

Phytoextraction of Heavy Metal from Contaminated Soils Using Genetically Modified Plants

Von der Fakultät für Mathematik, Informatik und Naturwissenschaften der
Rheinisch-Westfälischen Technischen Hochschule Aachen
zur Erlangung des akademischen Grades einer
Doktorin der Naturwissenschaften
genehmigte Dissertation

vorgelegt von

Master of Science

Hatice Daghan

aus

Adana, Türkei

Berichter: Universitätsprofessor Dr. rer. nat. Andreas Schäffer
Universitätsprofessor Dr. rer. nat. Rainer Fischer

Tag der mündlichen Prüfung: 07. Dezember 2004

Diese Dissertation ist auf den Internetseiten der Hochschulbibliothek online verfügbar.

LEBEN

Einzel und frei wie ein Baum
-Aber brüderlich wie der Wald
Das ist unsere Sehnsucht

YASAMAK

Bir agac gibi tek ve hür
Ve bir orman gibi kardescesine
Bu hasret bizim.

To LIVE

Like a tree single and at liberty
and brotherly like the trees of a forest
This yearning is ours.

Nazim Hikmet Ran

CONTENTS

	Page
I INTRODUCTION	1
I.1 Contamination by heavy metals in soils	2
I.1.1 Effect of soil properties on metal bioavailability	4
I.1.2 Health effect of heavy metals	5
I.1.3 Cadmium	5
I.2 Phytoextraction	7
I.2.1 Limitations of phytoextraction	9
I.2.2 Advantages of phytoextraction	9
I.2.3 Phytoextraction market	10
I.2.4 Ideal plants for phytoextraction	10
I.2.5 Uptake and accumulation of metals in plants	11
I.2.6 Definition and characteristics of metal hyperaccumulators	14
I.2.7 Metallothioneins	15
I.2.7.1 Structure and occurrence of MT	16
I.2.7.2 Classes of metallothionein	17
I.2.7.3 Physical and chemical characterization of MTs	20
I.3 Genetic engineering	21
I.4 Aim of this thesis	25
II MATERIALS AND METHODS	28
II.1 Materials	28
II.1.1 Chemicals and consumables	28
II.1.2 Enzymes and reaction kits	28
II.1.3 Primary antibodies, secondary antibodies and substrates	28
II.1.4 Bacterial strains	29
II.1.4.1 <i>Escherichia coli</i> Strains	29
II.1.4.2 Agrobacteria strains	29
II.1.5 Vectors	30
II.1.6 Oligonucleotides	30
II.1.7 Buffers, media and solutions	31
II.1.8 Matrices and membranes	32
II.1.9 Equipment and applications	32

II.2	Methods	34
II.2.1	Recombinant DNA technologies	34
II.2.1.1	Preparation of heat-shock competent <i>E.coli</i> cells	34
II.2.1.2	Transformation of <i>E. coli</i> by heat-shock	35
II.2.1.3	Preparation of electrocompetent <i>E. coli</i> cells	35
II.2.1.4	Transformation of <i>E. coli</i> by electroporation	35
II.2.1.5	Preparation of electrocompetent <i>Agrobacterium</i> cells	35
II.2.1.6	Transformation of <i>Agrobacterium</i> by electroporation	36
II.2.1.7	Preparation of heat-shock competent <i>Agrobacterium</i> cells	36
II.2.1.8	Transformation of <i>Agrobacterium</i> by heat-shock	37
II.2.1.9	Determination of the efficiency of recombinant bacteria transformation	37
II.2.1.10	Culturing of <i>E. coli</i> and glycerol stock preparation	37
II.2.1.11	Growth of recombinant <i>A. tumefaciens</i> and preparation of glycerol stocks	37
II.2.1.12	Isolation of plasmid-DNA from <i>E.coli</i>	38
II.2.1.13	Agarose gel electrophoresis of DNA	38
II.2.1.14	Preparative agarose gel electrophoresis	39
II.2.1.15	PCR amplification	39
II.2.1.16	Splice overlap extension PCR	40
II.2.1.17	DNA sequencing by LICOR and ABI Method	41
II.2.1.18	Sequence analysis	41
II.2.2	Generation and characterisation of transgenic plants	42
II.2.2.1	Transient assay in tobacco leaves by vacuum infiltration	42
II.2.2.2	Preparation of recombinant <i>Agrobacteria</i>	42
II.2.2.3	Vacuum infiltration of intact leaves	42
II.2.2.4	Recombinant <i>agrobacterium</i> -mediated stable transformation of tobacco plants	43
II.2.2.5	Growth of <i>N. tabacum</i> cv. Petite Havana SR1	44
II.2.2.6	Preparation of total soluble proteins from plant leaves	44
II.2.3	Protein analysis	45
II.2.3.1	Quantification of proteins	45
II.2.3.2	SDS-PAA gel electrophoresis and Coomassie brilliant blue staining	45
II.2.3.3	Immunoblot analysis	46
II.2.4	Immuno localisation	47
II.2.4.1	Immunogold labelling	47
II.2.4.2	Immunofluorescence labelling	48

II.2.4.3	Fluorescence microscopy	48
II.2.5	Hydroponic experiments	48
II.2.5.1	Plants growth	48
II.2.5.2	Tissue preparation	49
II.2.5.3	Dry digestion	50
II.2.5.4	Atomic absorption spectrometry (AAS)	50
II.2.5.5	Analysis of SH-Groups	50
II.2.5.6	Analysis of phytochelatins	51
II.2.6	Soil experiments	51
II.2.6.1	Soil properties	51
II.2.6.2	Soil preparation	52
II.2.6.3	Soil analysis	52
II.2.6.4	DTPA- method	53
II.2.6.5	Aqua regia-method	53
II.2.6.6	Pot experiments	53
II.2.6.7	Tissue preparation	53
II.2.6.8	Plant analysis	54
III	RESULTS	55
III.1	Cloning of metallothionein II	55
III.1.1	Cloning of the Chinese Hamster Metallothionein II vacuolar targeting cassette	55
III.1.2	Cloning of <i>Saccharomyces cerevisiae</i> and <i>Chinese hamster</i> Metallothioneins	56
III.2	Generation and characterization of transgenic plants	59
III.2.1	Transient expression of metallothioneins	59
III.2.2	Fluorescence microscopy analysis	60
III.2.2.1	GFP Analysis	60
III.2.3	Immunofluorescence analysis	61
III.2.4	Generation and screening of transgenic tobacco plants expressing Metallothioneins II	61
III.2.5	Immunogold labelling	64
III.3	Hydroponic experiments	66
III.3.1	Symptoms of cadmium toxicity on shoots and roots	66
III.3.2	Immunoblot analysis of Cd treated plants	67
III.3.3	Analysis of the plant dry weight	69

III.3.4	Analysis of the cadmium contents	70
III-3-5	Analysis of total soluble protein	71
III.3.6	Analysis of free sulfhydryl groups	71
III.3.7	Analysis of phytochelatins in shoots	72
III.4	Soil experiments	74
III.4.1	Symptoms of Cd toxicity on leaves	74
III.4.2	Immunoblot analysis	75
III.4.3	Analysis of the plant dry weight	76
III.4.4	Analysis of cadmium content	77
III.4.5	Analysis of free sulfhydryl groups	78
IV	DISCUSSION	80
IV.1	Generation and characterization of transgenic plants	81
IV.2	Hydroponic experiments	83
IV.3	Soil experiments	88
IV.4	Conclusion and future	91
V	SUMMARY	93
VI	REFERENCES	95
VII	APPENDICES	106
VII.1	Abbreviations	106
VII.2	Nucleic acid and amino acid sequence of Chinese Hamster Metallothionein II	108
VII.3	Nucleic acid and amino acid sequence of <i>Saccharomyces cerevisiae</i> Metallothionein II	108
VII.4	Nucleic acid and amino acid sequence of Sweet potato sporamin A tuberous root storage protein	109
VII.5	List of Figures	109
VII.6	List of Tables	111

I INTRODUCTION

Our modern society produces large amount of wastes and pollutants. Primary sources for pollution are the burning of fossil fuels, mining and melting of metalliferous ores, municipal wastes, fertilizers, pesticides, and sewage sludge. Contamination of soil, aqueous streams and ground water with toxic metals poses a major environmental problem and a serious danger to human health that still need an effective and affordable technological solution.

