CLONING, CHARACTERIZATION AND EXPRESSION OF A NOVEL METALLOTHIONEIN GENE (*mt-d*) FROM *Triticum durum*

by

HASAN ÜMİT ÖZTÜRK

Submitted to the Graduate School of Engineering and Natural Sciences
in partial fulfillment of
the requirements for the degree of
Master of Science

Sabancı University
July 2003

CLONING, CHARACTERIZATION AND EXPRESSION OF A NOVEL METALLOTHIONEIN GENE (*mt-d*) FROM *Triticum durum*

APPROVED BY:

Assoc. Prof. Zehra Sayers …….
(Dissertation Supervisor)

Prof. İsmail Çakmak …….

Prof. Altan Eraslan …….

DATE OF APPROVAL: …….

ABSTRACT

Two different metallothionein genes, labelled as *mt-d* and *mt-a* were identified in wheat (*Triticum durum* and *Triticum aestivum*) genomic DNA sequences were characterized. *mt-d* and *mt–a,* were found to contain 416 and 399 nucleotides, respectively. Nucleic acid sequence alignment showed 95 % similarity between the two. Sequencing results showed that the difference resulted from two extra TTTTTA repeats in the intron regions.

cDNAs encoding mt-genes were identifed by RT-PCR. Gene alignment algorithms strongly suggested that both of these cDNAs (*mt-a* and *mt-d*) encoded an open reading frame of 75 amino acids with two cysteine-rich domains featuring Cys-X-Cys motifs at the amino- and carboxy termininus. The deduced amino acid sequences of *mt-a* and *mt-d* genes show striking similarity to the MT-like proteins described within the Class II as Type 1 MTs and showed 100 % similarity with each other as deduced from cDNA sequencing results. These results indicate that *mt-d* from *T.durum* forms a "novel type 1" MT.

For further studies of *mt-d* expression, localization of the *durum* metallothionein protein (dMT) and its interactions with other proteins *mt-d* gene was inserted into the 5' MCS of pGFPuv vector. Verification was based on sequence data and restriction enzyme analysis. However, expression could not be validated by neither by visual detection of GFP expression nor by SDS-PAGE analysis. A more detailed sequence analysis indicated that the problem was due to a point mutation within the coding

sequence of the GFPuv, resulting in a stop codon and premature termination of the fusion protein.

Results presented here show the presence of metallothionein gene in the wheat *Triticum durum*. Although our attempts to express the gene as a fusion protein together with GFP to facilitate its localization in different systems was not successful it will be important in future studies to pursue this goal and achieve expression of labelled protein in plant systems to gain insights into its exact function in plants.

ÖZET

mt-d ve *mt-a* olarak isimlendirilen iki farklı metallothionein geni buğday (*T.aestivum* ve *T.durum*) genomik DNAsında belirlenerek karakterize edilmiştir. Karşılaştırmalı olarak bakıldığında *mt-d* ve *mt-a* genlerinin 416 ve 399 nükleotide sahip olduğu belirlenmiştir. İki genin nükleik asit düzeyinde birbirleriyle %95 benzerliğe sahip oldukları gösterilmiştir. Dizi analizi sonuçunda bu farkın *T.durum'* un intron bölgesinde bulunan 2 ekstra TTTTTA tekrarından kaynaklandığını belirlenmiştir.

mt-genlerini kodlayan cDNA'ler RT-PCR yöntemi kullanılarak belirlenmiştir. Gen dizi algorithmaları hem *mt-a* hem de *mt-d* cDNA'larının açık okuma bölgelerinin 75 amino asiti amino ve karboksi- uçlarının Cys-X-Cys motifine sahip olacak şekilde kodladıklarını göstermektedir. *mt-d* ve *mt-a* genlerinden elde edilen amino acid dizileri Sınıf II Tip 1 grubunda yer alan diğer MT proteinleri ile dikkat çekici bir benzerlik göstermektedir. Ayrıca bu iki gen protein düzeyinde birbirlerine 100% benzerlik göstermektedirler. Bu sonuçlarında belirttiği gibi *T.durum*'dan elde edilen *mt-d* geni "yeni bir tip 1" MTdir.

Durum MT protein (dMT)'in ekspresyonu, lokalizasyonu ve diğer proteinler ile olan etkileşmlerin araştırılması için yapılacak ileriki çalışmalar için *mt-d* geni pGFPuv vectörünün 5' coklu klonlama bölgesine eklenmiştir. Klonlamanın doğrulanması enzim kesim analizleri ve dizi analizi sonuçlarına bakılarak yapılmıştır. Ancak, ne doğrudan GFP ekspresyonu ile ne de SDS-PAGE analizi ile *mt-d* genin ekpresyonu gözlenememiştir. Detaylı bir dizi analizi bu probleme GFPuv kodlama dizisi üzerinde oluşan bir nokta mutasyonunun sebep olduğunu göstermiştir. Nokta mutasyonu erken

bir sonlanma kodonu oluşmasına, bu da füzyon proteininin erken sonlanmasına sebep olmuştur.

Burada sunulan sonuçlar ile *T.durum* da metallothionein genin varlığı gösterilmiştir.GFP kullanarak meydana getirilen füzyon proteinin ekspresyon çalışmalarında başarısız olunsa da bu amaçın takip edilmesi ve ekspresiyonda başarı sağlanması metallothioneinin bitki sistemlerindeki gerçek rolüne ışık tutması açısından çok önemlidir.

.

To my family

ACKNOWLEDGEMENTS

Three years long journey is coming to the end. A journey is easier if you are not alone. During this thesis I have been supported and encouraged by many people and now I would like to express my gratitude to all those who gave me this opportunity to complete this master thesis.

I am deeply indepted to my supervisor, Assoc.Prof. Zehra Sayers, for her guidance, support, stimulating advices, encouragement on every part of this thesis. I am grateful to her for giving me responsibility and chance to give my own decision during my thesis work. I am really glad that I have to get know Zehra Sayers and I hope this relationship continue throughout my life.

I would like to send my special thanks to Prof. İsmail Çakmak who is also great supporter during this thesis work. Additionally, he is a great mentor and gave me the opportunity to go and work in Germany. This experience was very valuable for me to work and meet with scientists coming from different countries.

I would like to express my special thanks to Kıvanç Bilecen, who is my lab parter. It is a great pleasure for me to work and making scientific discussions with him. I also want to thank to my collegues who are working at Sayers Lab: Süphan Bakkal, Mert Şahin, Çağdaş Seçkin, Erinç Şahin and undergraduates Tolga Sütlü and Doğanay Duru for their support and friendship. Their friendship is very important to me and I wish them best in life.

Especially, I would like to give my special thanks to Süphan Bakkal because of her enthusiasm on me not to get lost during the development of this thesis. She always was available when I needed her help and advices. It was a great pleasure for me to meet with her. I hope she can reach all her goals in life one by one and become an excellent scientist.

I am very grateful to my boss at TUBİTAK Research Institute for Genetic Engineering and Biotechnology, Yavuz Darendelioğlu for making life easier for me. I learned a lot from him. Besides being a good working partner, we were as close as a relative and a good friend. I am very glad to know him.

I want to thank to my friends; Koray Balcıoğlu and Kamuran Türkoğlu for all their help, support, interest and valuable hints. Their friendship is very valuable for me. I wish them all the best in their life. I believe time only strengthen our friendships.

This special thank goes from my heart to my family for trusting, respecting, believing, supporting and encouraging me in all period of my life.

TABLE OF CONTENTS

ABBREVIATIONS

ABA: Absisic acid

AtPCS: *Arabidopsis thaliana* phytochelatin synthase;

ATP: Adenosine triphosphate

cDNA: Complementary DNA

Diethyl pyrocarbonate :(DEPC)

EST: Expressed sequence tags

EXAFS: Extended X-ray absorption fine structure

GS: Glutathione synthetase

GSH: Glutathione

GUS: Glucuronidase

IME: intron-mediated enhancement

IPTG: Isopropyl β-D-Thiogalactopyranoside

MT: Metallotheionein

NMR: Nuclear magnetic resonance

NOS: nopaline synthase

nt : nucleotide

PC: Phytochelatin

PCR:Polymerase chain reaction

RE: Restriction Enzyme

ROS: Reactive oxygen species

RT: Reverse transcription

TaPCS: *Triticum aestivum* phytochelatin synthease

TIGR: The institute for genomic research

LIST OF FIGURES

LIST OF TABLES

1 INTRODUCTION

Metallothioneins (MTs) have been the subject of studies from different disciplines such as biochemistry, molecular biology, biophysics and structural biology over the past 40 years. Complementary information from different fields was required to gain insight into their unusual metal binding capacity, high cysteine content, physiological functions such as transport of essential metals e.g. Group IB and IIB metals (zinc and copper) and detoxification of the nonessential ones. (Stillman *et al*., 1992; Romero-Isart N., and Vasak. M., 2002)

MTs belong to a superfamily of ubiquitously observed low molecular mass proteins (6-7 kDa) with high cysteine content (25-30 % of the residues), having two cysteine thiolate based metal clusters formed by the coordination of d^{10} metal ions.

There is still discussion on the nomenclature of MTs. According to Fowler *et al*, 1987 MTs should be classified into 3 groups: Class I consisting of all proteins with Cys distribution closely related to mammalian forms, Class II comprising proteins in which cysteines are distantly related to mammalian MTs (Mejare and Bülow, 2001). Class III encompasses enzymatically synthesized polypeptides such as poly(γ-glutamylcysteinyl) glycines known as phytochelatins.

The three-dimensional structures of mammalian MTs were determined in from rabbit (Arseniev *et al*., 1988), rat (Schultze *et al*., 1988), and human (Mesosere *et al*., 1990) liver by using X-ray diffraction and aqueous solution by multidimensional/multinuclear NMR spectroscopy. However, there is no available plant MT structure in the structural databases . This thesis study is organized as to give general information about the MT proteins in plant systems in the context of structural

and functional studies. The characterization of different types of plant mt-genes from different plant species, determination of the possible functions with physiological, molecular and biochemical studies are the main topic of the overview.

The result of different strategies aiming to determine the expression profiles of plant MTs are presented according to the articles in the literature.

2 OVERVIEW

2.1 Metallothioneins

Metallothioneins (MTs) constitute a superfamily of ubiquitously observed cysteine (up to 30% residues) and metal rich polypeptides with low molecular weight (6-7 kDa).

MTs are characterized by their cysteine thiolate metal cluster generally formed by the binding of Group IB and IIB metals such as Zn^{+2}, Cu^{+}, Cd^{+2} and Hg^{+2} (Ref) and have been isolated from a wide range of organisms including eukaryotic and prokaryotic microorganisms, mammals and higher plants.

MT was identified by Margoshes and Vallee in 1957 from equine kidney cortex, and although, MTs have been subject of intensive research for over 40 years, their primary physiological role of MTs has not been identified yet. They are traditionally thought to play an important role in modulation of cellular metal metabolism, i.e, in the storage, uptake, regulation of the intracellular concentration of biologically essential metal ions such as copper and zinc and also in heavy metal detoxification (cadmium and mercury). MTs also transport metal ions to other proteins for example to zinc finger proteins, which are important DNA binding regulatory proteins. Moreover, recent studies suggest that MTs are also involved in protecting the cells against deleterious effect of reactive oxygen species (ROS), in adaptation to stress, in anti-apoptotic

processes and regulation of neuronal outgrowth (Palmiter RD; 1998) and downregulation of Alzheimer disease. (Kagi *et al*., 1993; Stillman *et al*, 1992; Sato *et al*., 1993; Romero-Isart *et al*., 2002)

2.2 Nomenclature of Metallothioneins

The first nomenclature system for MTs was adopted in 1979 by Nordberg & Kojima and with identification of more and more proteins which fit into definition of MTs, 1985 an international nomenclature committee was established. It was recommended that MTs should be classified into 3 groups (Kojima, Y., 1997). Class I including all MTs with locations of Cys closely related to mammalian forms, and Class II comprising polypeptides in which cysteines are distributed differently compared to mammalian MTs (Mejare and Bülow, 2001). Enzymatically synthesized polypeptides such as poly(γ-glutamylcysteinyl) glycines known as phytochelatins and cadystins were designated as Class III (Klaasen, 1999) .

Consequent addition to databanks of new amino acid sequences of MTs from different species forced the development of a new classification system based on sequence similarities and phylogenetic relationships. This information is deposited in the Swiss-Prot Databases under the URL of http://www.expassy.ch/cgi-bin/lists?metallo.txt

2.3 Primary Structure of MTs

Class I MTs are in general composed of 60-68 amino acids, of which 20 are cysteine residues, with complete correspondence. Therefore, two isoforms differ in amino acid composition in residues other than the 20 cysteins that are shared and in their charge properties. MT sequences contain many Cys-Xaa-Cys (where Xaa is a noncysteine residue) motifs and also a number of Cys-Xaa-Xaa-Cys motifs. Positions of the cysteinyl residues along the polypeptide chain are highly conserved in evolutionary terms. Another important characteristic of Class I MTs is the complete lack of aromatic amino acids including tyrosine, tryptophan and phenylalanine (Poutney *et al.*, 1995; Stillman, M., 1995; Vasak , 1998).

The metallated proteins have been exhaustively characterized through structural and functional studies and metal binding features have been reported for both *in vivo* and *in vitro* systems. Class I MTs consist of two distinct structural domains that coordinate seven divalent metal ions and twelve monovalent metal ions where clusters encompassing 3 and 4 metals are shown in Figure 2.1 (Chan *et al*., 2002).

All Class I MTs examined contain two or more distinct MT isoforms designated as MT-1 through MT-4 as shown in Table 2.1. In mammals MT-1 and MT-2 genes are actively expressed at all stages of development in many cell types, in different organs and tissues, and also in most cultured cells whereas MT-3 and MT-4 show cell-type specific expression pattern (Andrews, GK., 2000) e.g. MT-3 is expressed predominantly in the brain but also in glia and male reproductive organ (Palmiter RD., 1998) and MT-4 is found in certain stratified tissue such as squamous epithelia (Pountney *et al*., 1995)

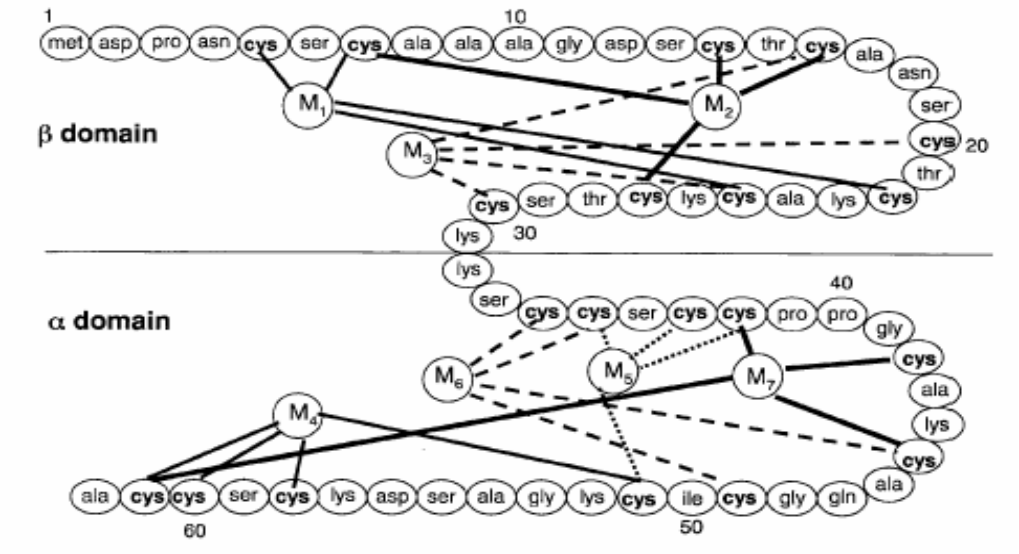


Figure 2.1 Amino acid sequence of rabbit liver MT-2. Cysteine residues are shown in bold (Chan *et al*., 2002).

Glucocorticoids, cytokines, reactive oxygen species (ROS), inflammatory stress signals and various metal ions induce MT-1 and MT-2 isoforms (Binz PA; Kagi HR ,1997). On the other hand, MT-3 and MT-4 are relatively less responsive to these inducers. The primary structure of brain MT-3 contains 68 amino acids with a novel primary sequence motif. Cys (6)–Pro-Cys (8)-Pro which is absent in all other MT families (Table 2.1). Additionally, MT-3 shows two insertions: in N-terminal at position 5 it has a Thr and in C-terminal at position 53 an acidic hexapeptide. Conserved proline residues and presence of these two novel residues has been shown to be decisive for the activity of MT-3 (Pountney *et al*., 1994). MT-3 exhibits inhibitory activity in neuronal assays however, the three dimensional structure and mechanism of the biological activity are currently unknown. In the primary structure of MT-4, there is a Glu insertion at position 5. When sequence similarities are compared, MT-1/ MT-2 isoforms show 70 % identity with MT-3 whereas in with MT-4 most of the non-cysteine residues are different (Quaife CJ. *et al.,* 1994).

The metal composition of purified MT isoforms are highly variable. Normally, the metal content differs according to the natural source and previous exposure to metals indicating that MTs isolated from different organisms and tissues may contain different metals. For example, inducible MT-1 and MT-2 from adult and fetal human livers contain mainly zinc but MTs isolated from adult human kidney contain mainly cadmium but also to some zinc and copper. It must also be noted that MTs are still the only known biological compounds that accumulate cadmium naturally. Both MT-3 and MT-4 isoforms contain zinc and copper. Probably the most interesting example for metal binding is seen in the terrestrial snail *Helix pomatia* (Stillman *et al., 1992)*. These snails have ability to leave at high concentrations of cadmium. They can also accumulate high concentrations of copper which is required for the biosynthesis of the oxygen carrier haemocyanin. Specific metal accumulation is observed in different tissues. Cadmium accumulates in the midgut gland whereas copper accumulates in foot by different tissue specific MT forms, both containing 18 Cys residues but differing in other amino acids. This case points out that MTs that binding to specifically to different metal, may also have different functions.

Mouse MT-1	MDPN·CSCSTGGSCTCTSSCACKNCKCTSCKKSCCSCCPVGCSKCAQGCVCKG·····AADKCTCCA	61
Rat MT-2	MDPN·CSCATDGSCSCAGSCKCKQCKCTSCKKSCCSCCPVGCAKCSQGCICKE·····ASDKCSCCA	61
Human MT-3	MDPETCPCPSGGSCTCADSCKCEGCKCTSCKKSCCSCCPAECEKCAKDCVCKGGEAAEAEAEKCSCCQ	68
Human MT-4	MDPRECVCMSGGICMCGDNCKCTTCNCKTCRKSCCPCCPPGCAKCARGCICKG·····GSDKCSCCP	62

Sea Urchin MTA	MPDVKCVCCKEGKECACFGQDCCKTGECCKDGTCCGICTNAACKCANGCKCGSGCSCTEGNCAC	64
Blue Crab MT-1	MPGPCCNDKCVCQEGGCKAGCQCTSCRCSPCQKCTSGCKCATKEECSKTCTKPCSCCPK	59
S.Cerevisae	QNEGHECQCQCGSCKNNEQCQKSCSCPTGCNSDDKCPCGNKSEETKKSCCSGK	53
N. Crassa	GDCGCSGASSCNCGSGCSCSNCGSK	25

Table 2.1 Representative amino acid sequences of mammalian and structurally characterized invertebrate MT forms (Romero-Isart *et al*., 2002)

2.4 Three Dimensional Structure

The metal-free protein also known as thionein or apoprotein, seems to have predominantly a disordered structure. This is indicated by experiments showing the rapid exchange rate of the amide protons in the apoprotein. When metal ions bind to apoMT, however, a well defined protein fold develops (Romero-Isart *et al.,* 2002).

The three-dimensional structures of mammalian MTs were determined in aqueous solution by multidimensional/multinuclear NMR spectroscopy using ^{113}Cd-reconstituted Cd_7-MT from rabbit (Arseniev *et al*., 1988), rat (Schultze *et al*., 1988), and human (Mesosere *et al*., 1990) liver. The first reported MT structure was native Cd_5,Zn_2-MT-2 (PDB ID : 4MT2) from cadmium over loaded rat liver by X-ray diffraction method (Vasak M, 1998).

Both studies, NMR in solution and X-ray diffraction in crystal, revealed an identical metal thiolate cluster structure and closely comparable polypeptide folds, displaying a monomeric dumbbell-like shape with 7 metal ions located in two separate metal thiolate clusters: (Figure 2.2)

1) Distorted chair (3-metal cluster) $M_3^{II}Cys_9$, located in N-terminal β domain (residues 1-30)
2) Adamantane-like (4-metal cluster) $M_4^{II}Cys_{11,}$ in the C-terminal α domain (residues 31-61)

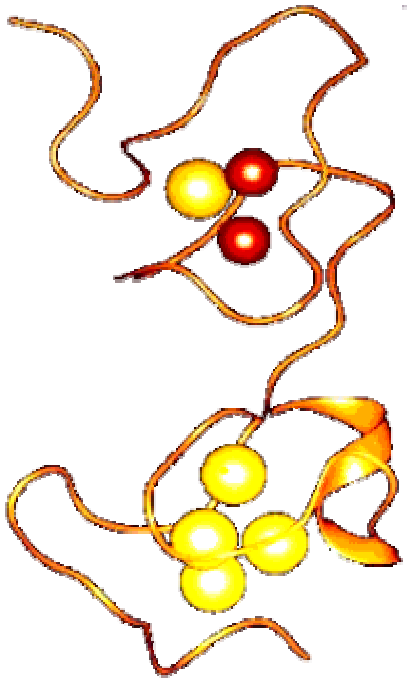


Figure 2.2 The Model of Cd5,Zn2-MT-2 (PDB ID: 4MT2) structure calculated from X-ray crystallography data

Structural information about Cu_4,Zn_3-MT-3 is available. Zn and Cu K-edge extended X-ray absorption fine structure (EXAFS) revealed the presence of two distinct homometallic metal-thiolate cluster containing three Zn(II) and four Cu (I) ions with tedrahedral and trigonal coordination geometry, Comparative studies with the chemically synthesized N- and C-terminal domains of human MT-3 suggest exceptional characteristic of this protein. According to these studies, Cu_4-cluster seemingly located in the N-terminal domain. This means α-domain contains only three Zn(II) ions.

Three dimensional structure of *Saccharomyces cerevisiae* MT is the only reported Cu(I) containing protein with 53 amino acids. NMR solution structure of both native copper containing form and ^{109}Ag(I) derivative displaying 10 out of the 12 available cysteines bind seven Cu(I) or Ag(I) ions in a single cluster.

