAC 3’
RP 5’TGC CGC CTC GAG TTT ACA AGT GCA AGG GTT GCA 3’

Underlined nucleotides are a restriction endonuclease recognition site introduced at 5’ end of primer for sticky end mediated directional cloning in pRSET A expression vector. GGA TCC, CTC GAG and AAG CTT are recognition sequences for BamHI, XhoI and HindIII restriction enzymes respectively.

4.2.2 DNA cloning
4.2.2.1 Plant leaf total DNA isolation from \textit{Croton bonplandianum} and \textit{Hibiscus radiatus}:

Plant DNA isolation was performed as per Porebski et al.[45]. The leaves of \textit{Croton bonplandianum} under the metal stress were collected and washed thoroughly with tap water and then with distilled water to remove the dust particles. 0.5 – 2.0 gm leaves were weighed and homogenized with 3-6 ml of extraction buffer (Appendix 6.5) in mortar and pestle, centrifuged at 10,000 rpm for 10 min at 4°C and the supernatant was collected. To this, equal volume (750 μl) of phenol: chloroform: iso-amyl alcohol (25:24:1v/v) was added, centrifuged at 10,000 rpm for 10min at 4°C and the upper aqueous phase was taken. 15 μl RNase (2mg/ml) treatment for 30min was given at room temperature to remove RNA. An equal volume of chloroform: iso-amyl alcohol (24:1 v/v) was added and centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant was collected and DNA was precipitated using absolute chilled ethanol, centrifuged at 10,000 rpm for 10min at 4°C. The pellets obtained were air dried and dissolved in T.E buffer. DNA obtained was stored at -20°C for further use. Quality of isolated DNA was checked on 0.8% agarose gel. \textit{Hibiscus radiatus} DNA was also isolated following the same procedure.

4.2.2.2 Metallothionein gene amplification and elution:

PCR amplification (Applied Biosystem 2720 thermal cycler) of the \textit{Croton bonplandianum} DNA using 1 μl of forward primer, 1 μl of reverse primer, 1 μl of
**Croton bonplandianum** DNA, 6.25 μl of Emerald master mix (Takara Bio, Japan) and 3.25 μl of nuclease free water with a total reaction volume of 12.5 μl was carried out under following PCR conditions for 30 cycles. After PCR amplification the product obtained was separated on 1.5% gel against 100 bp marker.

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For elution of DNA, the band with appropriate product size was excised from agarose gel, with a sharp scalpel and cut into small pieces. The pieces were taken in 1.5 ml microfuge tubes and to this 2.5 volume of sodium iodide solution was added. The tube was incubated at 50°C for 5-10 min to solubilize the gel. After that 10 μl silica suspension was added and incubated at room temperature for 20min with mixing at every 5 min interval. Then the tube was centrifuged at 12,000 rpm for 1 min and the supernatant was discarded. The pellets were washed with wash buffer once or twice and centrifuged at 12,000 rpm for 1 min. The supernatant was discarded and the pellets were air dried completely. The pellets were dissolved in 60 μl of TE buffer and incubated at 50°C for 5min. Again centrifuged at 12,000 rpm for 1 min and the supernatant was collected in another tube. The quality of eluted DNA was checked on agarose gel.

**Hibiscus radiatus** DNA metallothionein gene was successfully amplified and eluted using the same procedure for **Croton bonplandianum** DNA metallothionein gene.

**4.2.2.3 Cloning of metallothionein gene into pRSET A vector:**

**4.2.2.3.1 Restriction digestion of DNA metallothionein gene and pRSET A vector:**

The bacterial expression vector, pRSET A (Invitrogen, USA) with T7 promoter and six histidine tag at N-terminal was used for expression of metallothionein genes. Both the **C. bonplandianum** DNA MT insert and the vector were digested by BamHI (NEB Inc, USA) at 5’ end and XhoI (NEB Inc, USA) at 3’ end. The digestion was carried out by taking 1 μg of pRSETA vector and 1 μg of insert. Along with these both the enzymes with 2 μl each (20,000 units/ml) were added to 2μl BSA (10mg/ml) and 10μl of 10X buffer. The total volume of the reaction mixture was made 100μl with Milli Q and
incubated overnight in water bath at 37°C. Further incubation at 65°C for 10 min to deactivate the enzymes. The digestion was confirmed by separating the mixture on 1% agarose gel. The same procedure was followed to digest *H. radiatus* DNA MT insert.