In the past there has been little concern about pollution because the population on earth was not too large and there was plenty of space to dispose of the wastes. However, the rapid population growth and the global changes that have occurred in the last century in our society have dramatically increased the production of wastes and new types of pollutants (Tan, 1994). The soil has been traditionally the site for disposal of most of the wastes with the result that strong pollution and the risk of serious contamination have significantly raised in recent years. Various physical, chemical and biological processes are already being used to remediate contaminated soil. Phytoremediation represents one of the most promising, effective and technically affordable solutions.

Some plants have developed the ability to remove ions selectively from the soil to regulate the uptake and distribution of metals in their tissues. Most metal uptake occurs in the root system, usually via absorption, where many mechanisms are available to prevent toxic effects due to the high concentration of metals in the soil and water (EPA, 1996). Phytoremediation is the use of these plants to clean up the environment. The word phytoremediation comes from the Greek word phyton, "plant," and the Latin word "to remediate". Phytoremediation is an environmentally friendly, safe, cheap way to clean up contaminants. The idea of using plants to remove or inactivate pollutants from soils and surface waters was reintroduced and developed by Utsunomyia (1980) and Chaney (1983). In recent years phytoremediation has received increasing attention and extensive research has been conducted to investigate the biology of metal phytoextraction (Cunningham *et al.*, 1995; Salt *et al.*, 1995, 1998; Raskin 1996; Chaney *et al.*, 1983, 1997; Raskin *et al.*, 1997; Blaylock and Huang 2000; Pilon-Smits and Pilon 2000; Kayser, 2000; Krämer and Chardonnens, 2000).

Phytoremediation is of public acceptance and is an aesthetically pleasant, solar-energy driven, passive technique that can be used to clean up sites with shallow, low to moderate levels of contamination. Phytoremediation is not only a growing science; it's also a growing industry. This technique can be used along with or, in some cases, in place of mechanical cleanup methods (Kayser, 2000). Early estimates on the costs for remediating contaminated sites have shown that plants could do that same job as a group of engineers for one tenth of the cost. The

soil or water does not need to be gathered in and stored as hazardous waste, requiring large amounts of land, money, and manpower. Plants can be sown, watered, and then harvested with less manpower. The storage of the harvested plants as hazardous waste is seldom required and when needed is less demanding than traditional disposal techniques. However, the main drawback on the use of this novel technology is that it is not applicable to all sites.

Several mechanisms may be involved in the direct and indirect action of phytoremediation in contaminated soils. Therefore, phytoremediation of heavy metals can be divided into three groups (Figure I-1):

1. Phytoextraction; the use of metal-accumulating plants to remove toxic metals from soil,
2. Phytostabilization; the use of plants to eliminate the bioavailability of toxic metals in soils,
3. Phytorhizofiltration, the use of plant roots to remove toxic metals from polluted waters,

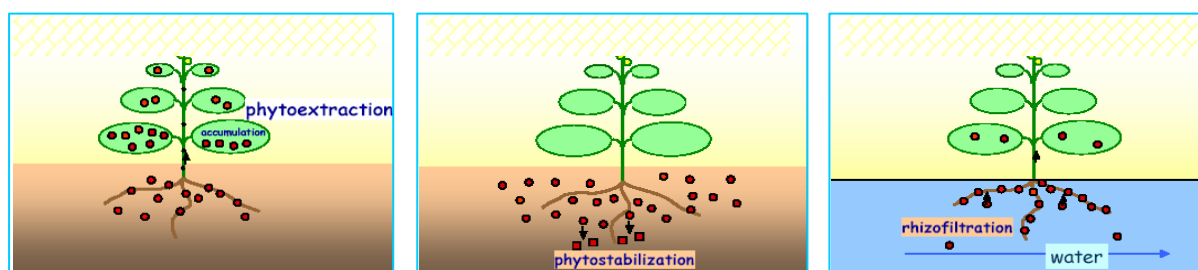


Figure I-1: Processes of Phytoremediation (adapted from Pilon-Simits, 2003).

The remediation of soils contaminated with heavy metals is based on mechanisms of phytoextraction and phytostabilization. In the present study a phytoextraction method was developed that uses genetically modified tobacco plants to remove heavy metals from contaminated soil.

I.1 Contamination by heavy metals in soils

Heavy metals are conventionally defined those elements with metallic properties and atomic number > 20 . These elements are natural components of soils in trace amounts. However, when their concentration exceeds thresholds of bio-tolerance, heavy metals are toxic and have to be regarded as dangerous soil pollutants.

The most common heavy metal contaminants are: Cadmium (Cd), Chromium (Cr), Copper (Cu), Mercury (Hg), Lead (Pb) and Zinc (Zn). Some of these elements, such as copper and zinc, are essential micronutrients in cellular metabolism and serve as structural and catalytic

components of proteins and enzymes. However, high concentrations of these micronutrients and heavy metals are extremely toxic to the cell metabolism. Therefore, contamination of the soil by heavy metals can result in unbalanced microflora and microfauna and toxic effects on the mesofauna and on plants. In particular, soils with high pollution by heavy metals show poor plant growth and low covering of the soil surface with the consequence that metals leach into runoff water with subsequent deposition into ground and surface waters (Lasat, 2000).

The most dangerous heavy metal pollutants are listed in Table I-1 where the limit of their tolerance is shown.

Table I-1: Currently found concentration ranges and regulatory guidelines for important metal contaminants in US soils in the order of relative importance (Salt *et al.*, 1998).

Metals	Soil Concentration range ^a (mg kg ⁻¹)	Regulatory Limit ^b (mg kg ⁻¹)
Pb	1.00-6,900	600
Cd	0.10-345	100
As	0.10-102	20
Cr	0.05-3,950	100
Hg	<0,1-1,800	270
Zn	150.00-5,000	1,500

^{a)} Riley *et al.*, 1992

^{b)} Nonresidential direct contact soil cleanup criteria (NJDEP, 1996).

In the soil metals exist as components of several fractions:

- 1) Free metal ions and soluble metal complexes in solution;
- 2) Metal ions occupying ion exchangeable sites and specifically adsorbed on inorganic soil constituents;
- 3) Metals bound to organic matter;
- 4) Precipitated or insoluble compounds, particularly in form of oxides, sulphides, carbonates and hydroxides; and
- 5) Metals entrapped in the structure of silicate minerals.