Fungal MT from *Neurospora crassa*, which consists of only 25 amino acids (Table 2.1), has less available detailed structural information. Limited NMR data conclude that fungal MT has a single protein domain with a Cu^{I}_{6}-thiolate cluster.

2.5 Biochemical Properties

2.5.1 Metal binding properties

Metal-free, apo-protein is colorless despite the lack of chromophoroses that absorbs light to the red there is no significant absorption band at 215 nm. The lack of aromatic amino acids in the polypeptide chain, refer that the lowest energy transition observed in the absorption spectrum arise from transitions located on the peptide chain (Stillman M., 1995).

Apo-MT binds varying number of metal between pH 1 and pH 12. MTs contain both terminal and bridging thiolate groups and as metals bind to the thiolate groups absorption spectrum between 230 and 400 nm changes (Stillmann *et al*., 1992). Metallothioneins are unique between metalloproteins since they can bind several other metals *in vivo and in vitro* such as Zn(II), Cd(II) and Cu(I), Ag(I), Au(I), Bi (III), Co(II), Fe(II), Hg(II), Ni(II), Pt(II), Tc(IV)O (Chan *et al*.,2002). The affinity of metal ions for the binding sites is ordered as follows; Hg(II) > Ag(I)~Cu (I) > Cd(II) > Zn (II) (Stillman M., 1995). Spectroscopic and volumetric measurements have been used in order to determine the average apparent stability constant for Zn (II) and Cd (II) also for Zn-MT complexes. These data are derived from ^{19}F NMR measurements where the

competition with a fluorinated complexing agent 5F-BAPTA (1,2-bis(2-amino-5-fluorophenoxy)ethane-N-N-N$^{'}$-N$^{'}$-tetraacetic acid) is used.

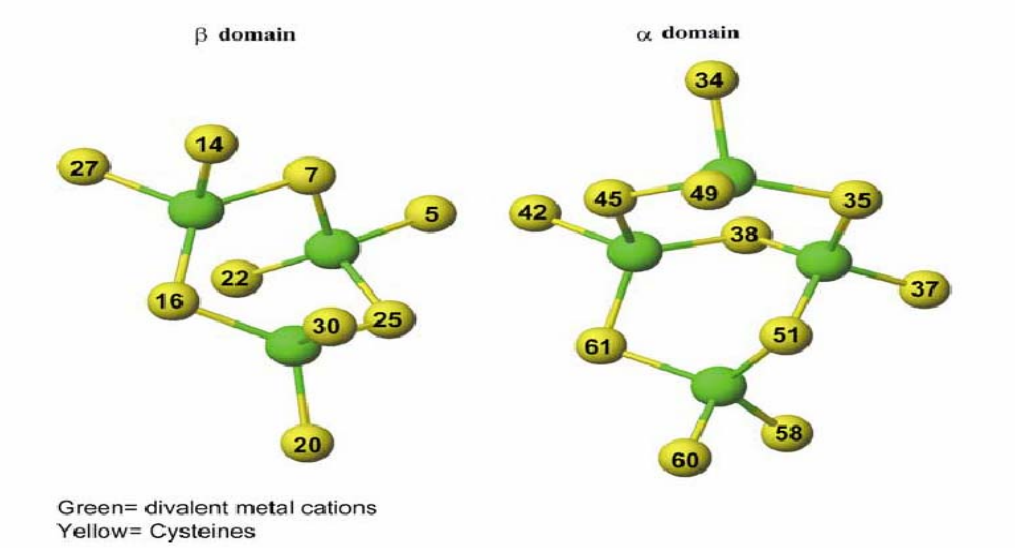


Figure 2.3 Metal-thiolate cores for Cd(II) and Zn(II) in β and α domains of mammalian based on the connectivities from NMR and X-ray (Chan *et al*.,2002)

Metallothioneins are kinetically very labile i.e. the thiolate ligands undergo both methylation and demethylation rapidly by following the order Hg(II) > Cd(II) > Zn(II)

2.6 Plant Metal Binding Proteins

The first direct evidence of the existence of MTs in plants was supplied by Lane *et al*. in 1987, 30 years after the first MT was detected in equine kidney, by isolating Ec protein and genes from wheat (Kawashima *et al*., 1992). Over 60 sequences of plant MT genes are now available in public databases. All types demonstrate three distinct domain: N-terminal and C-terminal domains; each containing 15-20 amino acids with Cys-rich MT-like pattern. A central domain; also called linker domain, consisting of 30-50 amino acids lacking cysteine residues. This is very different from the mammalian MTs in which the linker region is formed by 2 Lys residues (Charbonnel-Campaa *et al*., 2000).

According to the traditional classification based on the arrangement of Cys residues plant MTs had been grouped as Class II. MT-like proteins led to further subdivisions (Robinson *et al*., 1993) and with increasing number of reports on MT and MT-like genes other dicotyledonous and monocotyledonous species, which do not conform to these two types, alternative classification systems have been proposed (Binz PA and Kagi JHR, 2001; Murphy *et al*., 1995; Rauser, WE.,1999). The classification presented below is given by Cobbet and Goldsbrough (2002) and it enables the classification of all known plant MT genes into four types based on amino acid sequences as shown in Table 2.2.

Type 1 MTs posses a cysteine pattern exclusively in the form of Cys-Xaa-Cys. They contain 6 of them that are equally distributed in both N- and C-terminal. In the linker region separating the two domains there are approximately 40 amino acid. Interestingly, these linker regions contain aromatic amino acids. Within Type 1 MTs, the three belonging to family *Brassicaceae* show variation and stand out as a subtype because of a much shorter (only 7 amino acids) spacer region linking the two domains. Moreover, an additional Cys residue is predicted in domain 2. The last member of this type was isolated and characterized from heavy-metal tolerant plant *Festuca rubra cv.* Merlin (Ma *et al*., 2003).

E_c MT	2x**C**xxx**C**x**C**xxx**C**xxxx(x)**C**x**C**-12,14-**C**x**C**xxx**C**x**C**xx**C**x**C**-15-**C**x**C**xxx**C**x**C**xx**C**5x
Type 1	2-4x**C**x**C**xxx**C**x**C**xxx**C**x**C**--39 to 45--**C**x**C**xxx**C**x**C**xx**C**x**C**0-2x
	Subtype Brassicaceae
	5x**C**x**C**xxx**C**x**C**xxx**C**x**C**------7------**C**xx**C**x**C**xxx**C**x**C**xx(x)**C**x**C**
Type 2	xx**CC**xxx**C**x**C**xxx**C**x**C**xx(x)**C**xx**C**--37 to 49--**C**x**C**xxx**C**x**C**xx**C**x**C**0-2x
Type 3	xx**CC**xxx**C**x**C**xxx**C**x**C**xxx**C**xx**C**--37 to 40--**C**x**C**xx**C**x**C**xxx**C**x(x)**C**x**CC**x**C**x
Type 4	2-4x**C**xx**C**x**C**xxxxx**C**--32 to 34--**C**x**C**xxx**C**x**C**xx**C**x**C**0-2x

Table 2.2 Predicted cysteine motifs of Class II MTs from plants. The cysteines are arranged in two or three domains starting from amino-terminus at the left. X denotes any amino acids other than cysteine. (x) indicates deletion. The numbers between domains refer the amino acid residues linking the two domains (Rauser W., 2000)

Type 2 MTs have different number and motif of Cys in two domains. Eight cysteines are arranged in Cys-Cys, Cys-Xaa-Xaa-Cys and Cys-Xaa-Cys form in the N-terminal domain and 6 of them as Cys-Xaa-Cys in the C-terminus. Type 2 MTs also contain long spacer regions containing of approximately 40 amino acids but are much more variable between species. The first Cys pairs are present as Cys-Cys motifs in amino acid positions 3 and 4 of these proteins and Cys-Gly-Gly-Cys is found at the end of N-terminal. Moreover, MSCCGGNCGCS sequence is highly conserved in the N-terminal domain (Cobbet *et al*., 2002).

Type 3 MTs have truncated N-terminal domain 1 with only 4 Cys residues. First three cysteines are arranged in a Cys-Gly-Asn-Cys-Asp-Cys consensus sequence and the last Cys in the N-terminal domain is found within a highly conserved motif, Gln-Cys-Xaa-Lys-Lys-Gly. The C-terminal shows a great similarity with Type 1 and Type 2 MTs with 6 Cys amino acid residues and Cys-Xaa-Cys motif. In addition, two domains are separated from each other by nearly 40 amino acid residue long linker region. Recently, two new divergent Type 3 MTs from oil palm, *Elaesis guineensis* were added to the list (Abdullah *et al*., 2002).

Three cysteine rich domains distinguish Type 4 MTs from other plant MTs. Each domain posses 5 or 6 Cys residues and are separated by 10 to 15 residues. Despite the small number of known sequences that fit in this group differences between dicots and monocots are observed. Type 4 MTs in dicot plants contain extra 8 to 10 amino acid residues in N-terminal domain before the first Cys residue.

It has been suggested that the differences in the arrangement of Cys residues could modify metal specificities.

2.6.1 Early Cys-labelled (Ec) protein

Early Cys-labelled (Ec) protein, encoded by mRNA conserved in mature dry wheat embryos in alkylated form, has been isolated by Hanley-Bowdin L and Lane B

in 1983 and the first 59 amino acids were sequenced in 1984 (Lane B., 1987). Zn-containing Ec protein was the first higher plant protein to be classified as a MT-II (Kawashima *et al*., 1992). The amount of bound Zn in unalkylated Ec protein is approximately 5mol/mol protein (Rauser WB., 1999). Until now, three genes for the Ec protein from wheat and one gene from maize have been isolated. Unlike animal MT genes, which are found as multigene clusters, Ec MT genes are single copy and located on the long arm of the 1A, 1B, and 1D genomes of hexaploid wheat (*Kawashima et al.,* 1992 and White CN., 1995). The sequence of Ec protein is shown in Table 2.1. 17 Cys of the cDNA sequences are arranged into three groups of 6, 6, and 5 Cys with 12-15 amino acids separating the three groups. This property distinguishes Ec MT from the other plant-MT like genes where the Cys are grouped in two domains. These regions contain 7 pairs of Cys-X-Cys and 3 lone Cys motifs. Although, the number of Cys residues and distribution of Cys-X-Cys sequences are fewer in number, the partial amino acid sequence of Ec MT has remarkable similarity with crab MT (Lane B., 1987, Lerch, 1981). However, the predicted arrangement of Cys in full-length sequence is not homologous with MT-Is. In addition to conserved Cys residues, the Ec type proteins contain two conserved histidine residues which could be involved in Zn^{+2} co-ordination.

Ec MT genes contain promoter sequences with homology to ABA (absisic-acid)-responsive element on the 5`-flanking region like other plant genes, but no metal-responsive element TGCRCNCX (in which N is not A and X is G or C) found in animal MTs. The abundance of Ec mRNA increases by addition of ABA to the germination media whereas addition of Zn^{+2} does not show the same effect. Ec mRNA level increases during maturation of embryos and the highest levels of Ec mRNA were detected at the early stages of embryogenesis, shortly after the beginning of the rapid cell division and differentiation and decline during early germination (Kawashima *et al.*, 1992). The proposed homeostatic function of this embryo-specific Ec MT protein is endogenous control of zinc metabolism during embryogenesis. Therefore, Ec MTs control the accumulation and concentration of metals in grains they become very important in nutritional mean.

The majority of the plant MT genes have been identified in angiosperms. Some species such as Arabidopsis, rice, and sugarcane contain genes encoding all four types

of MTs. This points out that evolution of four plant MT types predates the separation of monocots and dicots. Majority of the flowering plants having four different types of MT strengthen this indication however there is little information about non-flowering plant species. There is a contrast between animal and plant MTs in the distinct arrangement of cysteines within four types of MTs. For example, mouse has four different MT types. Although, they show variation in tissue expression, all four mouse MT's contain the same conserved cysteine motif. It is thought that the diversity of the plant MT may differ not only in sequence but also in function (Cobbet *et al*., 2002).

2.6.2 Expression profile of metallothioneins

Numerous studies have been published on the expression on plant MT-like genes. Plant MTs transcripts have been detected in roots, stems, leaves flowers, fruits, and seeds and become evident under different condition. The expression patterns of MT and MT-like plant genes alter as a function of development implies the importance of their proteins in both stress and unstressed cells. Ec mRNA level is high in developing wheat embryos then, the protein level decrease during early germination (Kawashima *et al*., 1993). Type 1 expressed predominantly in the root, Type 2 expressed predominantly in aerial tissue and Type 3 expressed primarily in mature fruit. Expressed Sequence *Taq*s (EST) for MT genes are widespread in randomly sequenced cDNA libraries from a number of plants. For example, Hisada *et al*., have carried out random sequencing of citrus cDNA library derived from small seeds and from fruit at development phase. After comparing the random sequencing results of citrus cDNAs with MT-like genes among growth related genes, they saw that in small seeds MT-like genes appeared six times out of 192 clones (3.1%). In developing fruit 8 times (0,8 %) out of 950 clones and interestingly in the mature fruit frequency is high with 20.9%. Moreover, both type 2 and Type 3 MT-like genes were isolated both from small seeds and developing fruit but only Type 3 MT genes was detected in mature fruit (Moriguchi *et al*., 1998). Type 3 MTs have so far been isolated from fruit tissues in a wide range of plants, including kiwifruit (Ledger SE,1994), banana, apple (Reid and Ross,1997),Oil palm (Abdullah *et al*., 2002). The only exception being *Arabidopsis thaliana,* where it was isolated from seedlings (Murphy *et al*., 1997). In addition Type 2 MT gene constituted 0.4% of the

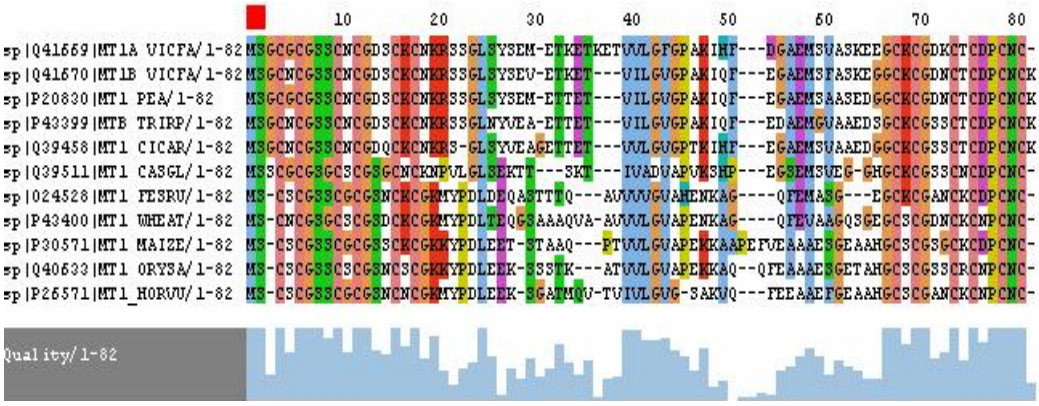
tomato ESTs, and 0.5% of maize ESTs were derived from Type 1 MT gene (TIGR Gene Indices, 2001, Cobbet *et al*., 2002). The differential screening of plant cDNA libraries used in order to determine MT genes, also indicate abundance of MTs in many other plant species.

Expression of plant MTs is induced by different stimuli such as metal exposure, hormone treatments such as ethylene (Coupe *et al*.,1995), cold (Reid and Ross,1997), osmotic stress, heat stress, sucrose starvation (Hsieh HM *et al*.,1995), viral infection (Choi *et al*.,1996), wounding (Choi *et al*.,1996), leaf senescence (Buchanan–Wollaston, 1994 and Clemens S, 2001) in different species. These are listed by Rauser and shown in Table 2.3. In leaf senescence there is a dramatic increase in MT mRNA levels. MT RNA expression in senescing leaves appears to be localized primarily to phloem tissue.

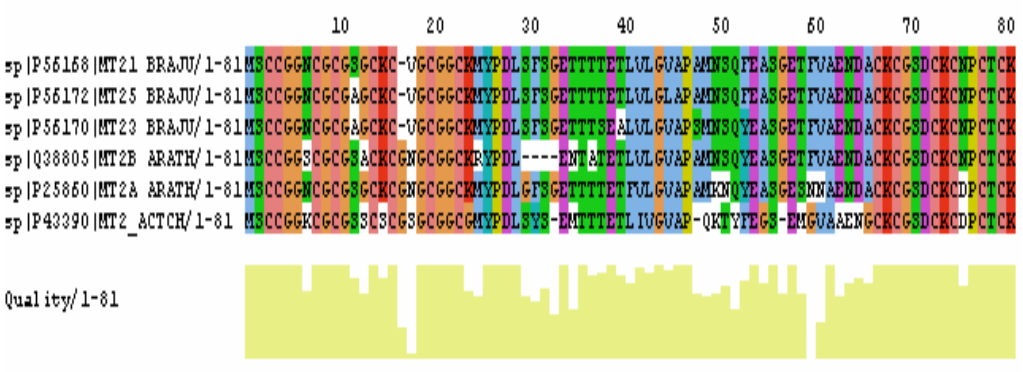
Plant Species	**Factor**
Rice (Hsieh *et al*., 1995)	Heat stress and sucrose starvation
Brassica napus (Buchanan-Wollaston V., 1994)	Leaf senescence
A. thaliana (Garcia-Hernandez *et al*., 1998)	Leaf senescence
Rice (Hsieh *et al*., 1995)	Leaf senescence
*Nicotinana glutinous (*Choi *et al., 1996)*	Wounding and viral infection
Zea mays L (Framaund et al.,1991)	Glucose starvation
Kiwi fruit (Actinidia deliciosa var deliciosa) (Ledger SE, 1994)	Fruit development
Norway spruce (Buschmann *et al*. 1998)	Ozone treatment
Raspberry fruit (Rubus idaeus cv Glen clova) (Jones *et al*. 1998)	Fruit ripening
Oil Palm *Elaesis guineensis* (Abdullah *et al*., 2002)	Fruit ripening

Table 2.3 Expression of plant MTs induced by different factors in different species

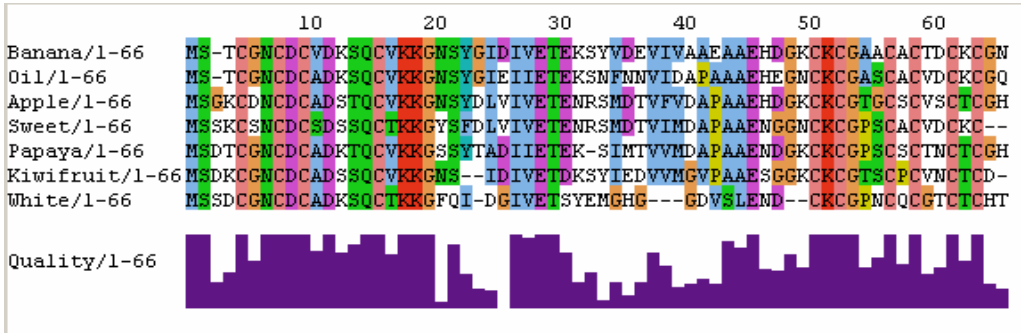
Type 1 MT



Type2MT



Type 3 MT



Type 4 MT

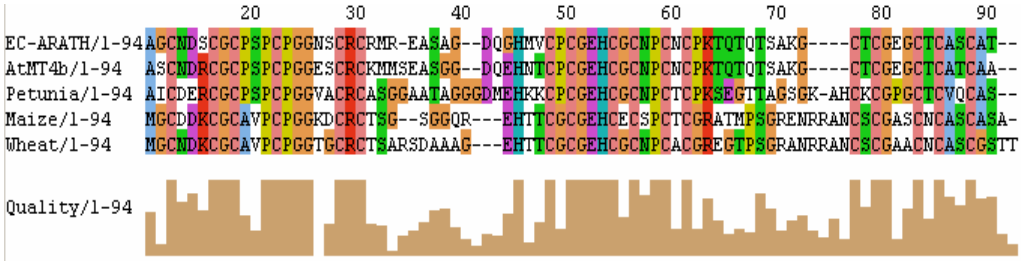


Figure 2.4 Alignment of plant MT types amino acid sequences. Cysteine residues are in pink and conserved sequences are colored.