4.2.2.3.2 Ligation of metallothionein gene and pRSET A vector:
The digested product on the gel was purified by gel extraction kit and its purity was checked on agarose gel. In 0.5 ml of microfuge tubes, 50 ng of digested pRSET A, 37.5 ng of purified digested *C. bonplandianum* DNA MT insert, 2 µl of T4 ligation buffer (10X), 1 µl of T4 DNA ligase enzyme (4,000,000 Units/ml) (NEB, USA) were added and the final volume was made 20 µl with milliQ water. Same mixture was prepared for *H. radiatus* DNA MT insert. These ligation mixtures were incubated overnight at 16°C water bath. After incubation the tubes are stored at -20°C till the transformation is carried out.

4.2.2.3.3 *E. coli* DH5α competent cell preparation and transformation:
To prepare competent cells, a well isolated single colony of *E. coli* DH5α was picked from the LA plate and inoculated into 10 ml of LB broth. This was incubated overnight into shaker at 37°C. 1 ml of this culture was further inoculated at 37°C in shaker until the O.D 600 has reached 0.2-0.4. The culture was chilled on ice for 10-20 min and then it was transferred to the sterile centrifuge tubes. The cells were pelleted by centrifugation at 6000 rpm for 10 mins at 4°C. The pellet was resuspended in 20 ml of sterile, cold 0.1M CaCl₂ solution and incubated on ice for 30 min. Another centrifugation was carried out same as above and the resulting pellet was resuspended in 4 ml of sterile, cold 0.1M CaCl₂ + 20% glycerol to yield final competent cell suspension. These cells can be stored at -80°C for 2 months.

For transformation, 10 µl of the ligated mixture was mixed with 100 µl of competent cells and incubated at -20°C for 20 min. After this 42°C heat shock for 2 min was given to the cells followed by incubation at -20°C for 10 min. To the sample 1 ml of sterile LB was added and incubated in a shaker at 37°C for 1 1/2 hr. Then the tube was centrifuged at 6,000 rpm for 3 min. 850 µl of the supernatant was discarded and the pellets were resuspended in 150 µl of remaining LB. These samples were plated on LA-Amp plates.
with the help of sterile spreader. The plates were incubated in inverted position overnight at 37°C. Next day the transformed colonies will grow on the plates.

4.2.2.3.4 Selection of positive MT clones and recombinant plasmid isolation:
After transformation, the transformed colonies were picked up and inoculated in 10ml of sterile LB-Amp tubes and incubated overnight in shaking condition at 37°C. After incubation the cell suspension was transferred to 2ml microfuge tube and centrifuged at 6,000 rpm for 3min. The supernatant was discarded and to the pellets 200 µl of solution I (Appendix 6.6) was added and mixed well by vortexing. To this, RNase treatment was given and the tubes were incubated for 5min at room temperature. Then 400 µl of solution-II (Appendix 6.6) was added to the tubes and incubated on ice for 10min. Followed by this 300 µl solution-III (Appendix 6.6) was added and incubation at room temperature for 10min was carried out. The cell debris and the proteins were separated by centrifugation at 12,000rpm for 10min at 4°C. The supernatant was collected in 1.5ml microfuge tubes and to this 0.8 volume of isopropanol was added and incubated at room temperature for 20min. After this, the tubes were centrifuged at 12,000rpm for 10min at 4°C and the pellets were air dried completely. Finally, the pellets were dissolved in 70 µl of TE buffer. The plasmid was then run on 1.5% agarose gel against 1 kb marker and pRSET vector.

4.2.2.3.5 Clones confirmation:
Single digestion:
After the results were observed on gel, single digestion of the C. bonplandianum DNA MT, cloned plasmid was used to confirm the cloning. A mixture of 2 µl of BamHI, 7µl plasmid, 2 µl BSA, 4 µl buffer was made up to 20 µl with MQ and incubated overnight at 37°C in a water-bath. Next day the digestion was confirmed on 1.2% agarose gel against 1 kb marker and pRSET vector.