Contamination of soils by heavy metals of anthropogenic source, generally results in higher level of metals occurring in the fractions (1)-(4) while metals in fraction (5) are indicative of background or autochthonous soil concentrations.

The effectiveness of phytoextraction for remediation of heavy metal contaminated soils is highly dependent on the availability of the metals for plant uptake. A major limitation to

phytoextraction is that only fraction (1) and, possibly some components of fraction (2) are readily available to plants. Therefore, manipulation of the soil environment to enhance the availability of metals is crucial to phytoremediation.

The chemistry and bioavailability of heavy metals in soils has been thoroughly investigated however most of this research has been directed towards studying methods for inhibition or reduction of metal availability in the soil. There is very little data available on practices designed to enhance metal uptake by plants, except for those cases where micronutrient deficiencies had to be overcome through addition of chelated micronutrient compounds (Salt *et al.*, 1995).

To balance the concentration of these toxic metals in cells, all organisms induce biosynthesis of low molecular weight, cysteine rich proteins called metallothionein (MT) (Liu *et al.*, 2000).

I.1.1 Effect of soil properties on metal bioavailability

Bioavailability and mobility of metals is strongly affected by physical and chemical characteristics of the soil, such as pH, content of clay minerals and organic matter, element concentrations (Figure I-2).

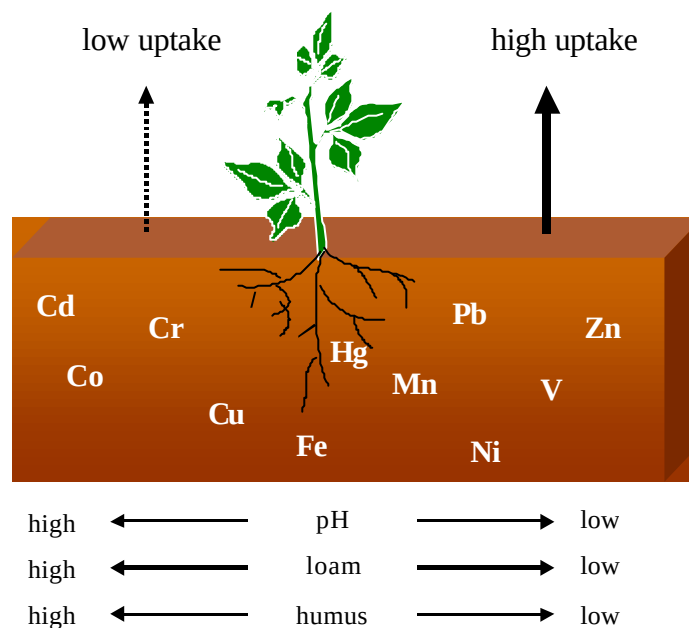


Figure I-2: General limitations of phytorextraction

The chemistry of metal interaction with the soil matrix is central to the phytoremediation concept. In general, sorption to soil particles reduces the activity of metals in the system. Thus, the higher the cation exchange capacity (CEC) of the soil, the greater the sorption and immobilization of the metals. In acidic soils, due to competition of H^+ for binding sites on the

colloidal components of the soil, metal desorption and release into solution is stimulated. Therefore, soil pH affects not only metal bioavailability, but also indirectly also the process of metal uptake into the roots. However, the effects on metal bioavailability appear to be related to the properties of each metal (Lasat, 2000).

I.1.2 Health effect of heavy metals

As a result of an exponential increase in the use of heavy metals in industrial processes and products, human exposure to heavy metals has risen dramatically in the last 50 years. Heavy metals may enter the human body through food, water, air, or absorption through the skin (Life Extension, 2003). Today cases of chronic exposure have been reported for mercury-amalgam dental fillings, lead in paint and tap water, chemical residues in processed foods, and personal care products (cosmetics, shampoo and other hair products, mouthwash, toothpaste, soap). In today's industrial society, there is no possibility to avoid exposure to toxic chemicals and metals.

The toxic effects of heavy metals result in damaged or reduced function of the central nervous system, reduced availability of biological energy, and damage to blood composition, lungs, kidneys, liver, and other vital organs. Long-term exposure to heavy metals may result in chronic physical, muscular, and neurological degenerative processes that mimic Alzheimer's disease, Parkinson's disease, muscular dystrophy, and multiple sclerosis. In addition, toxic metals can increase allergic reactions, cause genetic mutation, compete with good trace metals for biochemical binding sites, and act as broad range antibiotics against both harmful and beneficial bacteria (Farr, 2001). Heavy metal toxicity and the danger of their bioaccumulation in the food chain represent one of the major environmental and health problems of our modern society.

I.1.3 Cadmium

Cd is a nonessential heavy metal that is widespread in our environment because of contamination by power stations, metal industries and waste incineration. Toxicity to living cells is occurring at very low concentration, with suspected carcinogenic effects in humans. However, the biological effects of this metal and the mechanisms of its toxicity are not yet clearly understood (Suziki *et al.*, 2001). Cd is one of the increasingly frequent contaminants of agricultural soils, where it is usually present at 0.1–0.2 mg/kg but occasionally has been detected at much higher levels in some regions. Cadmium contamination in agricultural soils

is due to either excessive phosphate fertilization, use of sewage sludge as a soil amendment, or to naturally high background levels (de Borne *et al.*, 1998).

For human health and environmental restoration, the use of plants to clean up toxic metals from contaminated soil or water has been developed. Because a number of plant species are naturally capable of high levels of organic compound degradation or of heavy metal hyperaccumulation, plant biotechnology provides an opportunity to develop transgenic plants with increased metal binding or metal reducing capacity. Besides, phytoremediation is a low cost technology, and some extracted metals may be recycled (Liu *et al.*, 2000).

Table I-2: Cadmium accumulations in plants on a Cd contaminated soil (Kayser, 1999).

Plant	Concentration of Cd in soil (ppm)	Extracted Cd per ha and year (g/ha)	Years until Cd Conc. 0.8 ppm (mg/kg)
<i>Thlaspi caerulescens</i>	6.6	92	186
<i>Nicotiana tabacum</i>	6.6	92	186
<i>Zea mays</i>	6.6	38	450

Cadmium has no essential function in plants and at high concentrations is toxic to plants and animals. Uptake of Cd by plant roots depends on the concentration, the oxidation state of this metal in solution and on the physico-chemical characteristics of the soils such as pH, content of clay minerals and organic matter.

Besides the characteristics of the soil, the uptake of heavy metals by plants depends also on plant factors (Table I-2). Large genotypic differences in Cd contents have been described between plant species and even between cultivars and inbred lines of the same species, e.g. lettuce, wheat and barley, maize and tobacco. Differences in Cd contents between plant species may be due, at least in part, to differences in Cd translocation from roots to shoots. Most crop species tend to accumulate Cd at the highest concentrations in the root tissue, followed by leaves, then by seeds or storage organs. In contrast, some species, such as cabbage (*Brassica oleracea* L.), lettuce (*Lactuca sativa* L.) and tobacco (*Nicotiana tabacum* L.), accumulate high levels of Cd in leaves rather than in roots (Brandle *et al.*, 1993).