Table 2
MT-II Genes in Plants: Predicted Number of Cys Motifs, Distribution, and Regulation

Plant	Gene	Cys motif			Residues between domains	Transcript abundance				Regulation	Ref.
		CxC	CxxC	Other		Root	Leaf	Seed	Other		
Type 1											
Mimulus		6	0	–	39	High	Low	–	–	Down by Cu, Zn, Cd	(53)
Pea	$PsMT_A$	6	0	–	42	High	Low	–	–	–	(54)
Barley	*ids-1*	6	0	–	43	High	–	–	–	Up with Fe deficiency	(55)
		Sequence corroborated									(56)
Maize		6	0	–	45	High	Low	Low	Pith	–	(57)
		Sequence corroborated				Yes	–	–	–	Up with root tip excision, Up with glucose starvation	
Wheat	*wali1*	6	0	–	44	High	High	–	–	Up by Al in roots, not by Cd, constitutively high in leaves	(58)
										–	(59)
White clover		6	0	–	42	–	–	–	–	–	(60)
Arabidopsis	*MT1/MT1a*	6	0	1 lone C	7	High	Low	–	–	Up by Cu > Zn, Cd	(61,62)
		Sequence corroborated as *AtMT-q*									(63)
	MT1c	6	0	1 lone C	7	Yes	Yes	–	Siliques	–	(62)
Brassica napus		6	0	1 lone C	7	None	High	–	Flower	Up with leaf senescence	(64)
Rice	*OsMT-1*	6	0	–	43	High	Some	–	Cultured cells	Up by Cu, heat stress, succose starvation, leaf senescence, down with ABA	(65)
											(66)
		Sequence corroborated									
Cotton	*MT1-A*	6	0	–	42	High	Low/nil	–	–	Evidence for two further *MT1* genes	(67)
Fava bean	*MT1a*	6	0	–	45	High	High	–	Stem	Not by Cu, Cd, Zn, Fe	(68)
	MT1b	6	0	–	43	–	–	–	–	–	(68)
Red fescue		6	0	–	39	–	–	–	–	–	(69)
Type 2											
Soybean		5	1	1 CC	41	Low	High	–	–	Down by Cu	(70)
Arabidopsis		5	1	1 CC	43	–	–	–	–	–	(71)
	MT2/MT2a	Sequence corroborated				Low	High	–	–	Up by Cu > Zn, Cd	(61,62)
		Sequence corroborated as *AtMT-k*									(63)
	MT2b	5	1	1 CC	39	Yes	Yes	–	Siliques	Slightly up by Cu	(62)
Castor bean		5	1	1 CC	42	–	–	–	–	–	(72)
Fava bean		5	1	1 CC	39	Least	High	–	Flower Trichomes	Down by cold, salt, salicylic acid not by Cu, Zn, Cd	
Kiwi fruit	*pKIWI504*	5	1	1 CC	40	None	–	–	Fruit	Up early in fruit development	(73) (74)

Table 2.4 Number of Cys motif, transciption abundance and regulation of plant MT genes (Rauser, 1999)

Coffee		5	1	1 CC	42	–	From young leaves	–	–	–	(75)
Chinese cabbage		5	1	1 CC	42	–	From the inflorescence	–	–	–	(76)
Sambucus		5	1	1 CC	39	–	Yes	Abscission zone	–	Up with ethylene, leaflet senescence	(72)
Tobacco		5	1	1 CC	37	–	–	–	–	Modulated by cytokinin	(78)
		5	1	1 CC	42	–	Yes	–	–	Up with Cu, virus, wounding	(79)
White clover		5	1	1 CC	39	–	–	–	–	–	(80)
Brassica campestris		5	1	1 CC	42	–	–	–	Shoot apex	Up with vernalization	(81)
Rice	*OsMT-2*	5	1	1 CC	43	Low	High		Cultured cells	Up by heat shock, sucrose starvation	
										Down by Cu, Cd	(82)
Tomato (three sequences)		5	1	1 CC	35,36,43	Yes	–	–	–	–	(83)
	LeMT$_A$	5	1	1 CC	35	Low	High	–	–	–	(84)
	LeMT$_B$	5	1	1 CC	43	Low	High	–	–	–	(84)
Brassica napus		5	1	1 CC	19	–	Yes	–	–	Up with senescence	(85)
Brassica juncea (5 sequences)		5	1	1 CC	42,43	Yes	–	–	–	–	(86)
Apricot		5	1	1 CC	40	–	–	–	Fruit	–	(87)
Common ice plant		5	1	1 CC	42	–	–	–	–	Confers Cu tolerance	(88)
Type 3											
Rice		4	2	2 CC 1 lone C	40	–	–	–	–	–	(89)
		5	1	2 CC 1 lone C	37	–	–	–	Stem	–	(90)
		5	1	2 CC 1 lone C	37	–	–	–	–	–	(91)
Type 4											
Kiwi fruit	clone 503	4	0	2 lone C	32	None	–	–	Fruit	Up late in fruit development	(74)
Apple		4	0	2 lone C	34	–	–	–	–	Up with cold storage	(92)
Papaya		4	0	2 lone C	33	–	–	–	–	–	(93)
Banana	clone 3-6	4	0	2 lone C	34	No	Yes	–	Fruit	Up with fruit ripening	(94)
Rice (two sequences)		4	0	2 lone C	30,33	–	–	–	–	–	(95)
Sweet cherry		4	0	2 lone C	34	–	–	–	Fruit	–	(95a)
Others											
Arabidopsis	*MT1b*	4	0	2 lone C	7	–	–	–	–	Inactive gene	(62)
Arabidopsis	*MT3*	4	0	4 lone C	34	–	–	–	Seedlings	Up by Cu	(96)
Tomato		5	–	3 lone C	40	Yes	–	–	–	–	(97)
Brassica campestris		4	1	1 CC 1 lone C	42	–	–	–	Shoot apex	Up with vernalization	(81)
Douglas fir	*PM 2.1*	1	0	3 lone C	38	–	–	Low	–	Maximal at midembryogenesis	
										Up by osmoticum, ABA, Zn	(98)
Strawberry	*FMET1*	5	1	1 CC 1 lone C	40	–	–	–	Fruit	–	(99)
Banana	clone 3–23	5	1	2 lone C	41	No	Low	–	Fruit, corm	Down with fruit ripening	(94)

Number and type of cysteine motifs are shown with x denoting any amino acid other than Cys. The Cys-rich domains 1 and 2 are separated by a Cys-deficient region varying in the number of amino acid residues. The listing for a type of Cys motif is chronological.

Table 2.5 Number of Cys motif, transciption abundance and regulation of plant MT genes (continues) (Rauser, 1999)

2.6.3 Phytochelatins

Plants respond to heavy metal stress by developing a number of defense mechanisms. One such mechanism includes the chelation of heavy metals by a family of peptite ligands. MT III's, also referred as phytochelatins, are enzymatically synthesized Cys-rich polypeptides that are ubiquitously present in the cytoplasm of plant cells and possibly in animals (Grill *et al*., 1989; Clemens *et al*., 1999; Ha *et al*., 1999; Vatamaniuk *et al*., 1999). Phytochelatins (PCs) have been extensively studied regarding to their metal binding properties, biosynthesis, ATP-dependent transport of PC-metal complex, detoxification of heavy metals (Cobbett, 2000; Goldsbrough, 2000)

2.6.3.1 Types of phytochelatins

Five families of phytochelatin (PCs) are known. They are classified according to varying carboxy-terminal amino acid such as glycine (Gly), β-alanine (Ala), cysteine (Cys), serine (Ser), or glutathione (Glu). The chemical structures are shown in Fig. 2.9

Depending on the organism, medium and the metal supply, the number of (γ-Glu-Cys) repeats varies from two to eleven (Hall JL, 2002). Non defined repeats are marked by subscript n. The first (γ-Glu-Cys) peptide from (γ-Glu-Cys)-Gly family was characterized in fission yeast *Schizosaccharomyces pombe* and they were named as cadystins. The name "phytochelatin" (PCs) was suggested for the same peptides, independently characterized from four different cultured cells of plants. The argument on the trivial name still continuing but phytochelatin has gained popularity to call all MT-III's as PCs. PCs appear in both vascular and nonvascular plants also in mosses and algae. A plant species have different combinations of the various phythocelatins but they are rarer when compared with PC. In MT-III, most studies center on PCs since they occurred in all plants examined following exposure to Cd^{+2} and the principal detoxification mechanism in most plant species leads to PC.

Metal induction tests of PCs were performed on cell suspension cultures of *Rauvolfia serpentina* grown on Zn- and Cu- free medium (Grill, E,1987; Rauser WE.,1999). It was shown that salts of Cd, Pb, Zn, antimony (Sb), Ag, Hg, arsenate, Cu, tin (Sn), Au, and Bi induced the formation of PCs. The first five metals with an order are the best inducers but they were presented at varying concentration, ranging from 50-1000 μM. On the other hand, Al, Ca, Co, Cr, Cs, Mg, and Mn did not activate PCs synthesis.

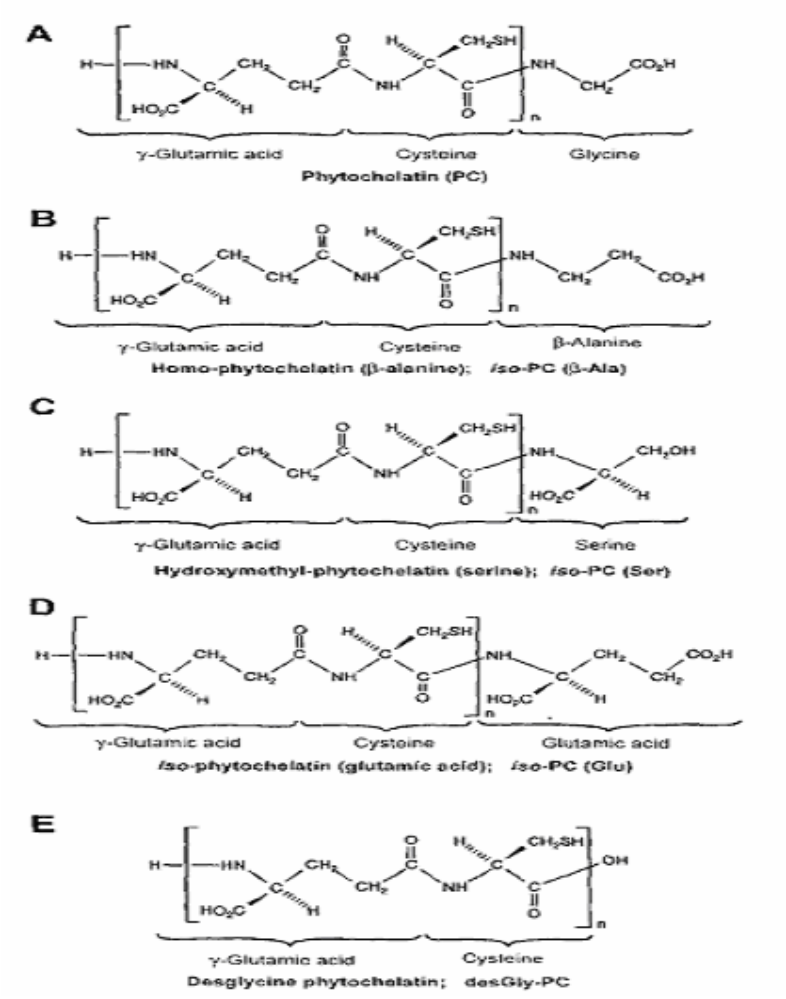


Figure 2.5 Chemical Structures of PC and *iso*-PC molecules

2.6.3.2 Biosynthesis of PCs

The enzyme catalyzing the phytochelatins biosynthesis from glutathione (GSH), named as γ-glutamylcysteine dipeptidyl transpeptidase (EC 2.3.2.15) or phytochelatin synthase, was first identified by Grill et.al in 1989 (Cobbet C, 2000). The enzyme catalyses the transpeptidation of the γ-Glu-Cys moiety of glutathione onto the another glutathione to form $(\gamma\text{-Glu-Cys})_2$-Gly and Gly or onto another $(\gamma\text{-Glu-Cys})_n$-Gly to form n+1 oligomer and Gly.

$$(\gamma\text{-Glu-Cys})\textbf{-Gly} + \textbf{(}\gamma\textbf{-Glu-Cys)}_{\textbf{n}}\textbf{-Gly} \longrightarrow \textbf{Gly} + (\gamma\text{-Glu-Cys})_{n+1}\text{-Gly}$$

where n= 1, 2, 3 …..

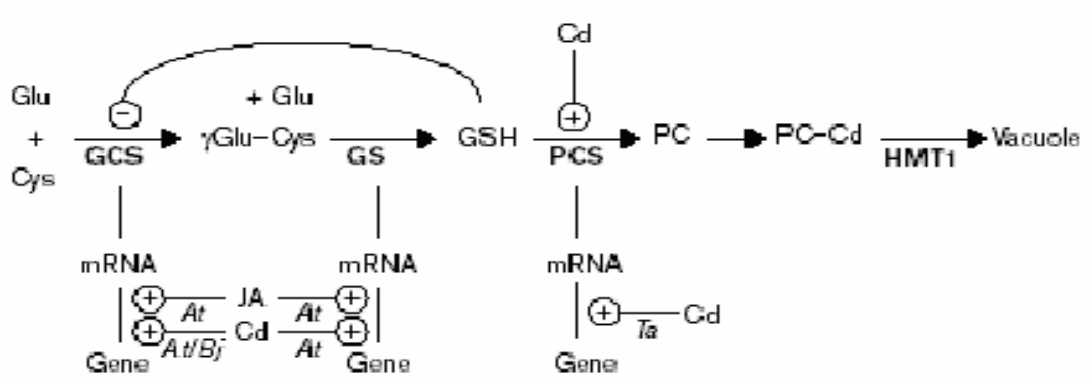


Figure 2.6 Biosynthetic pathway of phytochelatin. Positive and negative regulation of enzyme activity or gene expression indicated by ⊕ and θ, respectively. *A. thaliana (At), B.juncea (Bj), T.aestium (Ta)* indicate where particular regulatory influences have been observed in particular species. HMT1 is a vacuolar membrane transporter of PC-Cd complexes. JA, jasmonic acid; PCS, Phytochelatin synthase (Cobbet C, 2000)

The PC synthase enzyme is self-regulated, since its reaction product, the PC, start being synthesized within a few minutes (nearly no lag phase) after Cd supply, chelate the PC-synthase-activating metal and thus terminate reaction end unless further Cd is supplied (Toppi LS, and Gabbrielli R,1999).

The gene was first identified in cadmium-sensitive, *cad1* mutants of Arabidopsis. Mutants are PC deficient and lack PC synthase activity *in vitro* due to defect in the PC synthase gene but have wild-type levels of GSH. Three PC synthase gene, two from Arabidopsis one from wheat, were cloned independently in three laboratories (Cobbett CS, 2000). The CAD 1 gene of Arabidopsis has been isolated by positional cloning and was found to encode PC synthase. CAD 1 gene is also referred to as AtPCS1. AtPCS1 and TaPCS1 genes have been identified by expression of Arabidopsis and wheat cDNA libraries in *S.cerevisia*. Expression of these genes in yeast *S.cerevisia,* cause an increase in Cd^{+2} tolerance that is GSH dependent and correlates with the synthesis of PCs. Moreover, over-expression of ATPCS1 in *S.cerevisiae* cause an increase in Hg and arsenate tolerance.

A gene, SpPCS1 that is similar to the plant PC-synthase genes was identified in the genome of the fission yeast *S.pombe*. PC synthase deletion mutant of *S.pombe* were also Cd-sensitive and PC-deficient, verifying analogous functions of plant and yeast genes.

Recently, second PC synthase gene, AtPCS2, has been identified in *Arabidopsis* genome with significant homology to CAD1/AtPCS1 This is an unexpected result since after prolonged exposure to Cd, PCs were not detected in *cad1* mutants. This suggests the presence of single active PC synthase. However, low constitutive AtPCS2 expression is detected in all plant organs analyzed. In *S.cerevisiae* and phytochelatin synthase knockout strain of *S.pombe* AtPCS2 gene encodes functional phytochelatin synthase (Cazale *et al*., 2001).

AtPCS1 is a 55 kDa polypeptide of 485 amino acids. The amino acidcomparison between AtPCS1 and SpPCS1 exhibits that they have similar N-terminal regions with 45 % identity, whereas C-terminal region shows no obvious similarity. C-terminal has a characteristic feature by having multiple Cys residues, often as pairs. The C-terminal regions of *Arabidopsis* and *S.pombe* proteins contain 10 and 7 Cys residues, respectively, of which four and six are pairs (Cobbet *et al*., 1999).

3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals

All chemicals and growth mediums were supplied by Merck (Germany), SIGMA (USA), Fluka (Switzerland), and Riedel de Häen (Germany).

3.1.2 Buffers and Solutions

3.1.2.1 Growth culture medium

3.1.2.1.1 Liquid medium

For liquid culture of bacteria Luria-Bertani (LB Broth) from SIGMA was used. LB Broth includes tryptone, yeast extract, and sodium chloride mixed in an appropriate amount. For preparation of 1 L liquid medium 20 g of LB Broth is dissolved in deionized water. To sterilize, liquid medium was autoclaved at 121^0C for 20 min.

3.1.2.1.2 Solid medium

Solid medium for the growth of bacteria was prepared from Luria-Bertani Agar (LB Agar) from SIGMA. LB Agar composed of the tryptone, yeast extract, sodium

chloride, and agar in appropriate amounts. For preparation of 1 L solid medium 40 g of LB Agar is dissolved in correspondent amount deionized water by autoclaving at 121^{0}C for 20 min. Autoclaved medium was poured to petri plates (~20 ml/plate) after cooling. To prepare selective medium, add appropriate amount of antibiotic(s) to the medium before pouring to the petri plates.

3.1.2.2 Buffers for gel electrophoresis

All the buffers for gel electrophoresis were prepared according to the protocols from Sabrook *et al*., 1989.

3.1.2.1.1 Agarose gel electrophoresis

1 X Tris Acetate EDTA (TAE)

1 X Tris Borate EDTA (TBE)

1 X FA Buffer

3.1.2.1.2 Polyacrylamide gel electrophoresis

1 X Tris Glycine SDS

3 M Tris-HCl pH 8.9

1 M Tris-HCl pH 6.8

30 % Acryl – 0.8 % Bisacrylamide

5 X Loading Buffer Blue Staining Solution

Coomassi Blue Destaining Solution

Drying Solution

3.1.2.1.3 Plasmid isolation

TE Buffer

NaOH/SDS

Potassium acetate

3.1.3 Primers

Primers without restriction enzyme sites were designed according to the sequence reported by Kawashima (Kawashima *et al*., 1992) and synthesized by Integrated DNA Technologies, Inc., USA. Primers with restriction enzyme sites were purchased from SEQLAB (Germany) and SIGMA (USA).

3.1.4 Enzymes

3.1.4.1 Restriction enzymes

*Hind*III Restriction enzyme (Promega) – 10u/µl

*Xma*I Restriction enzyme (Promega) –

*Hind*III Restriction enzyme (Fermentas) – 10u/µl

*Sma*I Restriction enzyme (Fermentas) – 10u/µl

*Eco*RI Restriction enzyme (Fermentas) – 12u/µl

*Spe*I Restriction enzyme (Fermentas) – 5 u/µl

*Xho*I Restriction enzyme (Fermentas) – 10u/µ

*Not*I Restriction enzyme (Fermentas) – 10u/µ

3.1.4.2 Ligases

T4 DNA Ligase (Promega) – 3u/µl

T4 DNA Ligase (Fermentas) – 3u/µl

LigaFast™ Rapid Ligation System (Promega) – 3u/µl

3.1.4.3 *Taq* Polymerase

Taq DNA Polymerase in Storage Buffer A (Promega) – 5u/µl

Pfu DNA Polymerase (Promega) – 3u/µl

3.1.5 Vectors

pGEM®-T Easy (Promega)

pGEX-4T2 (Amersham Pharmacia)

pGFPuv (Clonetech)

pETM-11 (EMBL, Heidelberg)

pETM-30 (EMBL, Heidelberg)

pCR® II- TOPO® (Invitrogen)

pCR® -XL-TOPO (Invitrogen)

3.1.6 Cells

E.coli strains such as TOP10, XL-1 Blue, BL-21(DE3), JM109 were kindly provided by EMBL, Hamburg, and TOP10 F' (Invitrogen)

3.1.7 DNA, RNA and protein markers

MassRuler™ DNA Ladder, Low Range, ready-to-use (Fermentas)

MassRuler™ DNA Ladder, High Range, ready-to-use (Fermentas)

MassRuler™ DNA Ladder, Mix, ready-to-use (Fermentas)

GeneRuler™ 100bp DNA Ladder Plus (Fermentas)

GeneRuler™ 100bp DNA Ladder Plus (Fermentas)

GeneRuler™ 1kb DNA Ladder (Fermentas)

Protein Molecular Weight Marker (Fermentas)

3.1.8 Plant material

Triticum aestivum cv. Bezostaja and *Triticum aestivum* cv. BMDE-10

Triticum durum cv. Balcalı and *Triticum durum* cv. C-1252

All plant materials were kindly provided by Çukurova University Plant Breeding Department.

3.1.9 Commercial kits

Promega, PCR Core System II

QIAGEN, DNeasy® Plant Mini Kit (50)

QIAGEN, RNeasy® Plant Mini Kit (50)

QIAGEN, Oligotex® mRNA Mini Kit (12)

QIAGEN®, One-Step RT PCR Kit

QIAGEN, Qiaquick® PCR Purification Kit (250)

QIAGEN, Qiaquick® PCR Purification Kit (50)

QIAGEN, Qiaprep® Spin Miniprep Kit (250)

QIAGEN, Qiaquick® Gel Extraction Kit (250)

QIAGEN, QIAGEN® Plasmid Midi Kit (100)

TOPO® TA Cloning Kit (Invitrogen)

TOPO® XL PCR Cloning Kit (Invitrogen)

3.1.10 Sequencing

Sequencing analyses were commercially provided by SEQLAB GmbH (Germany) or MWG-The Genomic Company (Germany).

3.1.11 Equipment

List of equipments used in this study can be found in Appendix C.

3.2 Methods

3.2.1 Plant Cultures and growth conditions

Seeds of both durum wheat plants (*Triticum durum* cv. Balcalı and *Triticum durum* cv. C-1252) and aestivum wheat plants (*Triticum aestivum* cv. Bezostaja and *Triticum aestivum* cv. BMDE-10) were surface sterilized for 20 min. in 10% (w / v) H_2O_2 and then rinsed with distilled H_2O (dH_2O). Seeds were germinated in perlite moistened with saturated $CaSO_4$. After 4 days seedlings were transferred to continuously-aerated nutrient solutions containing the following nutrient elements: 0.88 mM K_2SO_4, 2.0 mM $Ca(NO_3)_2$, 0.25 mM KH_2PO_4, 1.0 mM $MgSO_4$, 0.1 mM KCl, 100 μM FeEDTA, 1 μM H_3BO_3, 0.5 μM $MnSO_4$, 0.2 μM $CuSO_4$. Seedlings were grown with a light/dark regimes of 16/8 h, at 25/20 ^{0}C. Nutrient solutions were changed every 3 days. For Cd^{+2} applications 5mM and 10 mM Cd^{+2} were added to the nutrent solution 3-4 days after the transfer.

3.2.2 Bacterial Culture growth

Cells were grown overnight (12-16 h) in Luria Bertani Broth (LB Broth) medium in sterile culture tubes by shaking at 250 rpm at 37^{0}C. Antibiotics were added prior to addition of cells. For plates selective and/or unselective Miller's LB Agar solid medium (Sigma) was used.

Liquid and solid culture growth, glycerol stocks and competent cell preparation were carried out according to the protocols from Sambrook *et al*., 2001.

3.2.3 Plant DNA isolation

Plants were harvested after 2 weeks. 100 mg of plant material was used for genomic DNA isolation and the rest was stored in -80^0C for further applications. Plant DNAs were isolated by using QIAGEN, DNeasy® Plant Mini Kit Polymerase Chain Reaction (PCR)

PCR was carried out using PCR Core System II (Promega). Final concentrations of PCR components such as $MgCl_2$ and *Taq* polymerase were optimized by trying different concentrations. Depending on the primers used (with and without restriction enzyme sites) different programs for the thermocycler were used in accordance with annealing temperatures calculated from the formula in the PCR Core System II (Promega) instruction manual. Following program is given as an example;

Heating Lid T = 105.0 ^{0}

1. Hot Start : T = 95.0 ^{0} 0:02:00 min
2. Pause to add *Taq* polymerase
3. Denaturation : T = 95.0 ^{0} 0:01:00 min
4. Annealing : T = 55.0 ^{0} 0:01:00 min

   + 0.0 ^{0} + 0:00

   R: 3.0 ^{0}/s + 0.0 /s

   G: 9.0 ^{0}/
5. Extension : T = 72.0 ^{0} 0:01:00 min

6. GOTO 4 Repeat cycle 39 times

7. Final Extension : T = 72.0 ^{0} 0:01:00 min

3.2.4 PCR product purification

PCR products were checked by electrophoresis on 1.5 % Agarose gels. Purification was carried out either by QIAGEN, Qiaquick® Gel Extraction Kit (250) from agarose gels or directly by QIAGEN, Qiaquick® PCR Purification Kit (250).