Plasmid PCR:
PCR amplification of C. bonplandianum DNA MT cloned plasmid was carried out by taking 12.5 µl of reaction mixture containing 1 µl of forward primer, 1 µl of reverse primer, 6.25 µl of master mix, 0.5 µl of plasmid DNA and 3.75 µl of Mili Q. Thermal cycling for 25 cycles was carried out at the same conditions which were used to amplify
the genomic DNA. The PCR product was checked on 1.5% agarose gel against 100 bp marker. Same procedure of single digestion and plasmid PCR was utilized for *H. radiatus* DNA MT clone confirmation.

### 4.2.2.3.6 Sequencing and sequence analysis:
Confirm *C. bonplandianum* DNA MT cloned plasmid and *H. radiatus* DNA MT cloned plasmid obtained were submitted for sequencing to Xcelris lab Ltd. Ahmedabad. The sequenced data were used for BLASTn analysis for similar sequence.

### 4.2.3 cDNA metallothionein gene cloning:
#### 4.2.3.1 Plant leaf total RNA isolation from *Croton bonplandianum* and *Hibiscus radiatus*:
Leaves of *Croton bonplandianum* treated with 100 mg/kg of Cd were collected and washed thoroughly with tap water followed by distilled water rinse for removal of dust particles. 0.5-2gm leaves were weighed and homogenized with 2-4ml of RNA extraction buffer (Appendix 6.5) in a mortar and pestle. Centrifuged at 12,000rpm for 10min at 4°C and the supernatant was collected for the twice extraction with an equal volume of chloroform: isoamylalcohol subsequently. Aqueous phase was taken and 0.25 volumes of 10M LiCl was added and incubated overnight at -80°C for precipitation of RNA. Next day RNA was pelleted out by spinning at 10,000rpm for 20min at 4°C. The RNA was resuspended in 50µl of DEPC treated water and precipitated with 0.1 volume sodium acetate (pH 5.2) and 2.5 volume 96% ethanol mixture. The pellet was obtained by centrifuge at 10,000rpm at 4°C for 20min, which was washed with 70% ethanol twice and allowed to air dry. The pellet was dissolved in 30µl DEPC treated water. The RNA obtained was stored at -20°C for the downstream application.

Total RNA was isolated following the same procedure from leaves of *Hibiscus radiatus* treated with 100mg/kg of Cd.

#### 4.2.3.2 cDNA preparation and metallothionein gene amplification:
100 ngm *C. bonplandianum* mRNA and 2µl oligo-DT were incubated at 65°C for 10min then followed by addition of 1µl RNAsin, 4µl reverse transcriptase buffer, 6µl of 30mM dNTP mix, 1.0µl 0.1M DTT, 0.5µl M-MULV reverse transcriptase. The mixture was
homogenized and incubated at 37°C for 1:30hr. temperature was then raised to 94°C for 2min to denature RNA-DNA hybrid.

PCR amplification of *C. bonplandianum* c-DNA was carried out in next step of RT-PCR reaction with 2µl each forward and reverse primer, 1µl of *C. bonplandianum* c-DNA, 6.25µl of master (1.3µl of 10x assay buffer, 1µl dNTP mix, 0.25µl Taq DNA polymerase, 3µl nuclease free water.) the PCR condition were:

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*H. radiatus* cDNA was prepared and used as template to amplify *H. radiatus* cDNA MT gene by following the same procedure as in case of *C. bonplandianum*.

### 4.2.3.3 Cloning of cDNA metallothiontein into pRSET A vector:

*C. bonplandianum* cDNA metallothiontein gene, *H. radiatus* cDNA metallothiontein gene and pRSET A vector were digested according to section 4.2.2.3.1. Digested products were separated on agarose gel and appropriate size were eluted. Ligation reaction was run according to 4.2.2.3.2 for cloning of *C. bonplandianum* cDNA MT insert and *H. radiatus* cDNA MT insert into pRSET A vector separately and transformed into DH5α according to 4.2.2.3.3. Next day after overnight incubation positive clones were identification and plasmid isolation were performed as per section 4.2.2.3.4. Positive clones of *C. bonplandianum* cDNA MT and *H. radiatus* cDNA MT were checked for clone confirmation as per section 4.2.2.3.5. Confirmed *C. bonplandianum* cDNA MT clone and *H. radiatus* cDNA MT clone were sent for sequencing and sequence analysis.