Considerable differences exist between the effects of various plant species on the rhizosphere pH of a given soil (Marschner, 1995). The production of trace elements chelating compounds, such as organic acids, amino acids in root exudates or mucilage affects the solubility and

increases or decreases the bioavailability of metal ions. For instance, more Cd was extracted from soils by root exudates of tobacco than maize.

The mobilization of Cd from the soil may be only of minor importance for the genotypic differences in shoot Cd contents between plant species, because the ranking order of plant species in terms of Cd contents in the shoot dry matter in soil experiments differs hardly from that in nutrient solution experiments.

Phytoextraction is apparently a viable option for remediating Cd contaminated soils. Addition of chelators such as organic acids, EDTA and EGTA, adjustments of the soil pH, adequate fertilization with NH_4 , K and P, addition of sulphur to increase glutathione activity, exploiting mycorrhizal and microbes uptake and the use of biotechnology to increase biomass of hyperaccumulators and/or increase accumulation in species that produce large biomass might be all valuable solutions for improving Cd-phytoextraction. In particular, some biotechnological solutions may include over expression of glutathione in plants, down regulation of $\text{H}^+/\text{Cd}^{2+}$ antiports, increase the production of organic acids, over expression of phytochelatin synthase and increasing antioxidant activity inside the cell. The most practical solution currently available for Cd-cleanup is arguably EGTA-assisted phytoextraction using high-biomass/high-accumulating species (Cox, 2000).

In plants Cd damages the light harvesting complex II and photosystems II, and I, which are active in photosynthesis. Total chlorophyll content is decreased by Cd treatment, and non-photochemical quenching is increased in *Brassica napus*. Probably Cd also interferes with movement of K^+ , Ca^{2+} and abscisic acid in guard cells, while inhibiting stomal opening. Cd alters the synthesis of RNA, inhibits ribonuclease activity, decreases catalase activity and increases ascorbate peroxidase activity in *Phaseolus aureus* (Shaw, 1995). It is highly probable that Cd interferes with transcription or signal transduction mechanisms. In *Schizosaccharomyces pombe*, fungi and plants, Cd can also be detoxified by chelation to glutathione (GHS) or phytochelatin. Cd-phytochelatin and Cd-GSH complexes are transported into the vacuole by ATP-dependent transporters (Suziki *et al.*, 2001).

I.2 Phytoextraction

Phytoextraction, or phytoaccumulation, is referred to as the uptake and translocation of metal contaminants in the soil via the roots into the aboveground portions of the plants. Certain plants called hyperaccumulators absorb unusually large amounts of metals in comparison to other plants (e.g. up to 0.1% chromium, cobalt, copper or nickel or 1% zinc, manganese in the

aboveground shoots on a dry weight basis). Such hyperaccumulators are taxonomically widespread throughout the plant kingdom (Cunningham *et al.*, 1995) and are relevant to phytoremediation. Little is known about their agronomic characteristics, pest management, breeding potential and physiology. Using wild plants as a seed source is, therefore, also unreliable (Cunningham *et al.*, 1995). These plants are often rare and grow in remote regions; in certain cases, their habitat is threatened by mining, development and other industrial activities.

Phytoextraction is the use of hyperaccumulating plant species to remove metals from the soil by absorption into the roots and shoots of the plant. To physically remove metals from the contaminated site the aboveground shoots of the hyperaccumulator plants are harvested and subsequently disposed of as hazardous wastes or treated for the recovery of the metals (Evanko *et al.*, 1997). Phytoremediation can be used to remove not only metals (e.g. Ag, Cd, Co, Cr, Cu, Hg, Mn, Mo, Ni, Pb, Zn) but also radionuclides (e.g. ^{90}Sr , ^{137}Cs , ^{239}Pu , ^{234}U , ^{238}U) and certain organic compounds (i.e., petroleum hydrocarbons) (Andrade *et al.*, 2002). Depending on the type of metal contaminants, single or combinations of hyperaccumulator species is selected and planted on the contaminated site. Preliminary field tests should be carried out to identify appropriate agricultural practices that may be required to ensure plant growth.

After several weeks or months of cultivation, the plants are analysed for the content in heavy metals and if appropriate, harvested and either incinerated or composted to recycle the metals. If necessary the process may be repeated to bring the level of the contaminant in the soil beneath limits of tolerance. As well, several species can be used onto a site, either at the same time or in sequence, to remove more than one contaminant. If the plants are incinerated, the ashes must be disposed of in landfill for hazardous wastes. The volume of ash that is produced by this method is less than 10% of the volume that would be produced if the contaminated soil is dug up for treatment (EPA, 1999).

Most plants that are growing on contaminated soils have developed the capacity to effectively exclude heavy metals from their tissues. Although plants take up and accumulate certain essential nutrients from the soil to concentrations as high as 1-3 %, the level of heavy metals only reaches 0.1 to 100 mg kg⁻¹ biomass. Some nonessential elements that are not particularly harmful (e.g. silicon and sodium) may accumulate to large amounts; on the contrary, hyperaccumulation of toxic metals in plants is rare.

I.2.1 Limitations of phytoextraction

The use of hyperaccumulators for phytoextraction is limited by several factors:

- Phytoextraction is slower than physico-chemical methods, and should be regarded as a long-term remediation process. Plants remove or degrade only small amounts of contaminants each growing season, so it can take several decades to adequately clean up a site.
- Chemical as well as physical factors such as climate, soil texture, pH, salinity, and concentration of pollutants and the presence of toxins must be within the limit of tolerance for the hyperaccumulator species. It has been reported that only lightly contaminated soils can be phytoremediated because most plants will not grow on heavily contaminated sites (Cox, 2000).
- Heavy metals in soils might be precipitated, incorporated into soil minerals or biota or sorbed to the soils matrix. At high pH, heavy metals are hardly bioavailable. In addition, metal availability might also be limited by diffusion kinetics.
- Contaminants that are highly water-soluble may leach outside the root zone and require containment.

Despite these limitations, in cases where large areas are polluted with relatively immobile contaminants at the soil surface, phytoremediation may still represent the most appropriate solution.

Heavy metal removal from soils by plants is equivalent to the product of dry weight plant biomass and metal concentration in the harvested parts of the plants. Improvements of phytoextraction may be achieved by increasing either of these factors. For example, new plant species may be selected with better genetic potential to accumulate metals, or plant husbandry and soil management practices may be improved to enhance metal uptake (Kayser, 2000).

I.2.2 Advantages of phytoextraction

Nevertheless, phytoextraction has some significant advantages (Cunningham *et al.*, 1995, Andrade *et al.*, 2002 and EPA, 2000);

1. can be used on large areas where other technologies are prohibitive
2. is a permanent solution, because organic pollutants for example may be mineralised
3. is aesthetically pleasant
4. the plant biomass may be converted into raw material for activities such as furniture making, power generation, fibre production, etc.

5. helps to reduce processes of erosion by wind and water, thereby reducing possible contaminations of lakes and rivers.
6. Phytoextraction requires low capital investments and operating costs. Plants are a cost-effective alternative to physical remediation systems.
7. the plant biomass containing the extracted contaminant can be a resource. For example, biomass that contains selenium (Se), an essential nutrient, has been transported to areas that are deficient in Se and used for animal feed.