3.2.5 Plant RNA isolation

Total plant RNA was isolated according to the method of Chomczynski and Sacch (1987) using QIAGEN, RNeasy® Plant Mini Kit. Poly(A)$^{+}$ RNA was isolated from total RNA using QIAGEN, Oligotex® mRNA Mini Kit as described by the manufacturer.

3.2.5.1 Preparing RNAse free environment

To prepare RNAse free water use dH_2O to make 0.1% diethyl pyrocarbonate (DEPC) solution. Stand it for at least 12 hours at 37 ºC and autoclave at 125 ºC for 15 minutes. Then use this RNase free water for your buffers, solutions and cleaning purposes.

3.2.6 RT-PCR

RT-PCR was carried out by following the procedure in QIAGEN®, One-Step RT PCR Kit to obtain cDNAs. QIAGEN®, One-Step RT PCR Enzyme Mix contained an

optimized combination of Omniscript Reverse Transcriptase, Sensiscript Reverse Transcriptase, and Hotstar*Taq* DNA Polymerase. Following RT-PCR conditions were programmed.

1. Heating Lid T = 105.0 ^{0}

2. Reverse Transcription : T = 50.0 ^{0} 0:30:00 min

3. Initial PCR activation : T = 95.0 ^{0} 0:15:00 min

   (*Taq* Polymerase is activated and reverse transcriptase are inactivated)

4. Denaturation : T = 94.0 ^{0} 0:01:00 min

5. Annealing : T = 53.5 ^{0} 0:01:00 min

6. Extension : T = 72.0 ^{0} 0:01:00 min

7. GOTO 4 Repeat cycle 39 times

8. Final Extension : T = 72.0 ^{0} 0:10:00 min

9. Hold 22 ^{0}

3.2.7 Cloning

Basic procedures were carried out according to Sambrook *et al*., 2001.

3.2.7.1 Subcloning

Different vector systems such as pGEM® -T Easy (Promega), PCR® II- TOPO® (Invitrogen), and PCR® -XL-TOPO (Invitrogen) have been used to subclone amplified MT cDNAs from *Triticum durum* and *Triticum aestivum*.

3.2.7.2 Ligation

Different ligation strategies were followed for different vector systems:

PCR amplified and purified MT fragment with restriction enzyme sites was ligated with pGEM® -T Easy vector (Promega) according to the specified amount (3:1 insert/vector ratio) in the pGEM® -T Easy Kit protocol. Ligation mixture was incubated o/n at 4^0C.

PCR® II- TOPO® (Invitrogen), and PCR® -XL-TOPO (Invitrogen) were ligated with PCR amplified and purified MT fragment containing different restriction enzyme sites, after at least 30 min incubation at room temperature (25^0C). Ligation reaction was stopped by the addition of 1 µl 6 X TOPO® Cloning Stop Solution.

Positive and negative controls of ligations were also prepared.

3.2.7.3 Transformation

Different endonuclease deficient strains of *E. coli*- XL1 Blue, TOP10, and BL21 (DE3) were used for transformations. Fresh or frozen stocks of competent cells were prepared and mixed with ligation mixtures according to standard procedures (Sambrook *et al*., 2001). Since the vectors contained different antibiotic resistance genes,

transformed cells and positive and negative controls were plated on appropriate antibiotic selective LB plates and were incubated over night at 37^0C.

3.2.7.4 Colony selection

Transformed colonies were selected and grown on appropriate antibiotic containing liquid and/or solid medium.

3.2.7.5 Preperation of glycerol stocks of cells

Glycerol stocks of *E.coli* containing different plasmids with *mt*-cDNA were prepared in 15 % glycerol in LB with antibiotics and kept at -80^0C according to the protocol from Sambrook *et al,* (1989).

3.2.7.6 Plasmid isolation

QIAGEN, Qiaprep® Spin Miniprep Kit or alkaline lysis miniprep standard protocol (Sambrook *et al*., 1989) were used in plasmid isolation. Isolated plasmids were checked by agarose gel electrophoresis.

3.2.7.7 Restriction enzyme digestion

Isolated plasmids were purified from cell cultures grown in the presence of appropriate antibiotics. To determine the presence of *mt* gene, purified plasmids were digested with appropriate restriction enzymes according to the manufacturers instructions and results were analyzed by agarose gel electrophoresis.

3.2.7.8 Agarose gel electrophoresis

Agarose gel electrophoresis was used in order to analyze purified and digested plasmids. Gels were prepared at 1.5% concentration using TAE buffer and were run in TAE buffer at 100 mV constant current for 40 minutes. Size and concentration determination were made by comparison of band intensities with appropriate DNA markers. Absorption spectroscopy is also used for concentration measurement and DNA/protein ratio ($OD_{260/280}$) determination.

3.2.7.9 Sequence verification

QIAGEN, Qiaprep® Spin Miniprep Kit or QIAGEN, QIAGEN® Plasmid Midi Kit purified MT-cDNA containing plasmids were sent to SEQLAB (Germany) or MWG-The Genomic Company (Germany) for sequence analysis.

3.2.7.10 Expression cloning

Different vectors such as pGFPuv, pGEX- 4T2, pETM-11, pETM-30 were used for expression cloning. Expression vectors and MT fragment from subcloning vectors were digested with the same restriction enzymes for preparing compatible ends, and ligated. Different fragment vector ratios (1:1, 2:1, 3:1, 4:1, 5:1, 6:1, 10:1, 15:1, 20:1, 25:1, 50:1, 1:2,1:3) were tried in ligation reactions. Different types of competent cells were transformed with ligation mixtures to. For sequence verification TOP 10 or XL1 Blue cells were used whereas for expression BL21 (DE3) competent cells were used. MT gene insertion was verified with restriction enzyme digestion and glycerol stocks were prepared from positive colonies.

3.2.7.11 Expression

Expression of MT gene in the construct with pGFPuv expression vector were carried out basically according to Sambrook *et al*., 1989. 15 ml of LB-Amp and LB liquid cultures were inoculated with 1 ml over night cultures of BL21 (DE3) , pGFPuv in BL21 (DE3) and pGFPuv-dMT construct in BL21 (DE3).Different Isopropyl β- D-Thiogalactopyranoside (IPTG) concentrations between 0.5 mM and 1mM were applied in order to induce recombinant protein expression. Aliquots, corresponding to a total OD_{600} of 1.4, were taken from induced and non induced cells at different time intervals (t_0, t_1, t_2, t_3....o/n).

Cells were pelleted in microcentrifuge at 13200 rpm at 4^0C and immediately stored at -20^0C. During MT expression studies cells were also grown in 50μM Cd containing LB medium. Pellets were prepared for SDS-PAGE gel according to the protocols in Sambrook *et al*., 2001. For SDS-PAGE analysis gels with 5% stacking and 12% separating (x) sections were used. Gels were run at 30mA current for 1 hour and stained in Coomassie blue solutions. Protein bands were seen after destaining. Protein molecular weight markers were used to identify the molecular weights of expressed proteins observed on gel.

5% Stacking Gel	**15% Seperating Gel**
50 mM Tris-HCl pH 6.8	375 mM Tris-HCl pH 8.9
5% Acryl-bisacryl (30%-0.8%)	15% Acryl-bisacryl (30%-0.8%)
0.04% 20 % SDS	0.1% 20 % SDS
0.075% 10 % APS	0.075% 10 % APS
0.05% TEMED	0.05% TEMED

4 RESULTS

4.1 Plant Growth and DNA Isolation

Triticum aestivum and *Triticum durum* species were grown basically as described in section 3.2.1. Different cadmium and zinc concentration were applied to observe the responses of these species. *Triticum durum* cv. Balcalı is resistant to higher Cd^{+2} concentration whereas *Triticum durum* cv. C-1252 is sensitive to Cd^{+2}. On the other hand, *Triticum aestivum* cv. Bezostaja can survive at low Zn^{+2} concentration but *Triticum aestivum* cv. BMDE-10 is sensitive to low Zn^{+2} concentration.

Genomic DNA was isolated from *T.aestivum* and *T.durum* grown as described in section 3.2.3 and purified DNA was analyzed by agarose gel electrophoresis as shown in Figure 4.1. Isolated DNA was used for screening for *mt* gene by PCR.

Figure 4.1 Genomic DNA isolated from *T.aestivum*, Bezostaja (left) and *T.durum*, Balcali (right). λ-DNA was used as marker

4.2 Optimization of PCR conditions for *mt-a* and *mt-d*

To determine the optimal PCR condition for *mt-a* and *mt-d,* the gradient facility of the thermocyler was used. A one step 2 dimensional optimization protocol was designed. In the vertical direction 3 different temperatures (51.7^0C, 53.7^0C, 56.4^0C) were tested according to the *Tm* values of the primers. In the horizontal direction three different Mg^{+2} concentrations (1.0 mM, 1,5 mM, 2.0 mM) were used. The sample S5 had the strongest band with 53.7^0C annealing temperature and 1,5 mM Mg^{+2} concentration as shown in Figure 4.2.

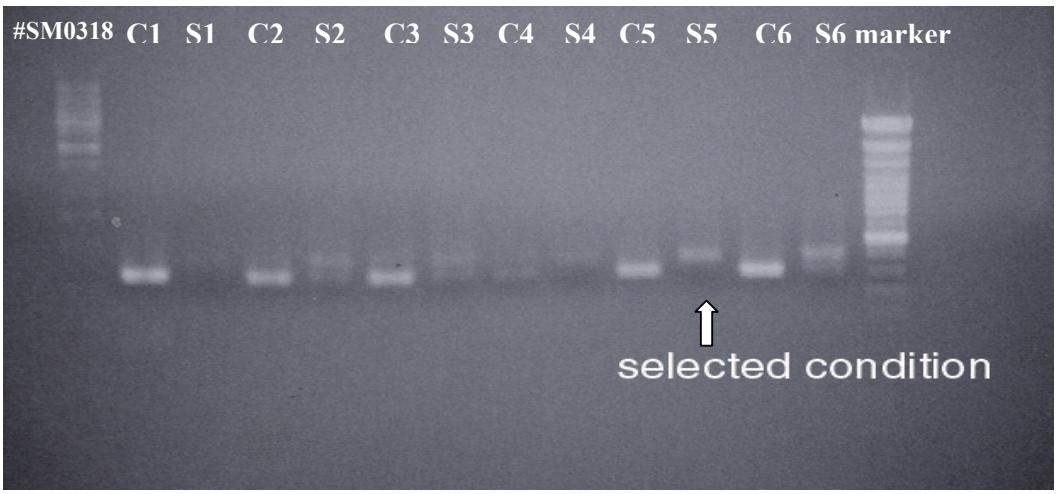


Figure 4.2 Agarose gel showing results of PCR optimization. DNA molecular weight markers and different samples.

4.3 Identification of *mt* genes

Three primers were designed without restriction enzyme sites according to the known sequence of *Triticum aestivum* (Snowden KC and Gardner RC 1993). The same primer set was used for *T.durum mt* gene identification.

Oligo 1 : 5' – ATG TCT TGC AAC TGT GGA - 3' (upstream)

Oligo 2 : 5' – TTA ACA GTT GCA GGG GTT - 3' (downstream)

Oligo 3 : 5' – ACA GTT GCA GGG GTT GCA - 3' (downstream with stop codon)

PCR was carried out by using primers labelled Oligo 1 and Oligo 3. *T.aestivum* and *T.durum* genomic DNAs were used as template and approximately 450 bp length PCR products corresponding to the *mt* gene were observed from both as shown in Figure 4.3.

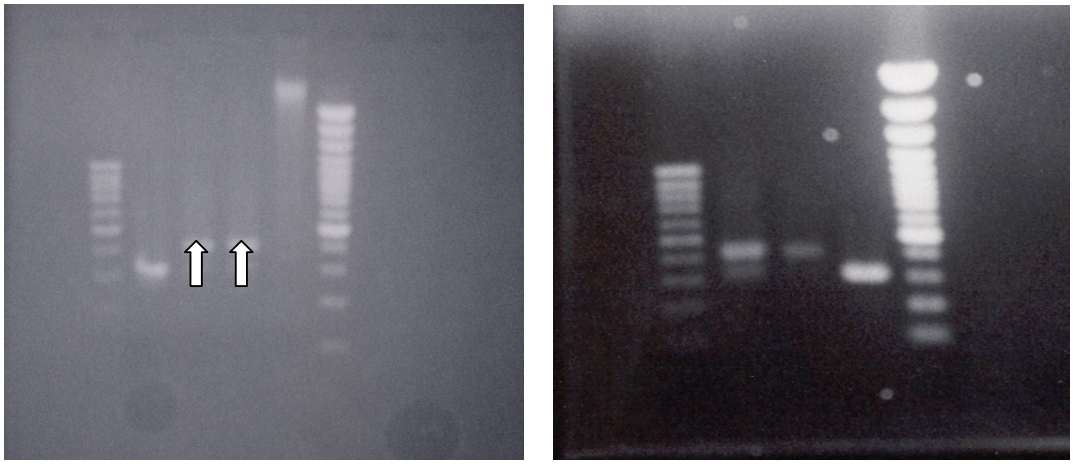


Figure 4.3 PCR results of *mt* gene identification in *Triticum aestivum and T.durum*

4.4 Subcloning and Sequence Verification of *mt-a* and *mt-d* in pGEM® -T Easy and pCR® II- TOPO® vectors

For sequence verification PCR products of *mt-a* were ligated into pGEM® -T Easy and *E.coli* JM109 cells were transformed with the constructs. pGEM® -T Easy has single 3'-T overhangs which promote efficiency of ligation and transformation. Multiple cloning site of pGEM® -T Easy contains a region encoding enzyme β-galactosidase and insertional inactivation of this gene allows directly identification by color secreening. 29 *mt-a* positive colonies were selected by blue white colony selection. To check the *mt-a* insertion pGEM® -T Easy-*mt-a* construct was digested with *Bam HI* and *Sal I*.

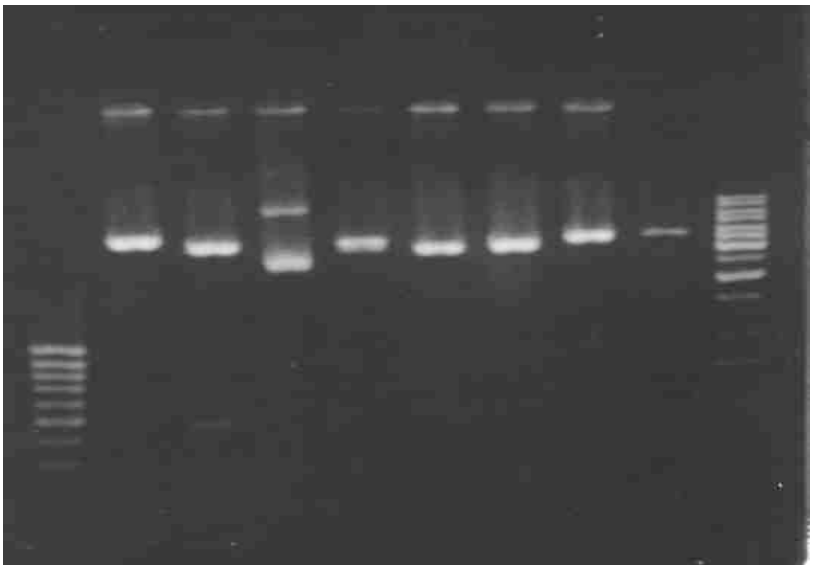


Figure 4.4 Restriction enzyme digestion analyses of pGEM® -T Easy-*mt-a* construct with *Bam*HI and *Sal*I

On the other hand, *mt-d* was inserted into pCR® II- TOPO® vector and *E.coli* TOP 10 cells were transformed.. *mt-d* gene fragment was checked by digestion of isolated plasmids with *Eco*RI The positive plasmids were sequenced to determine the *mt-d* DNA sequence.

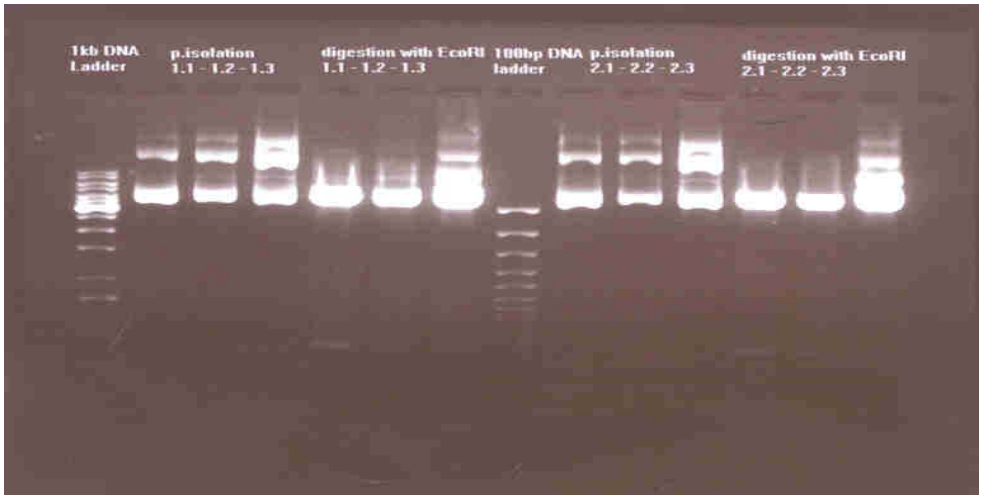


Figure 4.5 Restriction enzyme digestion analyses of pCR® II- TOPO® -*mt-d* constructs with *Eco*RI

4.5 Characterization of *mt-a* and *mt-d* genes

The pairwise comparison of complete *T.aestivum* and *T.durum* genomic MT gene nucleotide (nt) sequence shows 95 % similarity as shown in Figure 4.6. *mt-a* and *mt-d* are 399 bp and 416bp long respectively and the difference comes from an extra T(5)AT(6)A sequence in the intron part of *mt-d* gene. Both *T.aestivum* and *T.durum* contain an open reading frame (ORF) of 228 nucleotides which encode a 75 residue polypeptide with a deduced molecular mass of 7.35 kDa. *mt-a* and *mt-d* genes contain 5' and 3' untranslated regions of 172 and 188 nucleotides respectively. The ORF region of *T.durum mt-d* Exhibits 56.5 % G+C content. Alignment of genomic sequence of *mt-a* and *mt-d* with their cDNAs showed that both of the wheat cultivar *mt* genes have 2 exons and 1 intron as shown in Table 4.1 and 4.2.

```
MT_aestivum   ATGTCTTGCAACTGTGGATCCGGTTGCAGCTGCGGCTCAGACTGCAAGTGCGGGTATGGA
Mt-durum      ATGTCTTGCAACTGTGGATCCGGTTGCAGCTGCGGCTCAGACTGCAAGTGCGGGTATGGA 60
              ************************************************************

MT_aestivum   TGTTTTTTTT------------TCAAT---CATTAATGGATGCTTCTCCTTGCAAAA-TA
Mt-durum      TGCTTTTTTTATTTTTTATTTTTTGATTGACGTTAATGGATGCTTCTCCTTGCAAAAATA 120
              ** *******            *  **   * ************************ **
```

```
MT_aestivum   CTCTCGTGCTATTTATGTACTACTTCATCCGGTGATCCCTTTAGCTGATCAAGCATCAAT
Mt-durum      CTCTCGTGCTATTTATCTACTACTTCATCTGGTGACCCCTTTAGCTGATCAAGCATCAGT 180
              *************** *********** ***** ********************** *

MT_aestivum   TCCTATTGTGGTGATGAACGATTGATTTGATTCGCCGACCGGTTGCCTTCGCCCGTTGCA
Mt-durum      TCCTTTTGTGGTGATGAACTATTGATCTGATTCGCCGACCGGTTGCCTTCGCCCGTTGCA 240
              **** ************** ****** ********************************

MT_aestivum   GGAAGGTGTACCCTGATCTGACGGAGCAGGGCAGTGCCGCCGCCCAGGTCGCCGCCGTGG
Mt-durum      GGAAGATGTACCCTGATCTGACAGAGCAGGGCAGCGCCGCCGCCCAGGTCGCCGCCGTGG 300
              ***** *************** *********** ************************

MT_aestivum   TCGTCCTCGGCGTGGCGCCTGAGAACAAGGCGGGGCAGTTCGAGGTGGCCGCCGGGCAGC
Mt-durum      TCGTGCTCGGCGTGGCTCCTGAGAACAAGGCGGGGCAGTTCGAGGTGGCCGCCGGCCAGT 360
              **** *********** ************************************ ***

MT_aestivum   CCGGCGAGGGCTGCAGCTGCGGCGACAACTGCAAGTGCAACCCCTGCAACTGTAA-
Mt-durum      CCGGCGAGGGCTGCAGCTGCGGCGACAACTGCAAGTGCAACCCCTGCAACTGTTAA     416
              ************************************************** *
```

Figure 4.6 Pairwise alignment between *T.aestivum* and *T.durum mt* gene DNA sequences