### 4.2.4 Expression of cDNA metallothiontein gene:

#### 4.2.4.1 BL21 competent cell preparation and transformation:

BL21 competent cells were prepared following protocol as per section 4.2.2.3.3 and transformation of *C. bonplandianum* cDNA MT clone and *H. radiatus* cDNA MT clone were performed as per section 4.2.2.3.3. Transformed colonies were checked for
positive cloned MTs plasmid. Positive clones were stored as glycerol stock until use in -80°C.

4.2.4.2 Tolerance and accumulation of heavy metals by MTs:
cDNA MT clones of Croton bonplandianum and Hibiscus radiatus were checked for their heavy metal tolerance[46] and accumulation[46] to Cd and Ni metals. For tolerance studies, C. bonplandianum cDNA MT BL21 clone and H. radiatus cDNA MT BL21 clone along with BL21 as a control were streaked on LA+Amp plates containing 200µM, 400µM, 600µM, 800µM and 1000µM concentrations of Cd and Ni heavy metals each. LA-Amp plates devoid of Cd and Ni heavy metals used as control plate. After overnight incubation at 37°C plates were checked for tolerance of cDNA metallothionein clones.

For accumulation studies of C. bonplandianum cDNA MT BL21 clone, H. radiatus cDNA MT BL21 clone and BL21 (Control), active cultures were inoculated in 200ml LB-Amp medium containing 400µM of Cd and 1000µM of Ni concentrations separately. After overnight incubation at 120rpm and 37°C. Fresh weight and dry weight of collected pellets were checked. Samples were prepared for ICP-OES analysis as per section 2.1.2 and subjected for ICP-OES analysis.

4.3 Results and discussion:

4.3.1 DNA cloning:

4.3.1.1 Plant leaf total DNA isolation from Croton bonplandianum and Hibiscus radiatus:

![Image](M: λ DNA Mlu I digest; 1: Croton bonplandianum DNA)

![Image](M: λ DNA Mlu I digest; 1: Hibiscus radiatus DNA)
After ethanol precipitation step white fluffy mass of DNA was observed in *C. bonplandianum* and *H. radiatus*. As shown in figure 4.1 and 4.2 good quality of DNA observed under UV transilluminator against nucleic acid marker without any RNA and protein contamination isolated from *C. bonplandianum* and *H. radiatus* respectively. 269/280 nm ratio of these DNA fall in range of 1.6 – 2.0 indicating good quality for downstream processing. Same approach has been utilized by Kivanc et al.[47] for amplification of metallothionein gene from genomic DNA from *Triticum durum*.

### 4.3.1.2 *C. bonplandianum* and *H. radiatus* DNA MT gene amplification:

Attempts for amplification of metallothionein gene from *C. bonplandianum* DNA and *H. radiatus* DNA were made using different metallothionein gene specific primers sets in conventional PCR. Finally, *C. bonplandianum* DNA metallothionein gene of approximately 250bp amplified (Figure 4.3) using JC primer and *H. radiatus* DNA metallothionein gene of approximately 250bp amplified (Figure 4.4) using MAL GHL primer. 450 bp long amplicons were obtained from *T. durum* and *T. aestivum*[47]. Amplified genes were successfully eluted. As restriction digestion recognition sequences for BamHI and XhoI introduced in primers used, amplified genes had BamHI restriction site at 5’ end and XhoI restriction site at 3’ end.
4.3.1.3 Cloning of metallothionein into pRSET A vector:

4.3.1.3.1 Restriction digestion of *C. bonplandianum* DNA MT and *H. radiatus* DNA MT gene and pRSET A vector:

pRSET A vector is prokaryotic inducible his tagged expression vector. Parmar et al.[46], Yeon-Ok Kim et al.[48] and Ramos et al.[49] used pRSET A vector for cloning and expression. pRSET A vector has multiple cloning site for directional cloning. As gene of interest had BamHI at 5’ position and XhoI at 3’ position, both vector and inserts were subjected for restriction double digestion using BamHI and XhoI restriction enzymes. Single band of 2.9kb and ~250bp confirmed successful digestion of pRSET A vector and *C. bonplandianum* DNA MT gene (Figure 4.5) respectively. Same strategy utilized for *H. radiatus* DNA MT gene (Figure not shown). Eluted pure digested vector and insert were subjected for T4 DNA ligase mediated ligation reaction overnight followed by transformation in DH5α.

![Figure 4.5: Restriction digestion of C. bonplandianum DNA MT gene and pRSET A vector](image)

**Figure 4.5:** Restriction digestion of *C. bonplandianum* DNA MT gene and pRSET A vector

(M1: Step up 100 bp ladder; 1: Double digested *C. bonplandianum* DNA MT gene; M2: Step up 1 kb ladder; 2: Double digested pRSET A vector)

4.3.1.3.2 DH5α competent cell preparation and transformation:

DH5α is Non-pathogenic *E.coli* strain developed for laboratory cloning use[50]. Competent cells of DH5α were prepared and used for transformation of *C. bonplandianum* DNA MT and *H. radiatus* DNA MT gene ligated product. After overnight incubation as shown in figure 4.6 and 4.7 pinpoint colonies of transformed DH5α observed with *C. bonplandianum* DNA MT clone and *H. radiatus* DNA MT clone respectively on LA-Amp plates. 10 colonies from each plant were processed for
growth and thereafter with plasmid isolation to get confirm ligated gene clones. Raghothama et al [51], Yan Fang et al [52] and many more researchers have used this strain for cloning purpose.

4.3.1.3.3 Clones confirmation:

Plasmids were isolated from cloned DH5α and electrophoresed, plasmid with size of ~3.1 kb were selected as positive clones and preserved using glycerol stock preparation. Band of ~3.1 kb showed in figure 4.8 and 4.9 against 1kb step up DNA marker are C. bonplandianum DNA MT clone and H. radiatus DNA MT clone respectively.
MT clones were again used for clone confirmation by restriction digestion and gene specific plasmid PCR method. Single nick cut by either of BamHI or XhoI enzyme linearize the plasmid so cloned plasmid will show higher band of ~3.1 kb than empty pRSET A vector which is of ~2.9kb. After single digestion using either of BamHI or XhoI showed expected single band of ~3.1kb in *C. bonplandianum* DNA MT clone (Figure not shown) and *H. radiatus* DNA MT clone (Figure 4.10).

These clones were used for PCR amplification using gene specific primer. After electrophoresis of PCR product as shown in figure 4.11 and 4.12 band of ~250bp observed on gel electrophoresis which again confirmed successful directional cloning of metallothionein gene into pRSET A vector.
4.3.1.3.4 Sequencing and sequence analysis:

Obtained sequences were processed for quality.

*C. bonplandianum* DNA MT sequence (234bp):

ATGTCTTGCTGTGGGAGGAAACTGTGGGCTGGCTGGTCCAGTGGTGCCCAGGCTGCTTGCCAGTGGC
AACGGCTGTGCAGGGTGCAAGATGTTCCCTGACATAGTTGAGAAACCCAC
ATCCGAGACTCTTTTCTTTCTGGGCGGACCCCCAGAAACGCCACTTTTGAGG
CATCTGAGATGGGAGTGGGCTTGAGAATGGATGCAAGTGGTGAGACTAC
TGCCCTGGCAACCTTTGCTGTATTTCCGTAA


*H. radiatus* DNA MT sequence (240bp):

ATGTCTTGCTGTGGGAGGAAACTGTGGGCTGGCTGGTCCAGTGGTGCCCAGGCTGCTTGCCAGTGGC
AACGGCTGTGCAGGGTGCAAGATGTTCCCTGACATAGTTGAGAAACCCAC
ATCCGAGACTCTTTTCTTTCTGGGCGGACCCCCAGAAACGCCACTTTTGAGG
CATCTGAGATGGGAGTGGGCTTGAGAATGGATGCAAGTGGTGAGACTAC
TGCCCTGGCAACCTTTGCTGTATTTCCGTAA

BlastN analysis of *H. radiatus* DNA MT sequence showed up to 90% identity and 100% query coverage with reported metallothionein gene sequences in NCBI database. Deduced amino acid sequence of 80 amino acids also showed up to 90% identity with metallothionein protein already in NCBI database. Protein Blast detected conserved domain from 24 – 79 amino acid as metallothionein -2 superfamily. ORF finder detected whole sequence as open reading frame.