I.2.3 Phytoextraction market

According to Glass (1999), the global phytoremediation market was approximately U.S. \$ 35 million in 1999 and has been predicted to grow ten-fold over the coming 5 years. It has good chances in the future to increase its share of the global remediation market, which was approximately U.S. \$ 18–19 million in 1998 (Krämer *et al.*, 2001).

Kidney (1997) has estimated the current domestic market for phytoremediation to be only \$2 to \$3 million for organics removal from groundwater, and \$1 to \$2 million for removal of heavy metals from soils. The same study indicates that by the year 2005, however, the market for phytoremediation of organics in groundwater will be \$20 to \$45 million, of metals in soils will be \$40 to \$80 million, and of radionuclides will be \$25 to \$50 million. Each application of plants will yield a separate performance evaluation including rate and extent of cleanup and cost (EPA, 2000).

I.2.4 Ideal plants for phytoextraction

Phytoremediation has a huge potential for growth, due to the large number of species that might be suitable to this technique. Currently there are around 250,000 described species of superior plants. These plant species comprise varieties or cultivars, most of which have not yet been tested for this purpose. There are also many more species that have yet to be "discovered". Furthermore, crossbreeding and selection, as well as genetic modifications may improve those species with potential for this type of use. In other words, there is still a lot of room for research, improvement and application of the technology (Andrade *et al.*, 2002).

Scientists have found that many plants naturally absorb metals from the ground and store them in their tissues. Plants, like animals, need metals such as zinc and copper for growth. According to Raskin (1994), plants cannot distinguish between heavy metals such as cadmium and those that are needed as nutrients.

Based on the result of a screening carried out on various plants, Ensley has shown that *Brassica*, the genus to which broccoli and Indian mustard belong, grow faster, produce more biomass and can remediate more metals from the soil than other species (Harvey B, 1995). Salt *et al.*, (1995), found that several crop species that produce high biomass, and are related to wild mustards, could accumulate heavy metals in their shoots. Of all the species screened, certain cultivars of *Brassica juncea* (Indian mustard) showed the highest accumulation of Pb in the shoot and the ability to accumulate and tolerate Cd, Cr (VI), Ni, Zn, and Cu. Their general screen demonstrated a high variability between species and between cultivars within a species for the accumulation of heavy metals.

Ideally, plant species suitable to phytoextraction should show the following characteristics (Kärenlampi, *et al.*, 2000):

1. Ability to accumulate and tolerate high concentrations of metals in harvestable tissue;
2. Rapid growth rate;
3. High biomass production (Miller, 1996);
4. Deep rooting;
5. Easily harvestable.

I.2.5 Uptake and accumulation of metals in plants

Plant growing in metal contaminated environment can accumulate toxic metal ions and efficiently compartmentalize these into various plant parts. Several studies indicated that the partitioning of heavy metals at the whole plant level could broadly be divided into three categories. For instance, Chaney and Giordano classified Mn, Zn, Cd, B, Mo and Se as elements, which were readily translocated to the plant shoots; Ni, Co and Cu, were intermediate, and Cr, Pb and Hg were translocated to the lowest extent (Alloway, 1995).

However, the uptake of toxic metals, their translocations to plant parts and the degree of tolerance to them are dependent on metal speciation, and on the metabolism of the plants (Prasad *et al.*, 1999). The accumulation of heavy metals may involve several processes, including metal binding in the apoplasm, the formation of soluble complexes and insoluble deposits in the cytoplasm and their secretion into vacuoles. Binding to the cell wall is not the only plant mechanism responsible for metal immobilization into roots and subsequent inhibition of ion translocation to the shoot. Metals can also be complexed and sequestered in cellular structures (e.g., vacuole) becoming unavailable for translocation to the shoot (Lasat *et al.*, 1998). In addition, some plants, referred to as excluders, possess specialized mechanisms to restrict metal uptake into roots. However, the concept of metal exclusion is not well

understood (Peterson, 1983). In the metallophyte *Thlaspi caerulescens* Cd accumulated mainly in the apoplasm and, to a lesser extent, in vacuoles, whereas Zn was principally found in vacuoles and to a lesser extent, in cell walls. These results may be due to differences in the metabolism of the two metals, to their concentrations in the plants, or to the different fixation and detection methods that were used by the various authors (Guo, 1995). Organic acids (citrate, malate) or phytic acid in the vacuole can act as effective metal ligands at vacuolar pH.

Uptake of metals into root cells, the point of entry into living tissues, represent the most important step for the process of phytoextraction. However, phytoextraction requires transport of the metals from the root to the shoot. Two processes primarily control translocation of metals in plants, i.e. movement of the metal-containing sap from the root to the shoot: root pressure and leaf transpiration. Following translocation to leaves; metals can be reabsorbed from the sap into leaf cells. A schematic representation of metal transport processes that take place in roots and shoots is shown in Figure I-3;

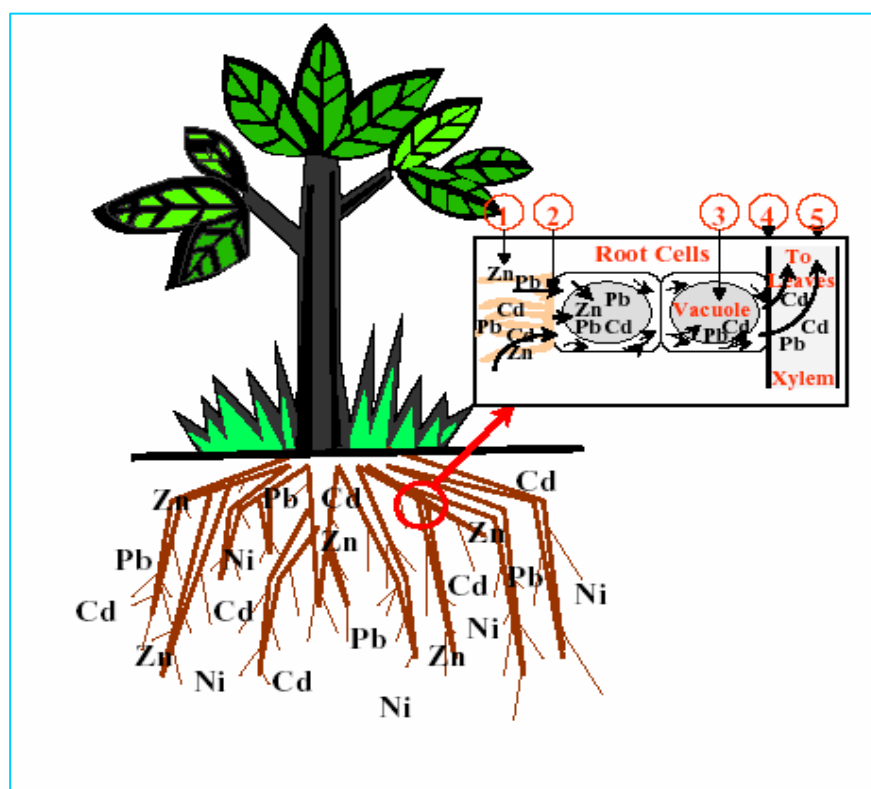


Figure I-3: Metal uptake and accumulation in plants (Lasat, 2000).

1. A metal fraction is sorbed at the root surface
2. The bioavailable metal moves across the cell membrane into root cells
3. A fraction of the metal absorbed into the roots is immobilized in the vacuole
4. The intracellular mobile metal fraction crosses the cell membrane and moves into the root vascular tissue (Xylem)
5. The metal is translocated from the root to the aerial tissues (stems and leaves).