Clustal W was used to carry out multiple sequence alignment on *T.aestivum* and *T.durum* (Figure 4.7). Since wheat-MT genomic sequence is not available in nucleic acid databases, Zea mays genomic sequence was used (NCBI :S57628) for comparison.

```
Mt-durum      ATGTCTTGCAACTGTGGATCCGGTTGCAGCTGCGGCTCAGACTGCAAGTGCGGGTATGGA
MT_aestivum   ATGTCTTGCAACTGTGGATCCGGTTGCAGCTGCGGCTCAGACTGCAAGTGCGGGTATGGA
MT_maize      ATGTCTTGCAGCTGCGGATCAAGCTGCGGCTGCGGCTCAAGCTGCAAGTGCGGGTA-ATA 60
                ********** *** *****  * *** ***********  *************** *

Mt-durum      TGCTTTTTTTATTTTTTATTTTTTGATTGA-CGTTAATGGATGCTTCTCCTTGCAAAAAT
MT_aestivum   TGTTTT------------TTTTTCAAT----CATTAATGGATGCTTCTCCTTGCAAAA-T
MT_maize      TATAATA----------ATATATAAGTGCACCGTGCATGATTAATTTCTCCAGCCTTCTT120
              *    *            * * *   *    * *  ***  *  **   *  **     *

Mt-durum      ACTCTCGTG-CTATTTA--TCTACTACTTCATCTGGTGACCCCTTTAGCTGATCAAGCA-
MT_aestivum   ACTCTCGTG-CTATTTA--TGTACTACTTCATCCGGTGATCCCTTTAGCTGATCAAGCA-
MT_maize      CTTGTCTTGTCTAGTTAATTTCCCTTCTTTATTTATTTTTTCCATT-GCAAAACAAACAA180
                * ** ** *** ***  *   ** *** **    *    ** ** **  * *** **

Mt-durum      -TCAGTTCCTTTTGTGGTGATGAACTATTGATCTGATTCGCCGACCGGTTGCCTTCGCCC
MT_aestivum   -TCAATTCCTATTGTGGTGATGAACGATTGATTTGATTCGCCGACCGGTTGCCTTCGCCC
MT_maize      ACAAAAAACAAAGTTAATCTGGATCGAGTAGTTCAATCCATTTGCGCGCTGTCCT-----240
                 *    *     *  *   ** * * *  *   ** *     *  * ** * *

Mt-durum      GTTGCAGGAAGATGTACCCTGATCTGACAGAGCAGGGCAGCGCCGCCGCCCAGGTCGCCG
MT_aestivum   GTTGCAGGAAGGTGTACCCTGATCTGACGGAGCAGGGCAGTGCCGCCGCCCAGGTCGCCG
MT_maize      -TTTCAGCAAGAAGTACCCTGACCTGGAGGAGACGAGCA---CCGCCGCGCAGCCCACC-360
              ** *** ***  ********* ***   ***  * ***   ******* ***  * **

Mt-durum      CCGTGGTCGTGCTCGGCGTGGCTCCTGAGAACAAGGCGGGGC---AGTTCGAGGTGGCCG
MT_aestivum   CCGTGGTCGTCCTCGGCGTGGCGCCTGAGAACAAGGCGGGGC---AGTTCGAGGTGGCCG
MT_maize      -----GTCGTCCTCGGGGTGGCCCCGGAGAAGAAGGCCGCGCCCGAGTTCGTCGAGGCCG400
              ***** ***** ***** ** ***** ***** * **   ******  * *****

Mt-durum      CCGGCCAGTCCGGCGAGG---------GCTGCAGCTGCGGCGACAACTGCAAGTGCAACC
MT_aestivum   CCGGGCAGCCCGGCGAGG---------GCTGCAGCTGCGGCGACAACTGCAAGTGCAACC
MT_maize      CGGCGGAGTCCGGCGAGGCCGCCCACGGCTGCAGCTGCGGTAGCGGCTGCAAGTGCGACC440
              * *   ** *********         *************   *  ********** ***
```

```
Mt-durum        CCTGCAACTGTTAA
MT_aestivum     CCTGCAACTGTAA-
MT_maize        CCTGCAACTGCTGA
                **********
```

Figure 4.7 Clustal W analysis of maize, *T.durum* and *T.aestivum* MT genomic sequences

Differences in genomic sequences between wheat and maize come from the intron region. In order to determine the type of MT, *mt-d* and *mt-a* sequences were translated and protein sequences were aligned with all known MT proteins (data not shown). Both proteins displayed the MT fingerprint Cys-Xaa-Cys motifs. It was found that they contained 6 of these motifs which were equally distributed in both N- and C-terminal as observed in plant Type 1 MTs. Linker region separating the two domains consisted of 44 amino acids. Protein sequences showed high level of similarity in the conserved regions and the differences were due to the amino acids found in the linker region as shown in figure 4.8.

```
MT_aestivum     MSCNCGSGCSCGSDCKCGKMYPDLTEQGSAAAQVAA-VVVLGVAPENKAGQFEVAAGQSG
Mt-durum        MSCNCGSGCSCGSDCKCGKMYPDLTEQGSAAAQVAA-VVVLGVAPENKAGQFEVAAGQSG
MT_barley       MSCSCGSSCGCGSNCNCGKMYPDLEEKSGATMQVTV-IVL-GVGSAKVQFEEAAEFGEAA
MT_maize        MSCSCGSSCGCGSSCKCGKKYPDLEETSTAAQPTVVLGVAPEKKAAPEFVEAAAESGEAA
                *** *** * *** * *** **** *   *       *                 *

MT_aestivum     EGCSCGDNCKCNPCNC
Mt-durum        EGCSCGDNCKCNPCNC
MT_barley       HGCSCGANCKCNPCNC
MT_maize        HGCSCGSGCKCDPCNC

                 *****  *** ****
```

Figure 4.8 Multiple sequence alignment result of *aestivum*, *durum*, barley and maize metallothionein protein sequences

ATGTCTTGCAACTGTGGATCCGGTTGCAGCTGCGGCTCAGA CTGCAAGTGCGGGTATGGATGCTTTTTTTATTTTTTATTTT TTGATTGACGTTAATGGATGCTTCTCCTTGCAAAAATACTC TCGTGCTATTTATCTACTACTTCATCTGGTGACCCCTTTAG CTGATCAAGCATCAGTTCCTTTTGTGGTGATGAACTATTGA TCTGATTCGCCGACCGGTTGCCTTCGCCCGTTGCAGGAAGA TGTACCCTGATCTGACAGAGCAGGGCAGCGCCGCCGCCCAG GTCGCCGCCGTGGTCGTGCTCGGCGTGGCTCCTGAGAACAA GGCGGGGCAGTTCGAGGTGGCCGCCGGCCAGTCCGGCGAGG GCTGCAGCTGCGGCGACAACTGCAAGTGCAACCCCTGCAAC TGTTAA	Gene 1..416 /gene="*mt-d*" mRNA 1..416 CDS 1…52 and 240…416 UTR 53….239 /translation="MSCNCGSGCSC GSDCKCGKMYPDLTEQGSA AAQVAAVVVLGVAPENKAG QFEVAAGQSGEGCSCGDNK CNPCNC

Table 4.1 Genecard for *T.durum* showing 2 exons (Turquoise) and 1 exon. Size and protein information are given on the right

ATGTCTTGCAACTGTGGATCCGGTTGCAGCTGCGGCTCAGA CTGCAAGTGCGGGTATGGATGTTTTTTTTTCAATCATTAAT GGATGCTTCTCCTTGCAAAATACTCTCGTGCTATTTATGTA CTACTTCATCCGGTGATCCCTTTAGCTGATCAAGCATCAAT TCCTATTGTGGTGATGAACGATTGATTTGATTCGCCGACCG GTTGCCTTCGCCCGTTGCAGGAAGGTGTACCCTGATCTGAC GGAGCAGGGCAGTGCCGCCGCCCAGGTCGCCGCCGTGGTCG TCCTCGGCGTGGCGCCTGAGAACAAGGCGGGGCAGTTCGAG GTGGCCGCCGGGCAGCCCGGCGAGGGCTGCAGCTGCGGCGA CAACTGCAAGTGCAACCCCTGCAACTGTAA	Gene 1..399 /gene="*mt-a*" mRNA 1..399 CDS 1…54 and 227…399 UTR 55….226 /translation="MSCNCGSGCSC GSDCKCGKMYPDLTEQGSA AAQVAAVVVLGVAPENKAG QFEVAAGQSGEGCSCGDNK CNPCNC

Table 4.2 Genecard for *T.aestivum* showing 2 exons (Turquoise) and 1 exon. Size and protein information are given on the right.

Although there are nucleotide differences in the coding sequences of the 3 different MT sequences, protein sequences are 100% identical due to the degeneracy of the genetic code.

```
MT_aestivum     ATGTCTTGCAACTGTGGATCCGGTTGCAGCTGCGGCTCAGACTGCAAGTGCGGGAAGATG
Mt-durum        ATGTCTTGCAACTGTGGATCCGGTTGCAGCTGCGGCTCAGACTGTAAGTGCGGGAAGATG
wheat_MT        ATGTCTTGCAACTGTGGATCCGGTTGCAGCTGCGGCTCAGACTGCAAGTGCGGGAAGATG
                ********************************************** ***************

MT_aestivum     TACCCTGATCTGACAGAGCAGGGCAGCGCCGCCGCCCAGGTCGCCGCCGTGGTCGTGCTC
Mt-durum        TACCCTGATCTGACAGAGCAGGGCAGCGCCGCCGCCCAGGTCGCCGCCGTGGTCGTGCTC
wheat_MT        TACCCTGATCTGACGGAGCAGGGCAGTGCCGCGCCCCAGGTCGCCGCCGTGGTCGTGCTC
                ************** *********** ***** ********************** ***

MT_aestivum     GGCGTGGCTCCTGAGAACAAGGCGGGGCAGTTCGAGGTGGCCGCCGGGCAGTCCGGCGAG
Mt-durum        GGCGTGGCTCCTGAGAACAAGGCGGGGCAGTTCGAGGTGGCCGCCGGGCAGTCCGGCGAG
wheat_MT        GGCGTGGCGCCTGAGAACAAGGCGGGGCAGTTCGAGGTGGCCGCCGGGCAGTCCGGCGAG
                ******** ************************************ ************

MT_aestivum     GGCTGCAGCTGCGGCGACAACTGCAAGTGCAACCCCTGCAACTGTTAA
Mt-durum        GGCTGCAGCTGCGGCGACAACTGCAAGTGCAACCCCTGCAACTGTTAA
wheat_MT        GGCTGCAGCTGCGGCGACAACTGCAAGTGCAACCCCTGCAACTGTTAA
                ************************************************
```

Figure 4.9 Multiple sequence alignment of *T.aestivum, T.durum* and wheat MT (AAA50846)

4.6 Subcloning of *mt-a* cDNA in *E.coli* with pGEM®-T Easy vector

mRNA was isolated form RT- PCR was performed to get cDNA from mRNA template. QIAGEN®, One-Step RT PCR Kit were used with Oligo 1 and Oligo 2

primers as described in section 3.2.7 and yielded 235 bp fragment as shown in Figure 4.10 were observed by agarose gel electrophoresis.

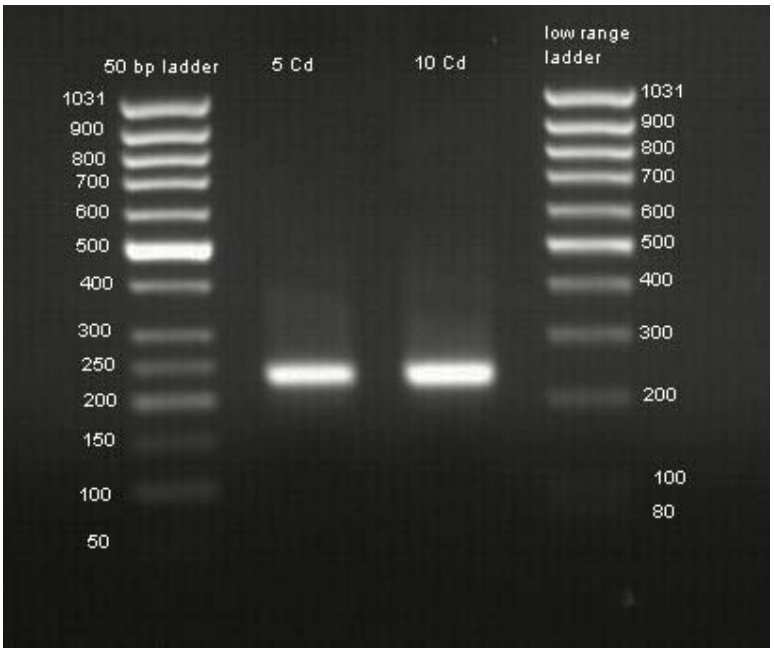


Figure 4.10 1.5% agarose gel analysis of RT-PCR result showing *T.aestivum* cv Bezostaja cDNA for *mt* gene

pGEM® -T Easy vector was again used for subcloning of purified cDNAs and *E.coli* TOP10 cells were transformed with pGEM® -T Easy-*mt-a*_cDNA constructs.

30 colonies grew on selective LB-Amp plates. Restriction enzyme digestion analysis was carried out on plasmids isolated from selected colonies (such as #2, #4, #7, #15, #16, #17 to check the *mt-a*_cDNA fragment. *Eco*RI was used in digestions since pGEM® -T Easy vector has two *Eco*RI recognition sites in the flanking regions. *mt-a*_cDNA fragments were observed between 200 and 300 bp bands as shown in Figure 4.11.

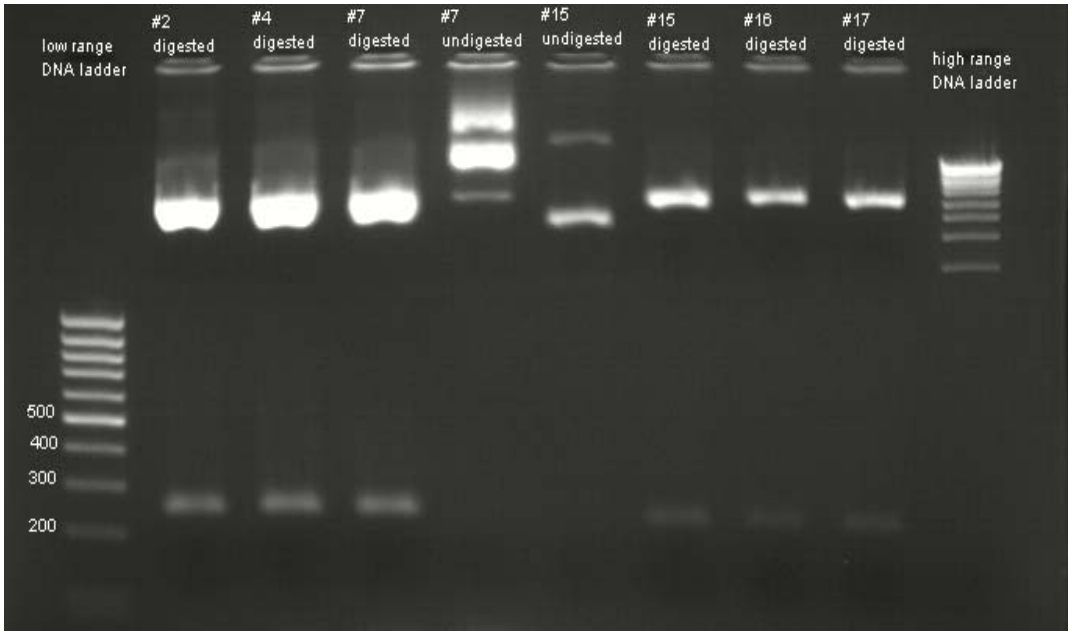


Figure 4.11 *Eco*RI digestion analysis showing the presence of *mt-a*_cDNA in pGEM®-TEasy vector. Undigested plasmids are shown in lane #7 and in lane #15 in.

4.7 Subcloning of *mt-d* cDNA in *E.coli* with pGEM®-T Easy

Triticum durum cultivar Balcali and Cesit-1252 were grown and treated as described above for T.aestivum cv Bezostaja. All conditions and procedures for mRNA isolation and RT-PCR were as described in section 3.2.5 .

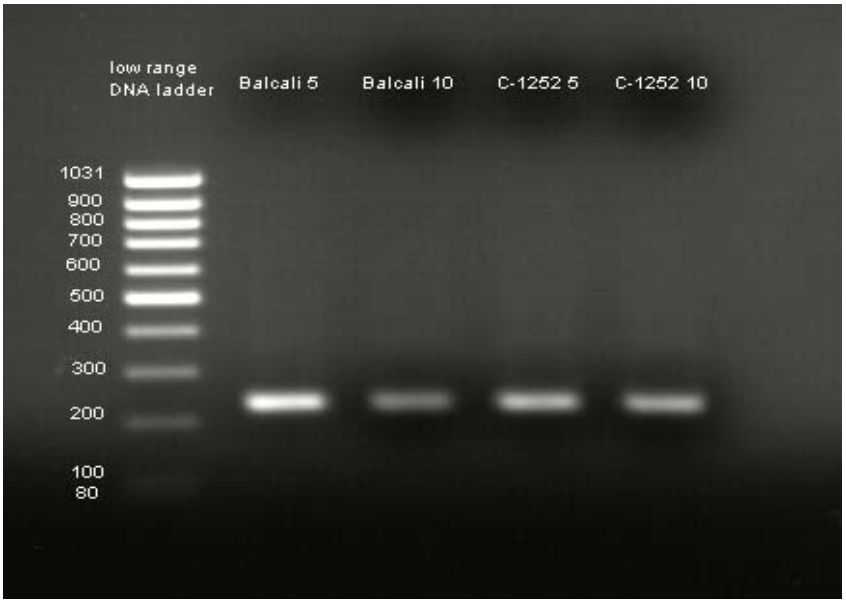


Figure 4.12 RT-PCR results showing *T.durum* cv Balcalı and *T.durum* cv Cesit-1252 cDNA for *mt* gene

Same cloning strategy was followed for *T.durum* cultivars. Purified *mt-d* cDNAs were ligated into pGEM-Teasy vectors and followed by transformation of *E.coli* TOP 10 cells with the constructs. Constructs were purified from colonies which grew on LB-amp plates and checked on 1,5 % agarose gel (data not shown). Digestion analysis with *Eco*RI was carried out to show the presence of the insert.

Figure 4.13 and 4.14 shows the electrophoretic analysis of selected clones of Balcali and C-1252 cultivars. B1, B2, B5, B6, B8, from Balcalı and C1, C2, C8 from C-1252 were chosen. To simplify comparison one undigested and one digested sample from the same clone were loaded to the gel. As expected *Eco*RI digestion yielded a fragment between 200 and 300 bp. Only B2 digestion gave no insert.

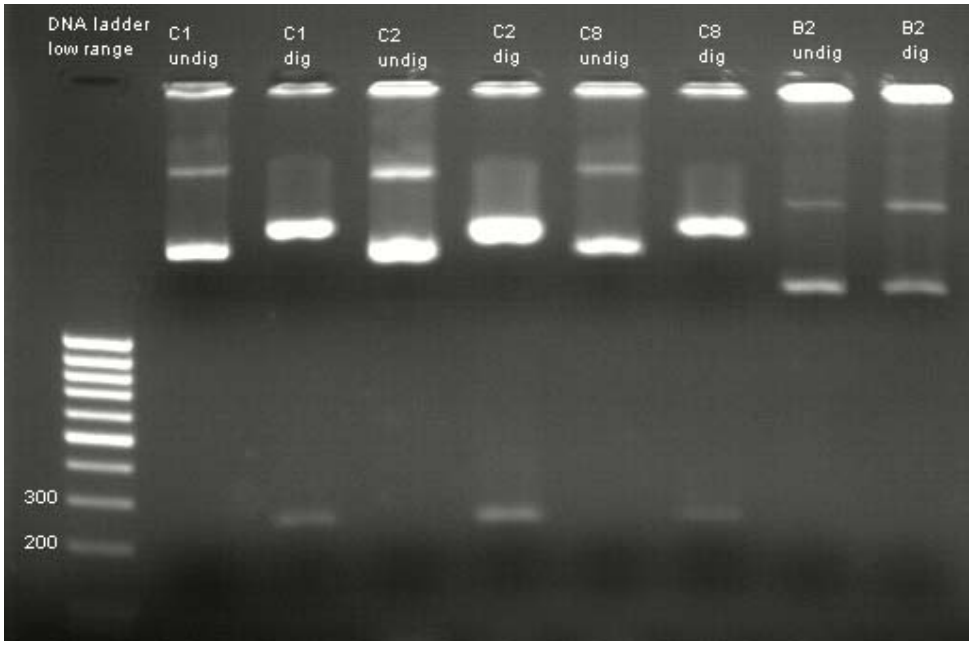


Figure 4.13 Restriction enzyme digestion analysis with *Eco*RI of pGEM® -T Easy-*mt-d*_cDNA constructs from C-1252.

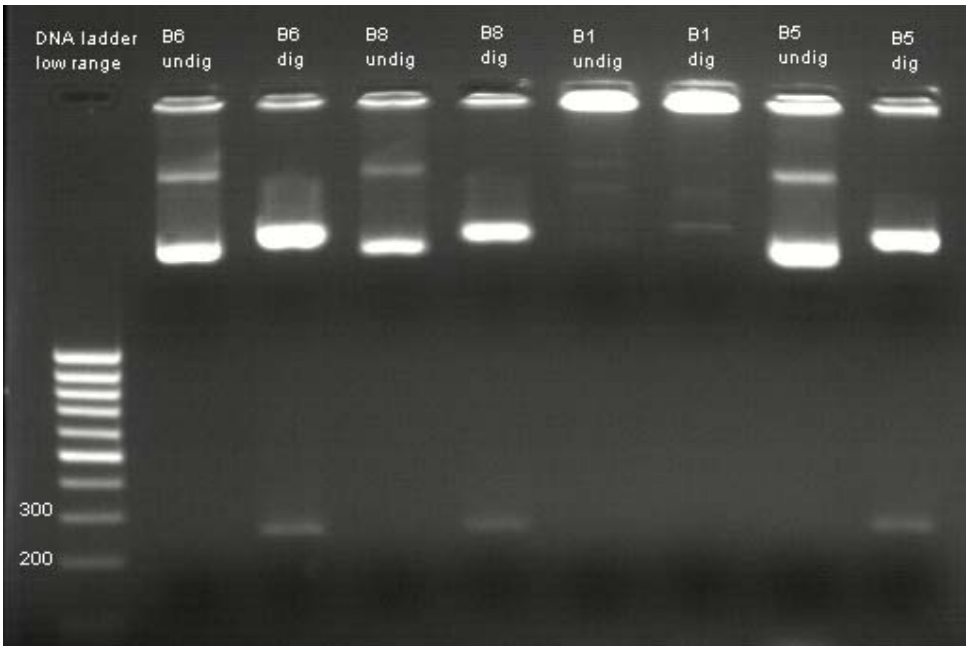


Figure 4.14 Restriction enzyme digestion analysis with *Eco*RI of pGEM® -T Easy-*mt-d*_cDNA constructs from Balcali.

4.8 Cloning of *mt-d* using pGFPuv expression vector

4.8.1 Isolation of *mt-d* cDNA from the subcloning vector

Digestion of *mt-d* fragment out of the subcloning vector (pGEM-Teasy) with appropriate restriction enzymes and insertion into expression vectors with the same restriction sites was the general strategy. *mt-d* cDNA fragment was isolated from pGEM® -T Easy constructs by digestion with *Eco*RI and *Spe*I. Inserts were checked and isolated from 1,5 % agarose gel. On the gel shown in Figure 4.15 #16 was is the uncut plasmid, #16 dig *Spe*I / *Eco*RI is the digested insert to be isolated and #16 refers to single digestion which generated the linearized vector as a control.