*C. bonplandianum* DNA MT gene sequence and *H. radiatus* DNA MT gene sequence were submitted to NCBI database with accession no KT928653.1 and KT928649.1 respectively.
4.3.2 cDNA cloning:

4.3.2.1 Plant leaf total RNA isolation from *Croton bonplandianum* and *Hibiscus radiatus*:

After successful isolation and cloning of metallothionein genes from *C. bonplandianum* DNA and *H. radiatus* DNA, an attempt to clone the same genes from RNA were made. To begin with the amplification and cloning of metallothionein genes from RNA, total RNA was isolated from 100mg/kg Cd heavy metal treated *Croton bonplandianum* and *Hibiscus radiatus*. As shown in figure 4.13 good quality of RNA isolated with three distinct bands of tRNA and rRNA observed. From the quality of these RNA, quality of mRNA was assumed to be intact and used for cDNA preparation.

4.3.2.2 cDNA preparation and metallothionein gene amplification:

![Figure 4.13: Separation of total RNA of *C. bonplandianum* and *H. radiatus* on agarose gel](image)

(1: RNA from *Croton bonplandianum*; 2: RNA from *Hibiscus radiatus*)

![Figure 4.14: Amplification of cDNA MT gene of *C. bonplandianum*](image)

(M: Step up 100 bp ladder; 1: *C. bonplandianum* cDNA MT amplicons)
cDNAs were prepared from good quality of RNA isolated from heavy metals stressed *Croton bonplandianum* and *Hibiscus radiatus* plant. *Croton bonplandianum* and *Hibiscus radiatus* cDNAs were used as a template to amplify metallothionein gene. Band of ~ 250 bp *C. bonplandianum* cDNA MT gene (Figure 4.14) was observed on agarose gel electrophoresis against 100bp DNA marker. Same result was obtained for *H. radiatus* cDNA MT (Figure not shown). Kivanc et al.[47] and Gisela et al.[53] also found similar results. Primer used for amplification of metallothionein were same as used in DNA metallothionein gene amplification i.e. JC primer set for *C. bonplandianum* cDNA and MAL GHL primer set for *H. radiatus* cDNA. Amplicons had BamHI and Xhol restriction sites at their 3’ and 5’ end respectively.

### 4.3.2.3 Cloning of metallothionein into pRSET A vector:

#### 4.3.2.3.1 Restriction digestion of *C. bonplandianum* cDNA MT and *H. radiatus* cDNA MT gene and pRSET A vector:

As shown in figure 4.15 after double digestion pRSET A vector linearized at 2.9kb and *C. bonplandianum* cDNA MT (Figure 4.15) & *H. radiatus* cDNA MT (Figure not shown) inserts showed band of ~250bp against DNA marker confirmed successful digestion of vector and inserts.

<table>
<thead>
<tr>
<th>Figure 4.15: Restriction digestion of <em>C. bonplandianum</em> cDNA MT gene and pRSET A vector (M1: Step up 100 bp ladder; 1: Double digested <em>C. bonplandianum</em> cDNA MT gene; M2: Step up 1 kb ladder; 2: Double digested pRSET A vector)</th>
</tr>
</thead>
</table>

#### 4.3.2.3.2 DH5α competent cell preparation and transformation:

DH5α competent cells were used for transformation of ligated cloned vector having *C. bonplandianum* cDNA MT and *H. radiatus* cDNA MT as an insert following procedure
as per section 4.2.2.3.3. After overnight incubation good numbers of pinpoint isolated colonies were observed on LA-Amp plate for *C. bonplandianum* cDNA MT clone (Figure 4.16) and *H. radiatus* cDNA MT clone (Figure not shown). These colonies were selected for clone confirmation.