Marschner (1995) identified various mechanisms of heavy metal tolerance in higher plants (Figure I-4). The relative importance of the various mechanisms is dependent on the plant species and on populations within the species, and whether they belong to the “excluder” or “includer” types. In *Silene cucubalus* the high copper tolerance of one population is closely related to its capability of restricting copper uptake and thereby preventing damage to the root cell plasma membranes, i.e. by a combination of the mechanism (1), (2), and (5) in Figure I-4 (4). Although immobilization in the cell walls might also play a role in copper (or other metals) tolerance the capacity of this mechanism is limited. Compartmentation, either as soluble or insoluble complexes, within the cytoplasm and in the vacuole is an important mechanism for copper tolerance. This is achieved by production of low-molecular-weight polypeptides such as phytochelatins (PCs) (Marschner, 1995).

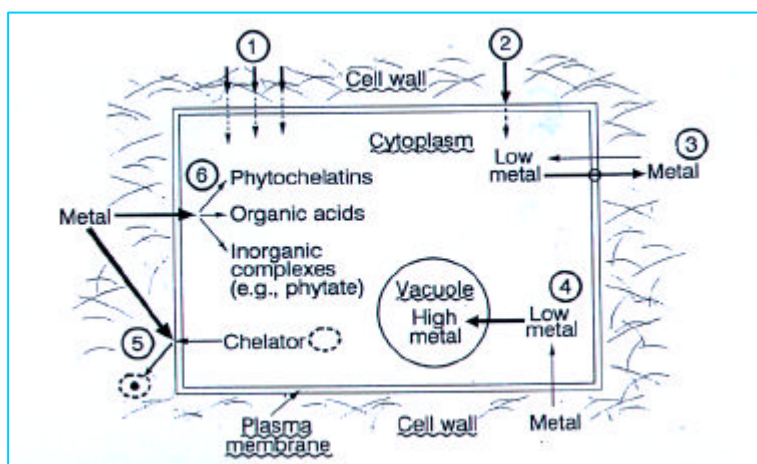


Figure I-4: Various heavy metal tolerance mechanisms in higher plants.

1. Binding to cell wall
2. Restricted influx through plasma membrane
3. Active efflux
4. Compartmentation in vacuole
5. Chelation at the cell wall-plasma membrane interface
6. Chelation in the cytoplasm

According to Vögeli-Lange and Wagner (1990), Cd-Phytochelatin complexes are believed to dissociate in the vacuole. At pH values of 3.5-5, Cd can be displaced from PCs and possibly the PCs are reshuttled into the cytoplasm. Cellular sequestration of Cd can have a large effect on the levels of free Cd in the symplast and, thus, can potentially influence movement of Cd throughout the plant. The concentration of ionic Cd^{2+} in the cytosol can be regulated by at least two processes: Cd^{2+} binding to phytochelatins (Grill *et al.*, 1985) and cellular compartmentation, (e.g. transports and accumulation in the vacuole). Although there is

evidence that Cd^{2+} binding to phytochelatins has little effect on xylem translocation to the shoots (Florijn *et al.*, 1993; Salt *et al.*, 1995), vacuolar compartmentation of Cd may be a more effective mechanism for inhibiting long-distance transport within the plant (Hart *et al.*, 1998).

There is relatively little information available concerning the movement of Cd into developing seeds. One recent study on Cd translocation into developing peanut fruits provided evidence that Cd accumulation occurred predominantly via the phloem. Several studies have been published describing Zn loading into developing wheat seeds. Herren and Feller (1994) reported that Zn entered wheatears mainly via the phloem when supplied at low concentrations. Similarly, Pearson and Rengel (1995) concluded that Zn enters wheat grains via the phloem (Hart *et al.*, 1998).

I.2.6 Definition and characteristics of metal hyperaccumulators

The ability of tolerating high levels of heavy metals, and to accumulate them to unusually high concentrations has evolved both independently and together in a number of different plant species (Kayser, 2000). Crop plants tolerate higher shoot Zn and Mn levels than Ni (about 300-500 mg Zn kg⁻¹ dry biomass), in general, however, such 'hyperaccumulators' contain >1 % Zn or Mn in the shoot. Shoot Cd levels in most plants are usually <1 mg kg⁻¹, whereas 'hyperaccumulators' accumulate and tolerate 100 mg Cd kg⁻¹; some hypertolerate >1% Cd.

How do hyperaccumulators achieve this remarkable bioaccumulation of soil metals? Research studies have pointed out the most important characteristics that make hyperaccumulation in plant possible:

- 1) There must be a rapid uptake rate for the element from the soil solution.
- 2) A plant must have the ability to translocate an element from roots to shoots at high rates.
- 3) The plant must be able to tolerate high levels of the element in root and shoot cells.

Hyperaccumulators have some limitations for phytoextraction of contaminated soils; most hyperaccumulators grow slowly and have small biomass. They often accumulate only a specific element and have not been found for all elements of interest. The optimum plant for the phytoextraction process should not only be able to tolerate and accumulate high levels of heavy metals in its harvestable parts but also have rapid growth rate and the potential to produce a high biomass in the field. Because most of the metal-accumulating wild plants are

relatively small size and have slow growth rates, their potential for phytoextraction is limited (Salt *et al.*, 1995).

But with genetic engineering improvement, plants metal accumulating ability can be increased. Some plants can synthesis phytochelatins (Class III MTs) under the stress condition. When MTI or MTII genes are transferred to these plants, metals can accumulate in vacuoles with PCs and MTI or MTII genes. This is the aim of genetic engineering.

I.2.7 Metallothioneins

The discovery of cadmium (Cd)-binding, cystein-rich protein from horse kidney by Margoshes & Vallee was the seminal finding that marked the birth of a field of research focused on the study of a new polypeptide super family, the metallothioneins (MTs). Metallothioneins (MTs) are a family of cysteine (Cys)-rich, low molecular weight (6-7kDa), nonenzymatic proteins that are found ubiquitously in animals, in higher plants, in eukaryotic microorganisms, and many prokaryotes (Table I-3). MTs have an unusual amino acid composition as they do not contain aromatic amino acids, and most important, one third of its residues are cysteines. Moreover, MTs show spectroscopic features of metal thiolates (mercaptides) (Kägi and Kojima, 1987; Kägi and Schäffer, 1988; Messerle *et al.*, 1990a-b). MTs can bind Zn, Cd, Ni, Pb, and Cu with affinities that depend on the concentration and bioavailability of the heavy metals.

Three major groups of scientist have contributed to our knowledge of MT:

- 1) Physiologists and toxicologists interested in its role in heavy metal metabolism and detoxification,
- 2) Protein chemists and spectroscopies intrigued by its usual structural features, and most recently,
- 3) Molecular biologist interested in gene regulation and the use of MT (promoter) sequences for genetic engineering experiments.

Nevertheless, although MTs were discovered over 40 years ago, their physiological functions are still unclear (Klaassen *et al.*, 1999).

Metallothionein have the following characterizations:

- (a) Low molecular weight
- (b) High metal content
- (c) Characteristic amino acid composition (high cysteine content, no aromatic amino acids nor histidine)

- (d) Unique amino acid sequence (characteristic distribution of cysteinyl residues such as cys-x-cys)
- (e) Metal thiolate clusters.