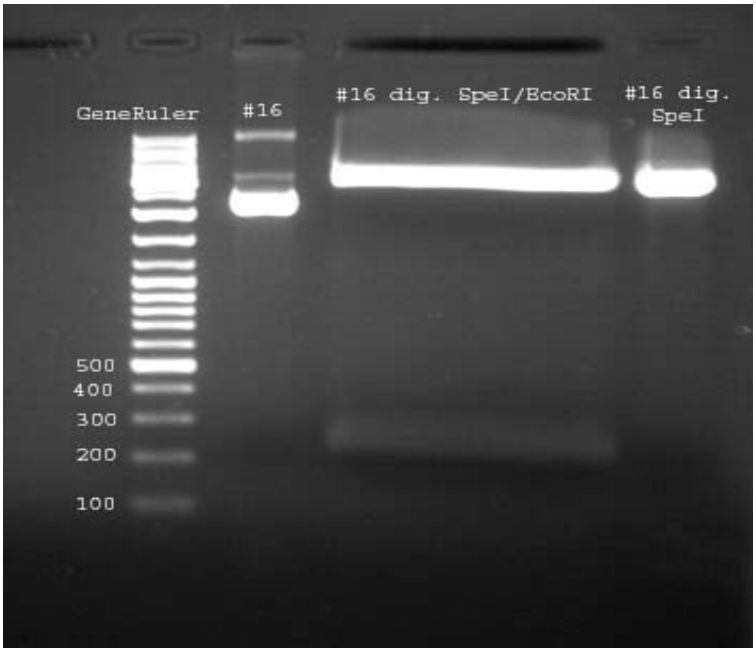


Figure 4.15 Agarose gel analysis showing double and single digestion results of pGEM® -T Easy vector containing *mt-d* cDNA insert

4.8.2 PCR amplification of *mt-d* cDNA with restriciton enzyme site primers

Initially *T.durum mt* gene was amplified with primers labelled as Oligo 1 and Oligo 3 which did not contain restriction enzyme sites. For insertion into different expression vectors such as pGEX-4T2 and pGFPuv new sets of primers were designed. For pGFPuv primers were labeled as Primer#2_L, Primer#2_R_non-stop. These contained restriction sites for *Hind*III (5') and *Xma*I (3') respectively as shown in Table 4.3. However, after subcloning and cloning it was noticed that the forward primer was wrongly designed. Therefore the gene was not inserted into pGEFPuv in frame with the start codon. The same problem was true also for pGEX-4T2 (see Chapter 5 discussion). So, new primers labelled P#4_F and P#4_R for were designed and are shown in Table 4.3. The strategy was first to amplify the *mt* gene with primers which did not contain RE sites. Then after purification, to use the first PCR products as template for a second PCR with RE sites containing primers.

Primer#2_L:	5`- CTATGAAGCTTATGTCTTGCAACTG – 3'`	***Hind* III**
Primer#2_Rnostop	5`-- CTATGCCCGGGACAGTTGCAGGGGT –3`	***Xma I***
Primer#4_F	5' - CTATGAAGCTTG ATGTCTTGCAACT - 3'	***Hind* III**
Primer#4_R	5' - CTATGCCCGGGTACAGTTGCAGGGG - 3'	***Xma I***

Table 4.3 RE sites containing primers designed for pGFPuv vector.

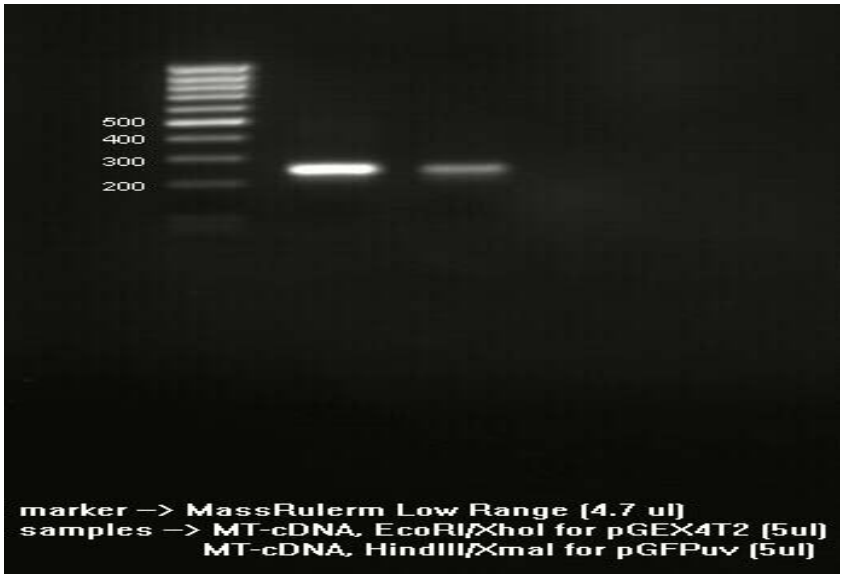


Figure 4.16 PCR results of *durum* cDNA with RE site containing primers

Purified *mt-d*_cDNA PCR products with the restriction enzyme sites were ligated to PCR-II-TOPO and *E.coli* XL1-Blue cells were transformed with the constructs. This subcloning was carried out because PCR-II-TOPO provides a high efficiency, 5-minute, one-step cloning strategy for the direct insertion of *Taq* polymerase-amplified products into a plasmid vector without requiring ligase and post-PCR procedures. 10 colonies were collected and glycerol stocks were labeled as "MT The Last Chance" were prepared.

For insertion into the pGFPuv vector, *mt-d*_cDNA containing PCR-II-TOPO constructs were isolated and digested with *Hind*III and *Xma*I to check the insert. As can be seen in figure 4. 17, no insert was found in MTLC-2.7. Clones MTLC-2.6, MTLC-2.9 and MTLC-2.10 were selected for further manipulations.

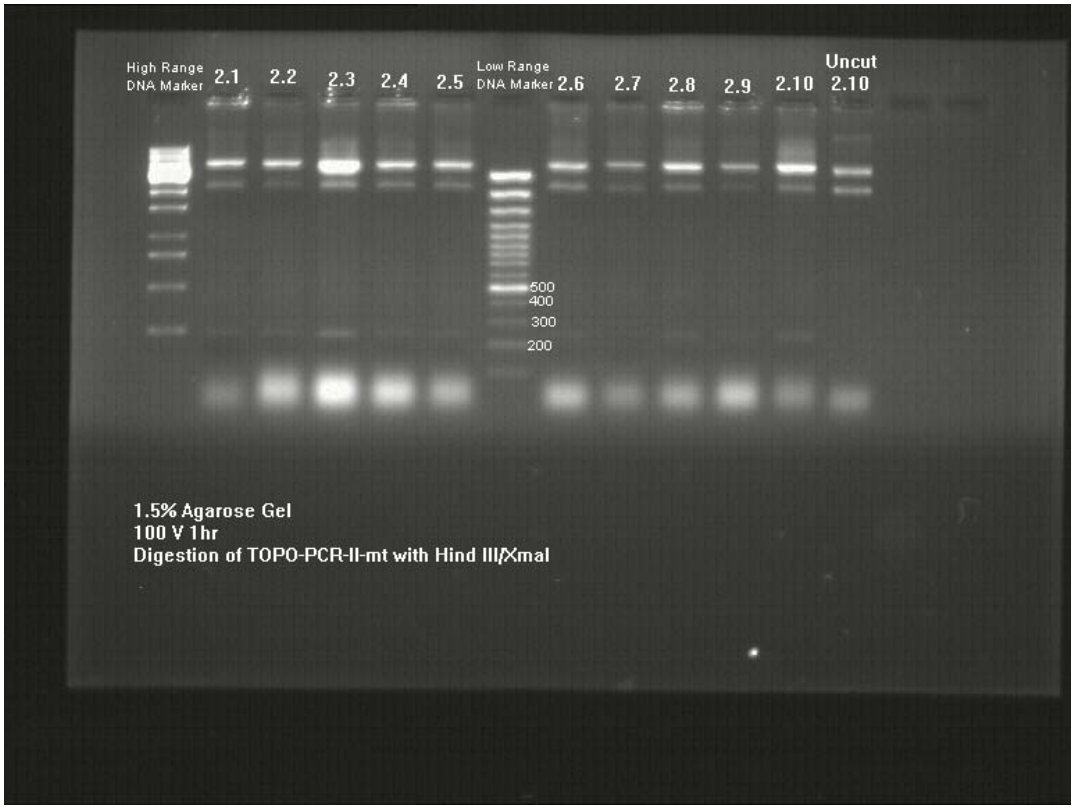


Figure 4.17 RE digestion results of PCR-II-TOPO vector to check the mt insert with *Hind*III/*Xma*I

Purified pGFPuv vector was digested with *Hind*III and *Xma*I and was purified from the agarose gel in preparation for ligation. RE digested *mt-d*_cDNA fragment from

MTLC-2.9 was also similarly treated as shown in figure 4.18. E. coli BL21(DE3) cells were transformed with constructs pGFPuv+*mt.*

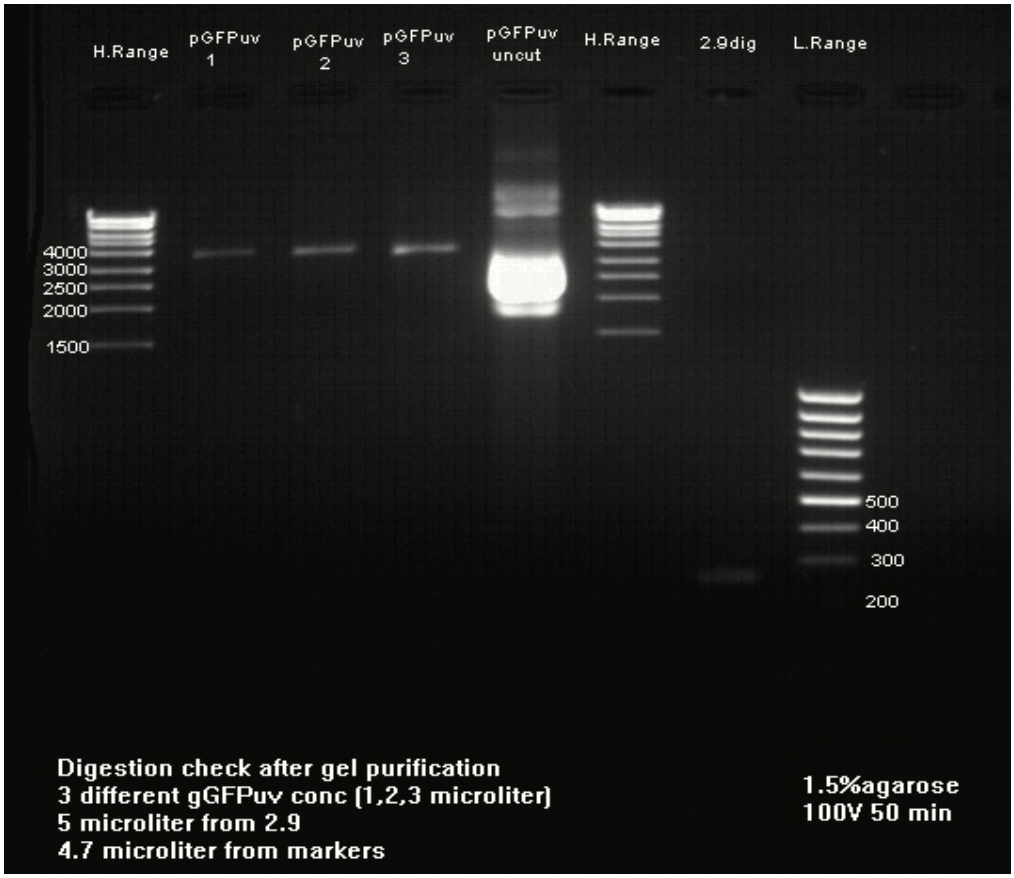


Figure 4.18 Double digestion (*Hind*III / *Xma*I)check after gel purification.

Five green colonies were selected and insert was checked in isolated plasmids by double digestion with *Hind*III and *Xma*I. No insert was observed. (data not shown).

The same procedure was repeatd with MTLC-2.10 clone. Isolated and purified *mt* gene from this clone was ligated to digested pGFPuv vector and both *E.coli* XL1-Blue and *E.coli* BL-21(DE3) cells were transformed with the constructs. 49 cells were selected from *E.coli* XL1-Blue plates whereas no growth was observed with *E.coli* BL-21(DE3) cells. 24 colonies were screened by minipreps And ten were selected for further digestion analysis. Six of them were digested with *Hind*III and *Sma*I and 4 of them were digested *Hind*III and *Xma*I as shown in Figure 4.19. #1 was chosen for transformation to *E.coli* BL21 (DE3)cells.

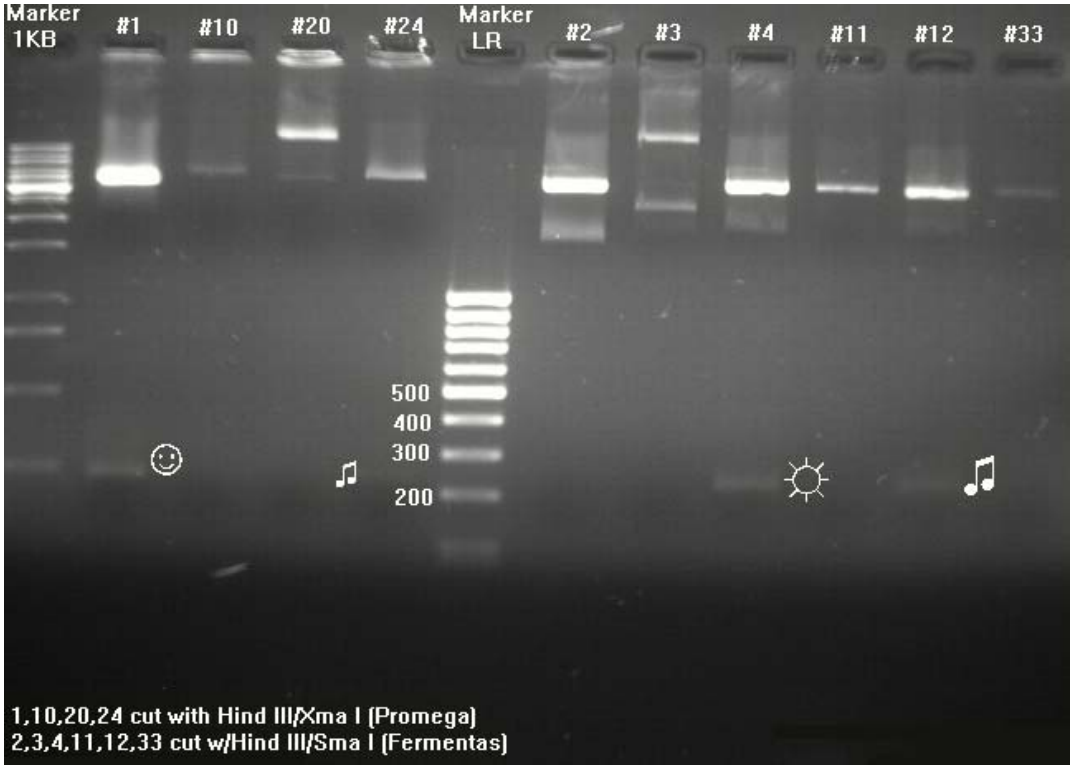


Figure 4.19Restriction enzyme analysis of pGFPuv_*mt-d* construct in XL1-Blue cells

4.9 Induction of metallothionein protein expression in *E. coli*

BL21 (DE3) cells containing the pGFPuv_*mt-d* construct as well as those with only pGFPuv and untransformed control were induced to monitor expression of GFP-dMT fusion protein. However, SDS-PAGE results showed that the fusion protein (GFPuv_*mt-d*) was not expressed as shown in figure 4.20.

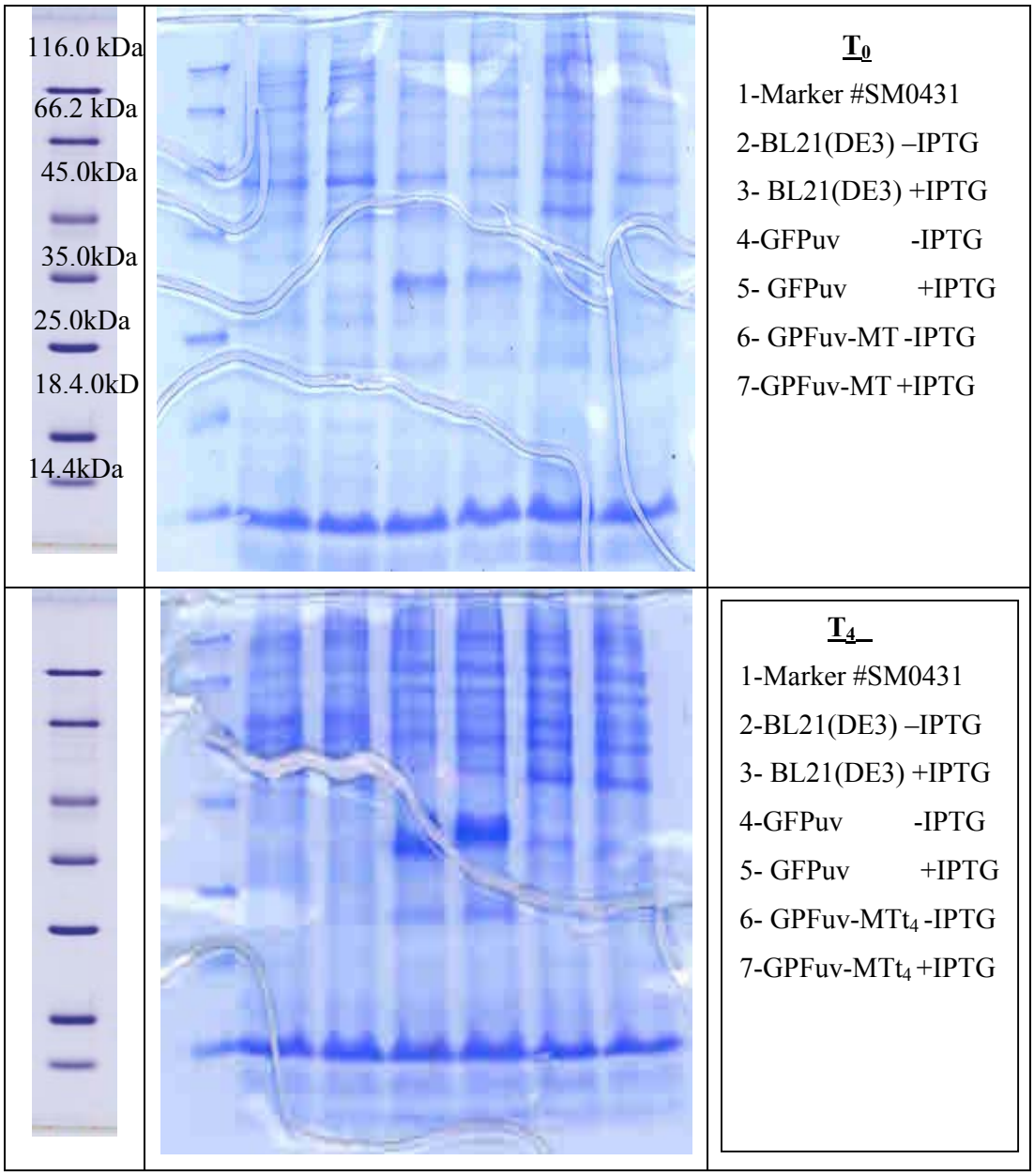


Figure 4.20 SDS-PAGE analysis to check the mt-d protein expression in induced and non-induced cells at 2 different time interval (0-4 hr). Samples were empty BL21(DE3), pGFPuv in BL21 (DE3), pGFPuv-mt-d in BL21 (DE3). The size of the marker indicated

Expression was repeated by adding 0.5 mM Cd^{+2} into the growth stablise the recombinant protein however, again no expression was observed (data not shown).