![Figure 4.16: LA-Ampicillin plates showing *C. bonplandianum* cDNA MT DH5α clone](image)

### 4.3.2.3.3 Clones confirmation:

![Figure 4.17: Confirmation of cDNA MT clone of *C. bonplandianum*](image)

![Figure 4.18: Confirmation of cDNA MT clone of *H. radiatus*](image)

Isolated cloned plasmids (Section 4.2.2.3.4) were checked on agarose gel electrophoresis and a shown in figure 4.17 and 4.18 band of ~3.1 kb was observed against empty pRSET A vector of 2.9kb and 1kb DNA marker. Thus, confirms successful cloning of metallothionein gene into vector.
Release of insert in double digestion of cloned vector as per section 4.2.2.3.5 again confirmed successful ligation of *C. bonplandianum* cDNA MT gene as shown in figure 4.19. *C. bonplandianum* cDNA MT gene specific cloned plasmid PCR showed band of ~250bp support the cloning confirmation results (Figure 4.20). Same results were obtained for *H. radiatus* cDNA MT clone (Figure not shown).

4.3.2.3.4 Sequencing and sequence analysis:

Obtained sequence were processed for quality.

*C. bonplandianum* cDNA MT (231bp):

ATGTCTTGCTGCGAGGAACTGTGGCTGCGCTTTCCCTGCAAGTGGCGG
CAGTGGCTGCAATGGATGTTGGATGCACCATCAACTGAGACCACCA
GAACGTGAGACCCTATTTGCTGGAGTGTGCTCCTTCCCAACAAGTCTATGAGA
GCTCAGAGATGAGCTTTGGAGCTGAGGGAGATGGGTGCAAGTGTGGATCA
AGCTGCAGCTGTGACCCATGCAACTGCTAA

BlastN analysis of *C. bonplandianum* cDNA MT sequence showed up to 87% identity and 100% query coverage with already reported sequences. Deduced protein sequence of 76 amino acids also showed up to 73% identity and 100% query coverage. ORF finder identify whole gene as open reading frame. Protein blast identified metallothionein-2 domain from 15 to 76 amino acid in protein sequence. Sequence was submitted to NCBI database with accession no KT928650.1
\[ H. radiatus \] cDNA MT (240bp):

\[
\begin{align*}
&\text{ATGTCTTGTGTGGAACACTGCTGGCTGCGTCTGGCTGCAAGTGC} \\
&CAGCGGCTTGAGGATGCAAGATGTAACCCCGACATGAACACTGCGGAG \\
&\text{ATTACCACAACCAGACTCTCGTCTCGGTGTCGCAACCCAGAAAGCACA} \\
&\text{CTTCGAGGGAGCTGAGATGGAATTTGGGCTGAGAACGACTGCAAGTGA} \\
&\text{AGTGTGAGGCGAACTCTGCAACCCCCTTGCAATTGTAA}
\end{align*}
\]

BlastN analysis showed up to 91% identity with already reported metallothionein genes from different plants. Deduced 80 amino acid sequence also showed 90% identity with metallothionein protein having metallothionein-2 motif from 24 to 79 amino acid. Sequence was submitted to NCBI database with accession no KT928651.1

Classification of metallothionein protein is based on arrangement of cysteine amino acid in alpha and beta domain. Metallothionein type 2 protein characterized by \( \text{CC} \text{xxx} \text{C} \text{xxx} \text{C} \text{xxx} \text{C} \text{xxx} \text{C} \text{xxx} \text{C} \) arrangement. Where ‘C’ designate cysteine amino acid, ‘x’ designate amino acids other than cysteine and ‘…….’ designate spacer region between alpha and beta strand. Deduced amino acid sequence of \( C. \) bonplandianum cDNA MT (MSCCGNNCGCGSSCKCGSCNGCGMYPDITETTRTETLIAGVAPSNKFYESSEMSFGAEKDGCCKCGSSCSCDPNC) mimic same cysteine arrangement having eight cysteine amino acids in alpha domain and six cysteine amino acids in beta domain. In case of \( H. \) radiatus cDNA MT, deduced amino acids sequence (MSCCGNNCGCGSCCKCGSGGCGCKMYPDNTAEITTTETLVLGVPQKAHFEGAEMEAENDCKCGGGCTCNCNCK) follows the same cysteine arrangement as in metallothionein type 2 protein but it has additional cysteine amino acid at 64\(^{th}\) position. That designate \( H. \) radiatus cDNA MT protein with sequence novelty.