I.2.7.1 Structure and occurrence of MT

The structure of MTs was first described by Winge and Miklossy (Figure I-5). MTs have two domains consisting of one cluster with three and one with four metal atoms. The gene is located on chromosome 16. This protein consists of a number of isoforms coded by various alleles. The ratio of mRNA for MT-I and MT-II genes remains constant during induction by metals, e.g. Cd, Zn, and Cu (Nordberg, 1998).

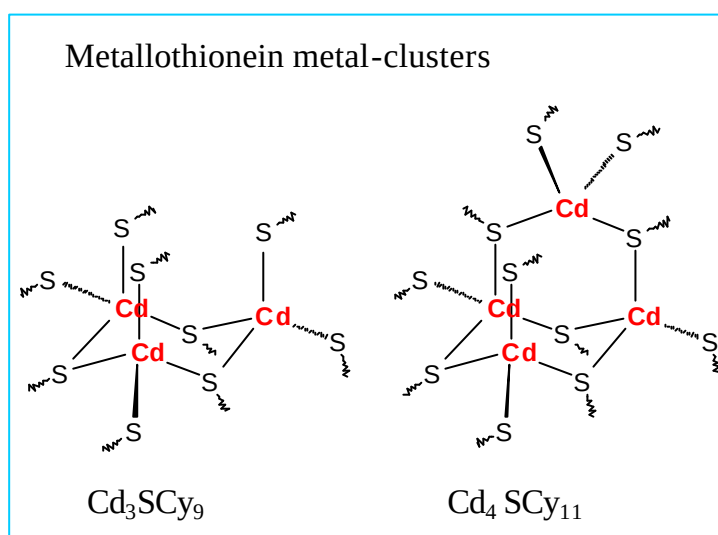


Figure I-5: Metallothionein metal-clusters (Kägi *et al.*, 1987). Metallothioneins from mammals contain 20 cysteines residues and bind seven cadmium or zinc ions in two discrete clusters. The thiolate side chains of these proteins act as bridging and terminal ligands to form Cd_3SCy_9 and $\text{Cd}_4\text{SCy}_{11}$ clusters.

The amino acid sequences of MTs from many mammalian sources reveal that all contain approximately 61 amino acids of remarkably similar composition. More important, all contain 20 cysteine residues that remain invariant along the amino acid sequence. All cysteines are known to participate in the coordination of 7 moles of Cd or zinc (Zn) per mole of MT. (Klaassen *et al.*, 1999).

MTs are cytoplasmic proteins; however, their occurrence in the nucleus during fetal development has been shown by immunohistochemistry. MTs from eukaryotic and prokaryotic microorganisms and from plants resemble mammalian MTs on their conspicuous

metal and cysteine content and on the occurrence of metal-thiolate cluster structures. Plant MTs first recognized in the roots of a copper-resistant strain of the grass *Agrotis gigantean* and in the roots of tomato plants exposed to elevated concentrations of CdCl_2 in the medium have now been found in a number of other plants including Cd-treated maize, cabbage, tobacco, rice and wheat and others, as well as in plant cell suspension cultures (Kägi, *et al.*, 1987).

I.2.7.2 Classes of metallothionein

Based on their structural similarities, MTs have been divided into three classes (Stillman *et al.*, 1992):

1. Class I: (Cys-Cys), occur in mammals, some fishes, crabs, oysters, mussels,
2. Class II: (Cys-x-Cys), occur in yeasts, plants, fungi, nematodes, cyanobacteria, drosophila,
3. Class III: (Cys-x-x-Cys) includes the phytochelatins that are occurring in all plants, algae and in some fungi

(x): any amino acids other than Cys

Metallothioneins belonging to class I and II are biosynthesized by direct translation of mRNA. On the other hand, class III metallothioneins are a group of metal-binding polypeptides containing γ -peptide bond that are biosynthesized as enzymatic products (Stillman *et al.*, 1992).

All vertebrates examined contain two or more distinct MT isoforms, which are grouped into two classes, designated MT-I and MT-II, depending on the elution position from DEAE-cellulose. In many cases each class actually consists of several different proteins, which are designated MT-I_A, MT-I_B, MT-I_C, etc. (Hamer, 1986).

1. Class I: Comprises mammalian metallothioneins with 20 cysteines and closely related metallothioneins such as the 18-cysteine metallothioneins of crustaceans (Stillman *et al.*, 1992). Mammalian MTs are a 61- or 62-amino-acid peptide containing 20 cysteines, 6-8 lysines, 7-10 serines, a single acetylated methionine at the amino terminus, and no aromatic amino acids or histidines. Metals are associated with MTs exclusively through thiolate bonds to all 20 cysteine residues.

Mammalian tissues usually contain two major fractions, i.e. MT-1 and MT-2, differing at neutral pH by a single negative charge. Within these fractions, isoforms identified by letters such as MT-1a, MT-1b could be resolved by high-performance liquid chromatography. An

excess of heavy metal ions such as Cd^{2+} or Cu^{2+} induces the expression of mammalian MTs. However, hormones, cytokines, growth factors, tumour inducers and chemical and physical stresses may also induce expression of MTs. This indicates that MTs play more than a simple protective role. For example, the fact that DNA damage caused by oxidative stress is reduced in the presence of MTs but it is enhanced when MTs expression is suppressed supports the protective ability of the sulfhydryl groups of these peptides (Kotrba *et al.*, 2000).

The metals in MTs are contained in two distinct, polynuclear clusters whose existence was initially inferred from ^{113}Cd -NMR studies. The A cluster contains 11 cysteines, binds four atoms of zinc or cadmium or five to six atoms of copper, and is located at the carboxy-terminal a domain extending from amino acid 31 to 61. The B cluster contains nine cysteines that bind four atoms of zinc or cadmium or six atoms of copper, and is located at the amino-terminal β cluster extending from amino acid 1 to 30 (Hamer, 1986).

MTs are commonly believed to be involved in metal homeostasis and the detoxification of heavy metals. Under normal conditions, MTs function in the storage and mobilization of essential metals such as zinc and copper. However, when the organism is exposed to a toxic metal such as cadmium, MTs act as scavengers to protect cells against metal toxicity (Yu *et al.*, 1998).

2. Class II: Metallothioneins isolated from non-animal sources such as the yeasts *Saccharomyces cerevisiae*, *Candida glabrata*, *Candida albicans*, algae, cyanobacteria (*Synechococcus* sp.) or plants (*Triticum aestivum*, *Zea mays*) belong to the class II of MTs. Thus far, the only lower organisms possessing a class I MT is the ascomycete *Neurospora crassa*. A well-known class II member is CUP1, the MT of *S. cerevisiae*. It is mainly responsible for copper tolerance and its structural gene is located in the CUP1 locus (chromosome VIII) that may amplify upon Cu^{2+} exposure. The CUP1 binds, however, both Cd and Zn (4 equivalents per molecule) besides 8 equivalents per molecule of monovalent ions. In the case of yeast protein there is no evidence of formation of two separate metal binding domains. In contrast to mammalian MTs, processing of the primary translation product results in the removal of 8 amino acids from the N-terminus. The processed CUP1 (6.6kDa) contains 12 cysteines of total of 53 amino acid residues. It is noteworthy that CUP1 seems to be solely involved in metal tolerance. On the contrary, other members of class II MTs, such as the wheat Zn^{2+} -binding EC protein (Fig. 1), function exclusively in metal homeostasis during seed development. Its expression only depends on the stage of

development and its level does not change as a function of the extracellular Zn^{2+} concentration (Kotrba, 2000).