Sequence analyses were performed and multiple sequence comparison showed that insertion was inframe but a mutation on the 60^{th} nucleotide position of pGFPuv vector had resulted in premature termination due to an unexpected stop codon formation. The alignment is shown in figure 4.21 .

```
                                  HindIII
1B                  BTGATTACA CCA ANC TTG ATGTCTTGCAACTGTGGATCCGGTTGCAGCTGCGGCTCAGAC
1X                  -TGATTACG CCA AGC TTG ATGTCTTGCAACTGTGGATCCGGTTGCAGCTGCGGCTCAGAC
wheat_MT_L11879     ------------------    ATGTCTTGCAACTGTGGATCCGGTTGCAGCTGCGGCTCAGAC
                     ****************************************

1B                      TGCAAGTGCGGGAAGATGTACCCTGATCTGACAGAGCAGGGCAGCGCCGCCGCCCAGGTC
1X                      TGCAAGTGCGGGAAGATGTACCCTGATCTGACAGAGCAGGGCAGCGCCGCCGCCCAGGTC
wheat_MT_L11879         TGCAAGTGCGGGAAGATGTACCCTGATCTGACGGAGCAGGGCAGTGCCGCGGCCCAGGTC
                        ******************************* *********** ***** *********

1B                      GCCGCCGTGGTCGTGCTCGGCGTGGCTCCTGAGAACAAGGCGGGGCAGTTCGAGGTGGCC
1X                      GCCGCCGTGGTCGTGCTCGGCGTGGCTCCTGAGAACAAGGCGGGGCAGTTCGAGGTGGCC
wheat_MT_L11879         GCCGCCGTGGTCGTCCTCGGCGTGGCGCCTGAGAACAAGGCGGGGCAGTTCGAGGTGGCC
                        ************** *********** ********************************

1B                      GCCGGGCAGTCCGGCGAGGGCTGCAGCTGCGGCGACAACTGCAAGTGCAACCCCTGCAAC
1X                      GCCGGGCAGTCCGGCGAGGGCTGCAGCTGCGGCGACAACTGCAAGTGCAACCCCTGCAAC
wheat_MT_L11879         GCCGGCCAGTCCGGCGAGGGCTGCAGCTGCGGCGACAACTGCAAGTGCAACCCCTGCAAC
                        ***** **************************************************

                              XmaI                    pGFPuv
1B                      TGTACC CGG GTA CCG GTA GAA AAA ATG AGT AAA GGA GAA GAA CTT TTC ACT GGA GTT GTC
1X                      TGTACC CGG GTA CCG GTA GAA AAA ATG AGT AAA GGA GAA GAA CTT TTC ACT GGA GTT GTC
wheat_MT_L11879         TGTTAA----------------------------------------------------------
                        ***
```

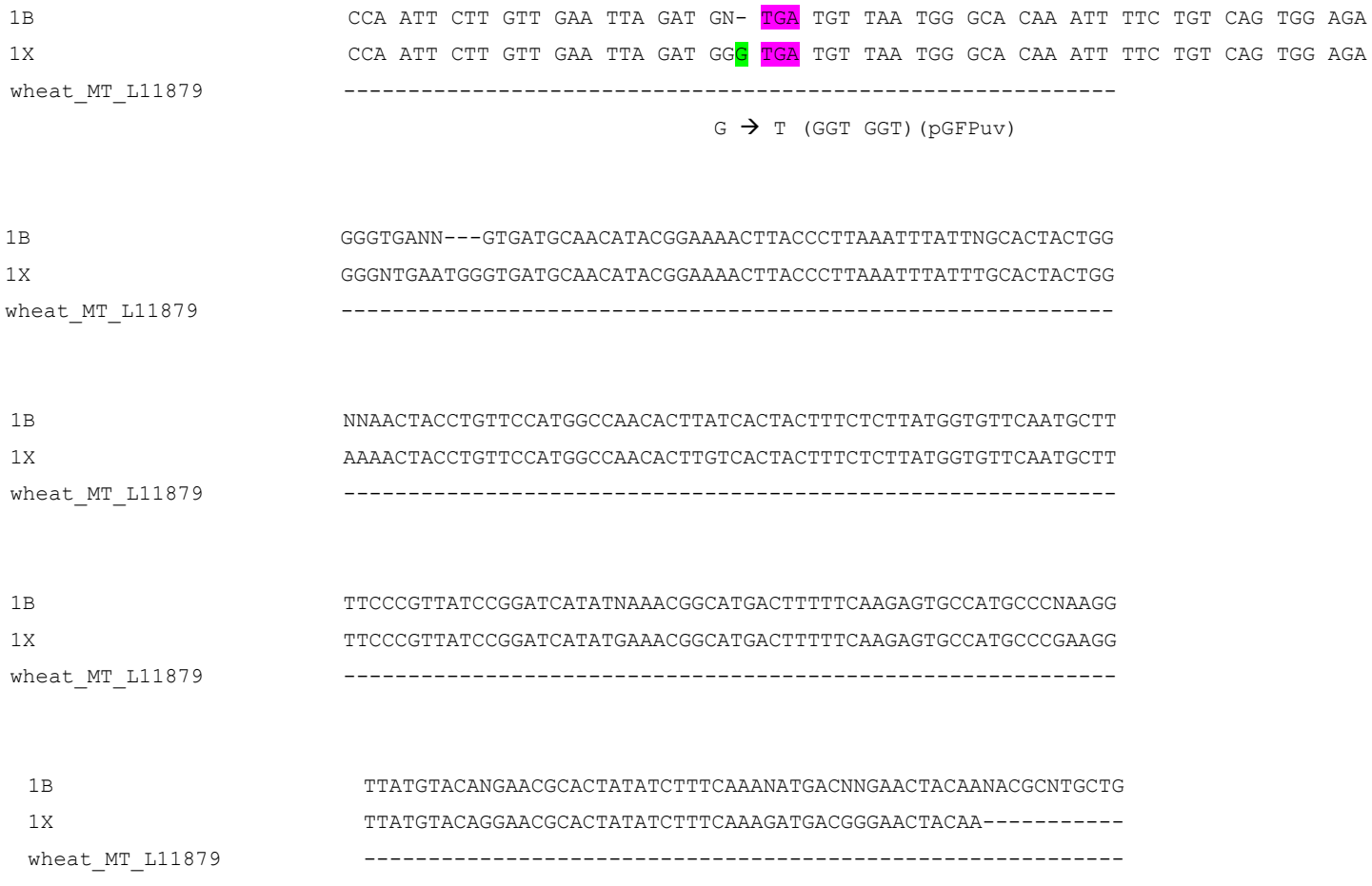


Figure 4.21Multiple sequence alignment of pGFPuv_*mt-d* contructs in *E.coli* XL1-Blue (1X) and *E.coli* BL21 (DE3) (1B) and wheat_MT RE sites were labeled the deletion and stop codon formation were showed in (pink)

5 DISCUSSION

Since their identification in plants in 1987 understanding the functional role of MT-like genes has been hindered due to the lack of protein data. Purification of MT from plant tissues has been unsuccessful with the exception of wheat E_c, (Kawashima *et al., 1992)* and more recently *Arabidopsis* MT1 and MT2 (Murphy *et al*., 1997). Only for wheat E_c has the association of the protein with metals been demonstrated (Robinson *et al*., 1993). The difficulty in obtaining purified plant MT has been attributed to the susceptibility to proteolysis because of the long linker region typical to plant MTs (Clemens S, 2001). In the mean time several studies have been published on the expression of plant MT-like genes. Evidence for developmental regulation was found as well as responsiveness to a variety of stimuli including metal exposure, hormone treatments, cold, osmotic stress, or heat stress (rewiewed and listed by Rauser 1999)

The main aim of the work presented in this thesis was to investigate the presence of *mt-lIke* genes and MT proteins in wheat *T. durum*. If the gene could be detected, further aims were cloning and characterization of the wheat *mt gene* and expression of the recombinant MT in a foreign host as fusion with a marker protein.

PCR was used for identification and amplification of the *mt-genes*. Optimization of the PCR reaction was performed by a one-step 2 dimensional PCR protocol. Different concentrations of Mg^{+2} and annealing temperatures were checked and after analysis of results by gel electrophoresis parameters that yielded most successful PCR were chosen. Since there are no available *T.durum mt* sequences in nucleic acid databases, characterization of *T. durum mt* gene was carried out using primers designed according to the *T.aestivum* MT mRNA sequence (NCBI Accession number: AAA50846). These primers were used for both wheat cultivars and gave positive results.

Sequence alignment of *mt* cDNAs and genomic DNAs from both species, showed %95 nucleic acid sequence similarity. The differences mainly came from the extra two "TTTTTA" repeats in the intron region of the *mt-d* gene as shown in figure 4.6. On the other hand the protein sequence of *mt-d* and *mt-a* showed the same amino acid composition. Multiple sequence alignment results of different plant MT protein sequences verified that *mt-d* and *mt-a* proteins are members of type1 MT family.

After verification of DNA sequences, *mt-d* cDNA was obtained by RT-PCR on mRNA isolated from wheat. Several studies had shown that deficiencies or heavy metal toxicities is one of the factors in the regulation of MT expression (de Miranda *et al*., 1990; Zhou *et al*., 1995). For this reason variable Cd^{+2} and Cu^{+} concentrations were applied to the growth medium of wheat cultivars before mRNA isolation. *mt-d* cDNA was subcloned using PCR-II-TOPO (Invitrogen). This vector was selected because it provides high efficiency; one-step cloning strategy by direct insertion of *Taq* polymerase amplified products into a plasmid vector without requiring ligase and post-PCR procedures.

After verification of the sequence, the cDNA was used as template for PCR amplification of the gene for directional cloning with expression vectors. Expression studies were carried out using pGFPuv (Amhersham Pharmacia) and pGEX-4T2 (Clonetech) vectors. pGFPuv was chosen to provide a fluorescent marker and pGEX-4T2 was chosen to achieve high level of expression as fusion with GST. Expression of MT as fusion was expected to provide not only stability to this low molecular weight protein but also to provide simple means of localization, purification, quantification and further characterization.

Although *mt gene* is of eukaryotic origin, gram-negative bacterium *E.coli* was selected as expression host in this study because *E.coli* is one of the most widely used hosts for production of heterologous proteins. Its ability to grow rapidly, its well-characterized genetics, availability of large number of cloning vectors and mutant host strains, and high level production of recombinant proteins make this organism as first choice in recombinant protein expression (Baneyx F, 1999). Production of different

plant *mt genes* in *E.coli* has also been reported in the literature previously (Evans *et al*, 1992; Kille *et al*,1991).

Marker genes have proved extremely useful for reporting gene expression in transformed plants, for example the β-glucuronidase gusA or GUS gene has been used extensively. However, for assaying primary transformants or for following time course expression in living plants using GUS is not suitable because transformed cells or patterns of gene expression within plants can be identified by a prolonged and lethal histochemical staining procedure which is a destructive test (Chalfie M and Kain S, 1996)

The green fluorescent protein (GFP), a cellular marker cloned from jellyfish *Aequoria Victoria,* shares none of these problems as its intristic fluoresence can be seen in living cells, and has allowed researchers to monitor dynamics of the plant cytoskeleton, endomembrane trafficking, organelle dynamics and macromolecular transport in living plant cells (Blancaflor EB and Gilroy S, 2000)

During expression studies initial work was hampered because of the wrongly designed primer which resulted in the insertion of *mt* gene into expression vectors out of frame with the fusion protein. As expected no expression was seen with these constructs. New primers were then designed by adding one nucleotide before ATG, and cloning for expression was tried again. *mt-d* gene was inserted directionally into the 5' multiple cloning site of the pGFPuv vector using *Hind*III and *Xma*I restriction sites. In the first attempts results were misleading due to false positives expressing GFPuv without the insert. It appears that these colonies contained either self-ligated vector or were contaminated with uncut vector.

Further screening revealed constructs which contained the insert and these were purified and used for transformation of expression cells. The presence of *mt-d* fragment which is about 300 bp length was demonstrated by agarose gel electrophoresis as shown in figure 4.19. Construct labelled as MTLC2.10 was used for plasmid isolation. The plasmid containing *mt-d* was eventually used for transformation of *E.coli* BL21 (DE3) cells for expression. Colonies were selected and clone #1 was used in expression

studies. Expression of GFPuv_*mt-d* was induced with 0.5-1.0 mM IPTG and the time course was followed by SDS-PAGE analysis of the cellular extracts.

The size of GPPuv and *mt-d* are 27k-Da and 7.35 kDa, respectively. Therefore, the size of the recombinant fusion protein is expected to be approximately 34.5 kDa. Comparison of the results shown in figure 4.20 for BL21(DE3) cells without the vector, BL21(DE3) cells with pGFPuv vector and BL21(DE3) with the construct revealed no difference indicating that the fusion protein was not being expressed. Thinking that the failure to observe the recombinant protein may be due the instability in MT structure in the absence of metal ions Cd^{+2} was included in the growth medium of the bacteria. However, again no expression of GFPuv-*mt-d* was observed in the samples. Sequence analysis of this construct showed that the insert was inframe with GPPuv but there was a point mutation on the pGFPuv sequence resulting in the premature termination of the fusion protein. G at the 60^{th} position of the GFPuv nucleotide sequence was deleted so that premature stop codon was formed. This data is shown in figure 4.21. The cause of this mutation may due to an UV attack during purification of vector from the gel after restriction enzyme digestion prior to ligation. Since *mt-d* is located at 5' end of GFPuv and is inserted into the vector in frame with promoter we would still expect MT protein to be expressed albeit without the marker. This could not be detected with SDS-PAGE analysis because of the very low molecular weight, about 7.5 kDa of the recombinant protein.

Due to lack of time the other PCRII-TOPO-*mt-d* constructs could not be screened for ligation with pGFPuv and sequenced. Since there are no reports in the literature of MT fusion with a marker protein it will be very useful to carry on this work till the end and obtain the labelled protein. Presence of the fusion protein may also protect MT from protease attacks at the hinge region and stabilize its structure. These are the experiments, which need to be carried out in the near future.

6 CONCLUSION

1. Metallothionein genes were identified in *T.durum* and *T. aestivum* genomes.

2. Gene sequences were about 400 nucleotides long and contained two exons separated by an intron.

3. The sequences showed 95% similarity to each other as well as to maize *mt gene* whose sequence is available in the literature.

4. The open reading frame consisted of 228 nucleotides which encoded a 75 residue polypeptide with a deduced molecular mass of 7.35 kDa.

5. Protein sequences were 100% identical due to the degeneracy of the genetic code.

6. Sequence analysis further showed that *T.durum* and *T.aestivum mt* genes coded for a protein that could be classified as plant Type 1 MT.

7. Although *mt-d* gene was inserted into the pGFPuv vector for GFP-MT fusion protein, this could not be achieved due to a mutation in the DNA sequence which caused premature termination. Since GFP expression on its own can be detected, the mutation is likely to be caused by UV exposure during preparation of the vector.

8. Labelling of MT proteins with fluorescent markers is an important factor in elucidation of functional roles of MT, and since there are no reports on this subject in the literature, should be pursued until a positive result is obtained

7 REFERENCES

1. Abdullah SNA., Cheah SC, Murphy DJ., "Isolation and characterisation of two divergent type 3 metallothioneins from oil palm, *Elaeis guineensis*" *Plant Physiol. Biochem.* 40 (2003) 255-263.

2. Andrews GK., "Regulation of metallothionein gene expression by oxidative stress and metal ions" *Biochemical Pharmacology* 59 (2000) 95-104.

3. Arseniev A., Schultze P, Wörgötter E., BraunW, Wagner G, Vasak,M, Kagi JHR,Wüthrich K "Three-dimensional structureof rabbit liver Cd-metallothionein-2a in aqueous solution determined by nuclear magnetic resonance. J. Mol. Biol. 201, (1988) 637±657.

4. Binz PA, Kagi HR. "Metallothionein: Molecular evolution and classification. *In*: Klaassen (ed): Metallothionein IV". Birkhauser Verlag, Basel Boston Berlin, (1997) 7-21

5. Blancaflor EB., Gilroy S., " Plant cell biology in the new millennium: New tools and new insights" *American Journal of Botany* 87:11 (2000) 1547-1560.

6. Bragigand V., Berthet B., "Some methodological aspects of metallothionein evaluation" *Comparative Biochemistry and Phisiology Part A* 134 (2003) 55-61.

7. Briat J., Lebrun M., " Plant responses to metal toxicity" *Plant Biology and Pathology* 322 (1999) 43-54.

8. Butcher H., Kennette W., Collins O., Demoor J., Koropatnick J., " A sensitive time-solved fluorescent immunoassay for metallothionein protein" *Journal of Immunological Methods* 9250 (2002) 1-10.

9. Chalfie M, Kain S, "GFP Green Fluorescent Protein Properties, Applications, and Protocols" Wiley-Liss, Inc.

10. Chan J., Huang Z., Merrifield ME., Salgado MT., Stillman MJ., " Studies of metal binding reactions by spectroscopic, molecular biology, and molecular modelling techniques" *Coordination Chemistry Techniques* 233-234 (2002) 319-339.

11. Charbonnel-Campaa L., Lauga B., Combes D., " Isolation of a type 2 metallothionein-like gene preferentially expressed in the tapetum in *Zea mays*" *Gene* 254 (2000) 199-208.

12. Chen W., Hsieh H., Huang PC., " Type2 rice metallothionein-like gene has two introns" *DNA Sequence-The Journal of Sequencing and Mapping* 8:4 (1998) 223-228.

13. Choi D., Kim HM., Yun HK., Park JA., Kim WT., Bok SH., " Molecular cloning of a metallothionein-like gene from Nicotina glutinosa L.and its induction by wounding and tobacco mosaic virus infection. Plant Physiology 112 (1996) 353-359

14. Clemens S., "Molecular mechanism of plant metal tolerance and homeostasis" *Planta* 212 (2001) 475-486.

15. Cobbett CS., " Phytochelatins and their roles in heavy metal detoxification" *Plant Physiology* 123 (2000) 825-832.

16. Cobbett C.S., " Phytochelatin biosynthesis and function in heavy-metal detoxification" *Current Opinion in Plant Biology* 3 (2000) 211-216.

17. Cobbett C., Goldsbrough P., " Phytochelatins and metallothioneins: roles in heavy metal detoxification and homeostatis" *Annu. Rev. Plant Biol.* 53 (2002) 159-182.

18. Coyle P., Philcox JC., Carey LC. & Rofe AM.. Metallothionein: the multipurpose protein *Cell Mol.LIfe ScI.*, **59:** (2002) 627-647.

19. Dallinger R., Berger B., Hunziker P. & Kagi JH. "Metallothionein in snail Cd and Cu metabolism" *Nature*, **388:** (1997) 237-238

20. Das P., Samantaray S., Rout G.R., " Studies on cadmium toxicity in plants: a review" *Environmental Pollution* 98 (1997) 29-36.

21. De Miranda JR, Thomas MA, Thurman DA, Tomsett AB., "Metallothionein genes from the flowering plant *Mimulus guttatus"* FEBS Lett. 260: (1990) 277-280.

22. Ebadi M., Leuschen M P., el Refaey H., Hamada FM. & Rojas P. The antioxidant properties of zinc and metallothionein. *Neurochem.Int.*, **29:** (1996) 159-166

23. Evans KM., Gatehouse JA., Lindsay WP., Shi J., Tommey AM. & Robinson NJ. "Expression of the pea metallothionein-like gene PsMTA in Escherichia coli and Arabidopsis thaliana and analysis of trace metal ion accumulation: implications for PsMTA function". *Plant Mol.BIol.*, **20:** (1992) 1019-1028.

24. Framond A.J., " A metallothionein-like gene from maize (Zea mays) cloning and characterisation" *FEBS* 290:1-2 (1991) 103-106

25. Garcia-Hernández M., Murphy A., Taiz L., "Metallothioneins 1and 2 have distinct but overlapping expression patterns in Arabidopsis" *Plant Physiol*. 118 (1998) 387-397.

26. Hall J.L., " Cellular mechanisms for heavy metal detoxification and tolerance" *Journal of Experimental Botany* 53:366 (2002) 1-11.

27. Hsieh H., Liu W., Huang P.C., " A novel stress-inducible metallothionein-like gene from rice" *Plant Molecular Biology* 28 (1995) 381-389.

28. Hudspeth R.L., Hobbs S.L., Anderson D.M., Rajasekaran K., Grula J.W., " Characterisation and expression of metallothionein-like in cotton" *Plant Molecular Biology* 31 (1996) 701-705.

29. Kagi, JHR "Metallothionein III" Birkhauser, Basel, (1993) 29-56.

30. Kawashima I., Kennedy T.D., Chino M., Lane B.G., "Wheat E_c metallothionein genes: Like mammalian Zn^{+2} metallothionein genes are conspiciously expressed during embryogenesis" *Eur. J. Biochem.* 209 (1992) 971-976.

31. Kojima Y., Binz PA., & Kagi HR. "Nomenclature of metallothionein: Proposal for a revision. *In*: Klaassen (ed): Metallothionein IV". Birkhauser Verlag, Basel Boston Berlin, (1997) 3-6.

32. Lane B., Kajioka R., Kennedy T., "The wheat-germ E_c protein is a zinc-containing metallothionein" *Biochem. Cell Biol.* 65 (1987) 1001-1005.

33. Ma M., Lau PS., Jia YT., Tsang WK., Lam SKS., Tam NFY., Wong YS. "The isolation and characterization of Type I metallothionein (MT) cDNA from a heavy-metal-tolerant plant, *Festuca rubra* cv. Merlin". *Plant Science*, 164 (2003) 51-60.

34. Mejáre M., Bülow L., " Metal-binding proteins and peptides in bioremedetion and phytoremediation of heavy metals" *Trends in Biotech*. 19:2 (2001) 67-72.

35. Messerle BA, Schaffer A, Vasak M, Kagi JH, Wuthrich K., "Three-dimensional structure of human [113Cd7]-metallothionein-2 in solution determined by nuclear magnetic resonance spectroscopy" J.Mol. Biol. 214 (1990) 765–779

36. Moriguchi T., Kita M., Hisada S., Endo-Inagaki T., Omura M., "Characterization of gene repertoires at mature stage of citrus fruits through random sequencing and analysis of redundant metallothionein-like genes expressed during fruit development" *Gene* 211 (1998) 221-227.

37. Nordberg M., " Metallothioneins: historical review and state of knowledge" *Talanta* 46 (1998) 243-254.

38. Palmiter RD., "The elusive function of metallothioneins" *Proc. Natl. Acad. Sci.* 95 (1998) 8428-8430.

39. Pountney DL, Fundel SM, Faller P, Birchler NE, Hunziker P, Vasak M., "Isolation, primary structures and metal binding properties of neuronal growth inhibitory factor (GIF) from bovine and equine brain" FEBS Letters 345 (1994) 193-197

40. Rauser WE "Structure and function of metal chelators produced by plants: the case for organic acids, amino acids, phytin, and metallothioneins" *Cell Biochem.Biophys.*, **31:** (1999) 19-48.

41. Rauser WE., "The role of thiols in plants under metal stress" *Sulfur Nutrition and Sulfur Assimilation in Higher Plants* (2000) 169-183.

42. Robinson NJ., Tommey AM., Kuske C., Jackson PJ., "Plant metallothioneins" *Biochem J.* 295 (1993) 1-10.

43. Romero-Isart N., Vasak M., "Advances in the structure and chemistry of metallothioneins" *Journal of Inorganic Biochemistry* 88 (2002) 388-396.

44. Romero-Isart N., Vasak M., "Advances in the structure and chemistry of metallothioneins" *Journal of Inorganic Biochemistry* 88 (2002) 388-396

45. Quaife C.J., Findley S.D., Erickson J.C., Froleick G.J., *et al.* Induction of a new metallothionein isoform (MT-IV) occurs during differentiation of stratified squamous epithelia. *Biochemistry*, **33:** (1994). 7250-7259

46. Sambrook J, Manniatis T, Fritsch EF, " Molecular Cloning: A Laboratory Manual" Cold Spring Harbor Laboratory Press,2nd Edition, (1989)

47. Sato M., Bremner I., " Oxygen free radicals and metallothionein" *Free Radical Biology &Medicine* 14 (1993) 325-337.

48. Schultze P, Worgotter E, Braun W, Wagner G, Vasak M, Kagi JH, Wuthrich K., "Conformation of [Cd7]-metallothionein-2 from rat liver in aqueous solution determined by nuclear magnetic resonance spectroscopy" J. Mol. Biol. 203 (1988) 251–268.

49. Snowden KC, Gardner RC, " Five genes induced by aluminum in wheat (Triticum aestivum L.) roots." Plant Physiology 103(3) (1993) 855-61

50. Stillman M.J, Shaw III FC, Suzuki KT, "Metallothioneins Synthesis, Structure and Properties of Metallothioneins, Phytochelatins and Metal Thiolate Complexes" VHC Publishers, Inc. (1992)

51. Stillman M.J., "Metallothioneins" *Coordination Chemistry Reviews* 144 (1995) 461-511.

52. Vasak M., " Application of ^{113}Cd NMR to metallothioneins" *Biodegradation* 9 (1998) 501-512.

53. Vasak M., Hasler DW., "Methalothioneins: New functional and structural insights" Current Opinion in Chemical Biology 4: (2000) 177-183

54. Yu L., Umeda M., Liu J., Zhao N., Uchimiya H., " A novel MT gene of rice plants is strongly expressed in the node portion of the stem" *Gene* 206 (1998) 29-35.