Splice site analysis and ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalo/) analysis with known metallothionein gene intron in \( Arabidopsis \) thaliana showed that both isolated \( C. \) bonplandianum and \( H. \) radiatus cDNA metallothionein genes were devoid of intron.

4.3.2.3.5 Expression of \( C. \) bonplandianum cDNA MT and \( H. \) radiatus cDNA MT in BL21:

Gene expression studies providing clues about the possible functions of the encoded
proteins presently constitute the major part of plant MT research. To check the correct functionality of a gene, it needs to be expressed in a suitable host expression system. One source of information is yeast complementation assays[54], in which the ability of a given MT to complement engineered metal ion sensitivities of yeast cells is probed, second is metal accumulation test[46]. Real time PCR[55] and transcriptome analysis[56] are next generation expression assays. *C. bonplandianum* cDNA MT and *H. radiatus* cDNA MT genes were expressed in BL21 expression system. Metal tolerance test results were detailed in Chapter 5 and Metal accumulation were detailed in table no 4.1.

Table 4.1: Accumulation of Cd and Ni by MT gene

<table>
<thead>
<tr>
<th>No</th>
<th>Name of Clone</th>
<th>Fresh weight (gm)</th>
<th>Dry weight (gm)</th>
<th>Metal accumulation (µg/gm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Cd (400 µM)</td>
<td>Ni (1000 µM)</td>
<td>Cd (400 µM)</td>
</tr>
<tr>
<td>1</td>
<td>BL21</td>
<td>0.558</td>
<td>0.558</td>
<td>0.0672</td>
</tr>
<tr>
<td>2</td>
<td><em>C. bonplandianum</em> cDNA</td>
<td>0.653</td>
<td>0.586</td>
<td>0.0632</td>
</tr>
<tr>
<td>3</td>
<td><em>H. radiatus</em> cDNA</td>
<td>0.606</td>
<td>0.544</td>
<td>0.0812</td>
</tr>
</tbody>
</table>

Both *C. bonplandianum* cDNA MT and *H. radiatus* cDNA MT clone showed good Cd accumulation compared to control BL21 as metallothionein can bind with a variety of metal ions and the preferred binding associates are Zn, Cu and Cd ions [57]. Up to 900-fold higher Cd accumulation in metallothionein clone indicates overexpression of metallothionein gene into bacterial cell. The same story repeated in case of Ni accumulation. Up to 600-fold accumulation were observed in MT clones compared to control BL21. The results are consistent with previous reports of metal tolerance of *E. coli*, expressing metallothionein genes[47], [58]–[62].

4.4 Conclusions:

234 bp and 240 bp metallothionein genes were successfully isolated from *Croton bonplandianum* and *Hibiscus radiatus* DNA respectively. Cloned DNA MT genes sequence showed positive match with metallothionein gene/protein. 231 bp and 240 bp metallothionein genes were successfully isolated from *Croton bonplandianum* and
*Hibiscus radiatus* cDNA respectively. Cloned cDNA MT genes sequence showed positive match with metallothionein gene/protein. Sequence novelty was found in *Hibiscus radiatus* plant metallothionein protein having an extra exceptional cysteine amino acid at 64th position. *C. bonplandianum & H. radiatus* DNA metallothionein genes and *C. bonplandianum & H. radiatus* cDNA metallothionein gene were submitted to NCBI database with accession no KT928653.1, KT928649.1, KT928650.1 and KT928651.1, respectively. Up to 900-fold Cd accumulation and 600-fold Ni accumulation were observed in MT clones compared to BL21 confirms functional intron-less plant metallothionein gene overexpression in prokaryotic system.

4.5 References:


Chapter 4