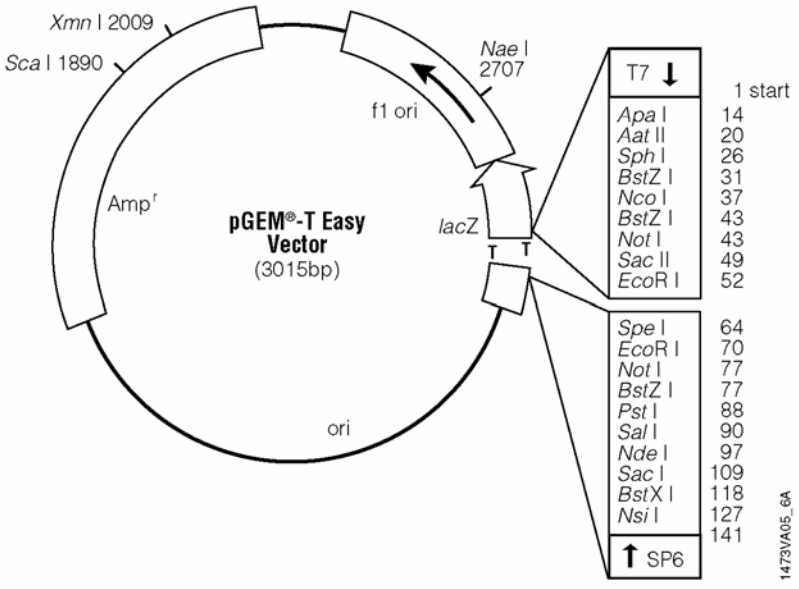
55. Zenk M.H., "Heavy metal detoxification in higher plants" *Gene* 179 (1996) 21-30.

56. Zhou J., Goldsbrough P.B., " Functional homologs of fungal metallothionein genes from Arabidopsis" *The Plant Cell* 6 (1994) 875-884.

57. Zhou J., Goldsbrough P.B., " Structure, organization and expression of the metallothionein gene family in *Arabidopsis" Mol. Gen. Genet*. 248 (1995) 318-328.

APPENDIX A

pGEM-T Easy vector circle map and sequence reference points

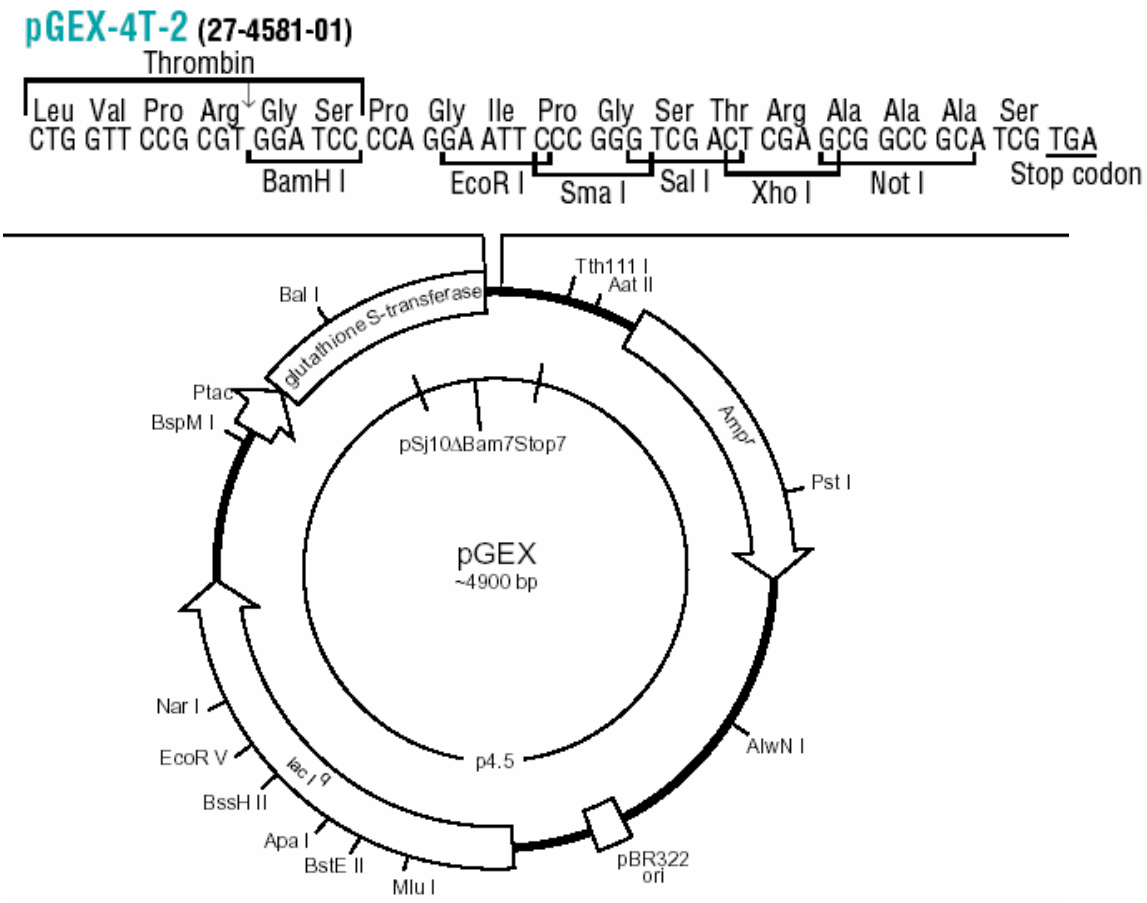


Enzyme	# of Sites	Location
Aat II	1	20
Acc I	1	76
Acy I	2	17, 1932
Afl III	2	99, 502
*Alw*26 I	2	1456, 2232
*Alw*44 I	2	816, 2062
*Alw*N I	1	918
Apa I	1	14
*Asp*H I	4	94, 820, 1981, 2066
Ava II	2	1533, 1755
Ban I	3	246, 1343, 2626
Ban II	3	14, 94, 2664
Bbu I	1	26
Bgl I	3	39, 1515, 2833
Bsa I	1	1456
*Bsa*A I	1	2589
*Bsa*H I	2	17, 1932
*Bsa*J I	5	37, 43, 241, 662, 2936
*Bsp*120 I	1	10
*Bsp*H I	2	1222, 2230
*Bsp*M I	1	62
*Bss*S I	2	675, 2059
*Bst*O I	5	242, 530, 651, 664, 2937
*Bst*X I	1	103
*Bst*Z I	2	31, 62
*Cfr*10 I	2	1475, 2690
Dde I	4	777, 1186, 1352, 1892
Dra I	3	1261, 1280, 1972
Dra III	1	2589
Drd I	2	610, 2544
Dsa I	2	37, 43
Eag I	2	31, 62
Ear I	3	386, 2190, 2878
*Ecl*HK I	1	1395
*Eco*52 I	2	31, 62
*Eco*ICR I	1	92
*Eco*R V	1	51 (see above)
Fok I	5	119, 1361, 1542, 1829, 2919
Fsp I	2	1617, 2840
Hae III	4	380, 750, 2740, 2748
Hga I	4	613, 1191, 1921, 2806
Hinc II	1	77
Hind II	1	77
*Hsp*92 I	2	17, 1932
Mae I	5	56, 997, 1250, 1585, 2740
Mlu I	1	99

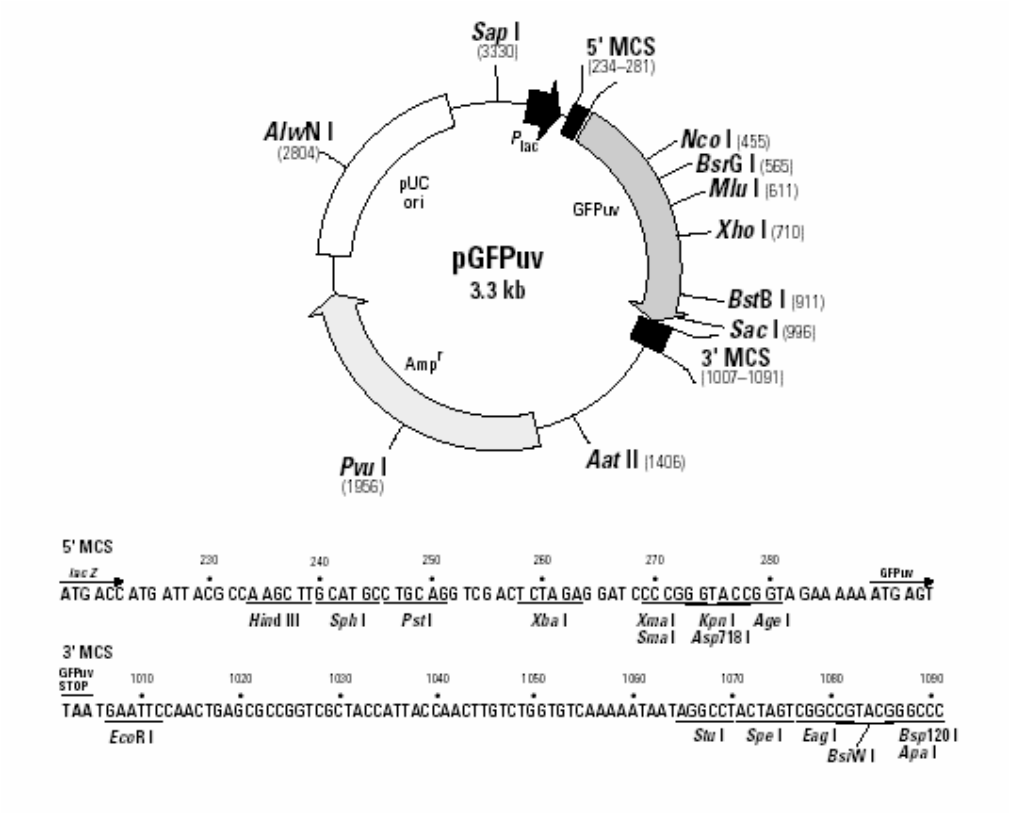
Total number of hits per restriction enzyme for pGEM- T Easy vector

pGEM®-T Easy Vector Sequence reference points:	
T7 RNA Polymerase transcription initiation site	1
SP6 RNA Polymerase transcription initiation site	141
T7 RNA Polymerase promoter (–17 to +3)	2999–3
SP6 RNA Polymerase promoter (–17 to +3)	139–158
multiple cloning region	10–128
lacZ start codon	180
lac operon sequences	2836–2996, 166–395
lac operator	200–216
β-lactamase coding region	1337–2197
phage f1 region	2380–2835
binding site of pUC/M13 Forward Sequencing Primer	2956–2972
binding site of pUC/M13 Reverse Sequencing Primer	176–192

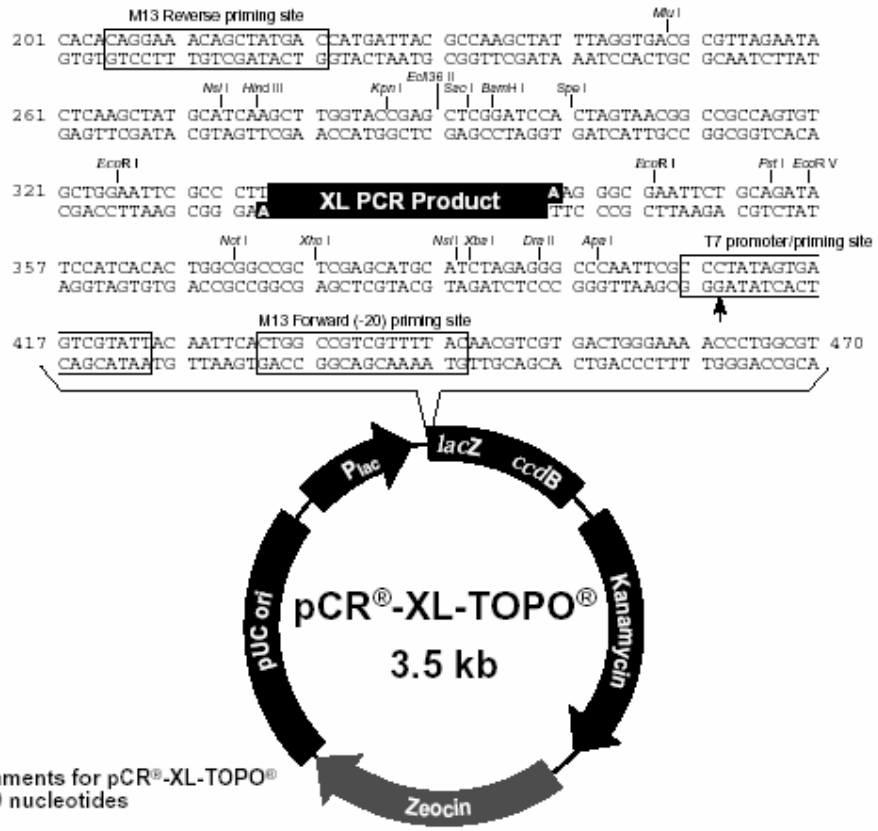
Vector map of pGEX-4T2



pGFPuv vector map showing 5’ and 3’ multiple cloning sites

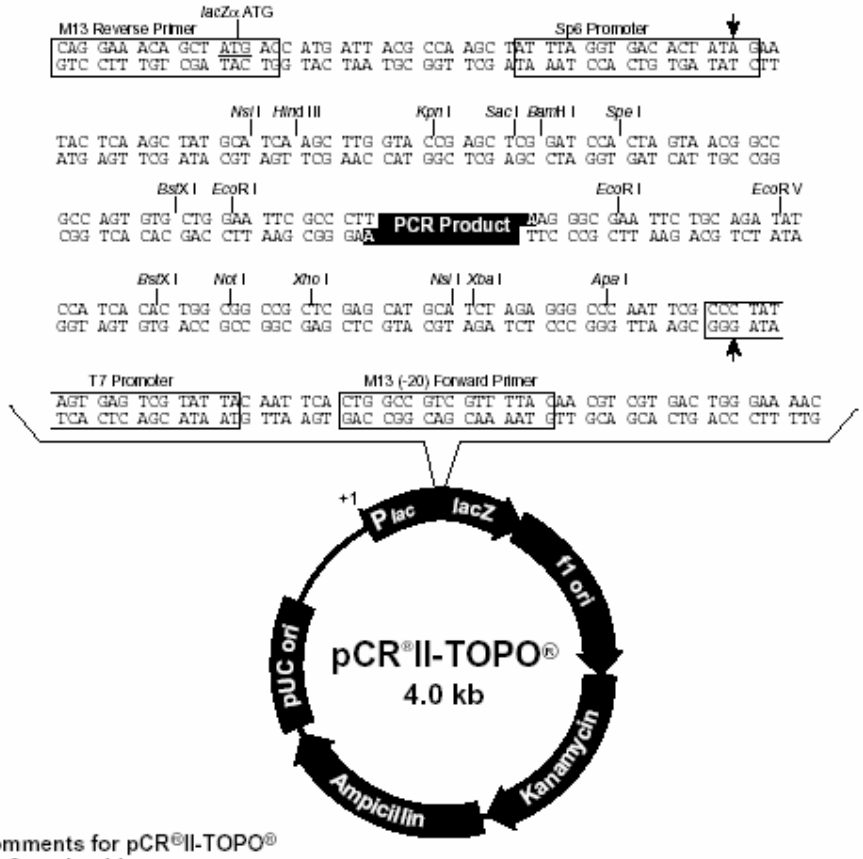


pCR-XL-TOPO vector map and sequence reference points



Comments for pCR®-XL-TOPO®
3519 nucleotides

Lac promoter/operator region: bases 95-216
M13 Reverse priming site: bases 205-221
Lac Za ORF: bases 217-576
Multiple Cloning Site: bases 248-399
TOPO® Cloning site: bases 336-337
T7 promoter priming site: bases 406-425
M13 Forward (-20) priming site: bases 433-448
Fusion joint: bases 577-585
*ccd*B lethal gene ORF: bases 586-888
Kanamycin resistance ORF: bases 1237-2031
Zeocin resistance ORF: bases 2238-2612
pUC origin: bases 2680-3393



Comments for pCR®II-TOPO®
3973 nucleotides

LacZα gene: bases 1-589
M13 Reverse priming site: bases 205-221
Sp6 promoter: bases 239-256
Multiple Cloning Site: bases 269-383
T7 promoter: bases 406-425
M13 (-20) Forward priming site: bases 433-448
f1 origin: bases 590-1027
Kanamycin resistance ORF: bases 1361-2155
Ampicillin resistance ORF: bases 2173-3033
pUC origin: bases 3178-3851

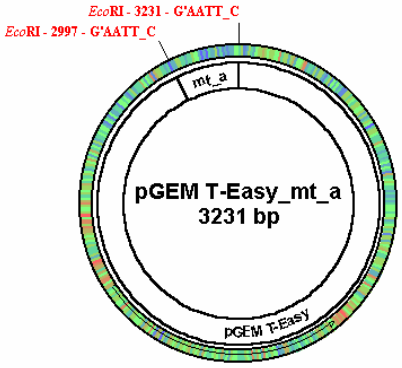
pCRII-TOPO vector map including multiple cloning site and sequence reference points

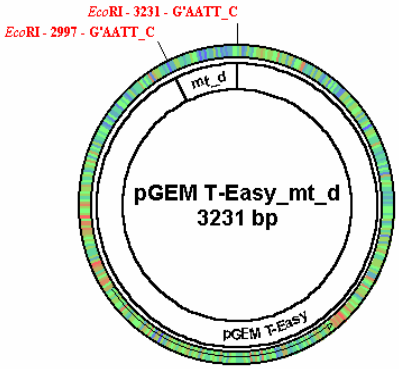
pCR T7/NT-TOPO vector map and sequence reference points

pTrcHis-TOPO vector map and sequence reference points

APPENDIX B

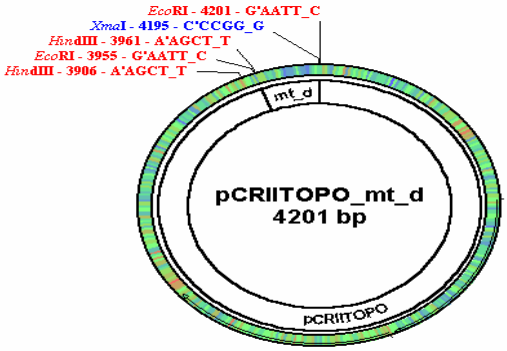
B.1 pGEM T-Easy_mt_a and pGEM T-Easy_*mt-d*





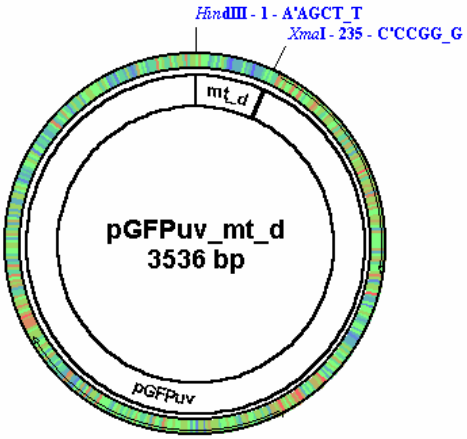
pGEM T-Easy_mt_a and *mt-d* constructs contain the mt_a and *mt-d* genes, respectively In between the *Eco* RI restriction enzyme sites.

B.2 pCRIITOPO_*mt-d*



pCRIITOPO_*mt-d* construct contains *mt-d* gene with *HInd* III at 5'-end and *Xma I* at 3'end, respectively

B.3 pGFPuv_*mt-d*



pGFPuv_*mt-d* construct contains *mt-d* gene derived from pCRII_*mt-d* construct with the *HInd* III and *Xma I* restriction enzymes and inserted into the pGFPuv vector

APPENDIX C

Autoclave:	Hirayama, Hiclave HV-110, JAPAN
	Certoclav, Table Top Autoclave CV-EL-12L, AUSTRIA
Balance:	Sartorius, BP211D, GERMANY
	Sartorius, BP221S, GERMANY
	Sartorius, BP610, GERMANY
	Schimadzu, Libror EB-3200 HU, JAPAN
Centrifuge:	Eppendorf, 5415C, GERMANY
	Eppendorf, 5415D, GERMANY
	Eppendorf, 5415R, GERMANY
	Kendro Lab. Prod., Heraeus Multifuge 3L, GERMANY
	Hitachi, Sorvall RC5C Plus, USA
	Hitachi, Sorvall Discovery 100 SE, USA
Deepfreeze:	-70^{o} C, Kendro Lab. Prod., Heraeus Hfu486 Basic, GERMANY
	-20^{o} C, Bosch, TÜRKİYE
Distilled Water:	Millipore, Elix-S, FRANCE

	Millipore, MilliQ Academic, FRANCE
Electrophoresis:	Biogen Inc., USA
	Biorad Inc., USA
Gel Documentation:	UVITEC, UVIdoc Gel Documentation System, UK
	Biorad, UV-Transilluminator 2000, USA
Ice Machine:	Scotsman Inc., AF20, USA
Incubator:	Memmert, Modell 300, GERMANY
	Memmert, Modell 600, GERMANY
Laminar Flow:	Kendro Lab. Prod., Heraeus, HeraSafe HS12, GERMANY
Magnetic Stirrer:	VELP Scientifica, ARE Heating Magnetic Stirrer, ITALY
	VELP Scientifica, Microstirrer, ITALY
Microliter Pipette:	Gilson, Pipetman, FRANCE
	Mettler Toledo, Volumate, USA
Microwave Oven:	Bosch, TÜRKİYE
pH meter:	WTW, pH540 GLP MultiCal®, GERMANY
Power Supply:	Biorad, PowerPac 300, USA

	Wealtec, Elite 300, USA
Refrigerator:	+4^{o} C, Bosch, TÜRKİYE
Shaker:	Forma Scientific, Orbital Shaker 4520, USA
	GFL, Shaker 3011, USA
	New Brunswick Sci., Innova™ 4330, USA
Spectrophotometer:	Schimadzu, UV-1208, JAPAN
	Schimadzu, UV-3150, JAPAN
	Secoman, Anthelie Advanced, ITALY
Speed Vacuum:	Savant, Speed Vac® Plus Sc100A, USA
	Savant, Refrigerated Vapor Trap RVT 400, USA
Thermocycler:	Eppendorf, Mastercycler Gradient, GERMANY
Vacuum:	Heto, MasterJet Sue 300Q, DENMARK
Water bath:	Huber, Polystat cc1, GERMANY