Polypeptides, such as yeast metallothioneins, show location of cysteines only distantly related to mammalian metallothioneins (Stillman *et al.*, 1992). *Saccharomyces cerevisiae* MT, purified from a genetically selected overproducing strain, consists of 53 amino acids and contains eight copper atoms ligated to 12 cysteine residues. Surprisingly, the purified protein lacks the eight amino-terminal residues predicted by the DNA sequence of the gene. The removed sequence is well conserved in MTs of different origins in that it contains four hydrophobic residues including two aromatics. There is little obvious conservation of the positions of the cysteines or of the primary structure of yeast and mammalian MT except for the hexapeptide Lys-Lys-Ser-Cys-Cys-Ser. The binding stoichiometry of 12 cysteines to eight copper atoms suggests an octahedral cluster structure involving exclusively sulphur bridges. The yeast protein can also bind in vitro to eight atoms of zinc or cadmium. Hence the lower eukaryotic MTs resemble mammalian MT in their ability to bind group 1b and 2b metals in distinct configurations (Hamer, 1986).

3. Class III: Enzymatically synthesized peptides such as the poly (γ -glutamylcysteinyl) glycines are known as phytochelatins (PCs) and cadystins (Stillman *et al.*, 1992). The synthesis of phytochelatins is induced not only by Cd^{2+} but also by other heavy metal ions such as Cu^{2+} , Hg^{2+} , Pb^{2+} , and Zn^{2+} . PCs have the general structure (γ -Glu-Cys) n -Gly, where $n = 2-11$, and they are also called class III metallothioneins (MTs). Although the synthesis of class I and II MTs in animals and yeast (*Saccharomyces cerevisiae*), respectively, is controlled by genes and performed on ribosomes, the γ -carboxamide bond involved in the class III MT is not synthesized on ribosomes. That is, the synthesis of PCs is not controlled genetically (Kuboto *et al.*, 1995).

Over the past decade, many genes encoding MT-like proteins have been found in plants. There has been considerable interest in their structure and expression, with the aim of better understanding their role in plant metabolism. Although the exact function of plant MTs remains unclear, it seems that plant MTs are not solely associated with metal ions, but may also be related to other factors such as senescence and non-metal stresses (Yu *et al.*, 1998).

PCs are synthesized from glutathione by the enzyme PC synthase, and consist of between two and nine repeated units of γ -glutamylcysteine with a terminal glycine, which can be missing or substituted by other amino acids in some plant species. PC-deficient *cad1* mutants of *Arabidopsis thaliana* are hypersensitive to cadmium and copper PCs can bind metals

possessing a high affinity to sulfhydryl groups, for example arsenic and cadmium. In response to metal exposure, the production of PCs is higher in metal-sensitive than in metal-tolerant plants. This indicates that the metabolically expensive synthesis of PCs is part of a basal metal tolerance present in all plants, whereas metal-tolerant plants adapted to metalliferous soils possess additional tolerance mechanisms (Krämer *et al.*, 2000).

I.2.7.3 Physical and chemical characterization of MTs

General characteristics of Metallothioneins are;

1. MT I and MT II's molecular weight is 6-7 kDa, 61 amino acids, 20 Cysteine (30%), N-acetylmethionine, C-alanine, no aromatics, no histidine
2. Unique amino acid sequence, tertiary structure/metal clusters
3. Metal content; Cd, Zn, Cu, Hg; 5-10% W/w
4. Light absorption 250 nm (Cd), 225 nm (Zn), 275 nm (Cu), 300 nm (Hg)
5. Induced synthesis by Cd, Zn
6. No disulfide bonds, heat stability
7. Cytoplasmic localization
8. Isoforms
9. Localization on chromosome

Degradation of MT protein is also an important aspect of MT regulation. There are tremendous differences in the half-lives of MT synthesized as a result of chemical induction of the MT gene. For example, the half-life of Zn-MT is approximately 18-20h, whereas that of Cd-MT is about 3 days (Klaassen, *et al.*, 1999).

All class II MTs and I characterized thus far single chain proteins with chain weights of 6000 or less. However, on gel filtration mammalian and crustacean MTs are eluted at a position corresponding to that of a globular protein of molecular weight 10,000. This discrepancy has been attributed to their nonglobular shape. Class III MTs may occur in oligomeric structures composed of two or more chains differing in chain weight from 500 to 2000 Daltons depending on the source. In these aggregates the chains proposed to be linked via metal-thiolate bridges and/or via disulfide bonds. Their apparent molecular size is strongly dependent on salt concentration.

In general, the affinity of metal ions for the binding sites follows the order typical of thiolate model complexes, i.e., Zn (II) < Pb (II) < Cd (II) < Cu (I), Ag (I), Hg (II), Bi (III) (Kägi and Kojima, 1987; Kägi and Schäffer, 1998).

MT is usually detected by virtue of its high content of metals (detected by atomic absorption spectrophotometer or labelling with radionuclides) or of cysteine (detected by ^{35}S -labeling). Commonly used separation methods include gel filtration, reversed phase high-pressure liquid chromatography been raised against several mammalian MTs and can be used in immunodiffusion, immunoelectrophoresis, and radioimmunoassay. These methods are particularly useful for detecting low levels of MT and for subcellular localization studies by immunofluorescence (Hamer, 1986).

The structure of MT has been studied by a variety of biophysical and biochemical techniques including UV, CD, ESR, and NMR spectroscopy, amino acid sequencing and partial proteolysis and, most recently, X-ray crystallography.

Functions of Metallothioneins are;

1. Transport and storage of metals
2. Protection from metal toxicity
3. Free radical scavenger
4. Immune response
5. Genotoxicity and carcinogenicity

I.3 Genetic engineering

Effective genetic engineering of metal transport and tolerance in plants requires a better understanding of the mechanisms involved in these processes. Nevertheless, increasing metal tolerance has already been obtained, both by the introduction of metallothioneins, and by the introduction of a semi-synthetic gene encoding MerA (bacterial mercuric ion reductase). The transgenic *Arabidopsis* tolerates levels of mercuric ion up to $100\mu\text{M}$ by reducing the toxic cation to Hg^0 , which is then volatilized. Other possible tolerance mechanisms under investigation include alterations in phytochelatins and metal-binding peptides. Ways of increasing the translocation of metals from root to shoot are also needed. Augmenting the uptake of relatively immobile contaminants is being attempted by altering root morphology through *Agrobacterium rhizogenes* transformation (Cunningham, *et al*, 1995).

Another goal of developing transgenic plants with increased metal binding capacity was to use these metal-binding factors to immobilize Cd in the plant roots, thus reducing Cd movement to the food chain or into products of tobacco. The vacuolar compartmentation of Cd in roots may reduce Cd translocation to shoots; however, expression in plants of the hmt1 vacuolar pump for Cd-PCs from fission yeast has not yet been successful and modification of the gene sequence may be required before its effectiveness can be tested. The expression of

MT as full size protein, and of the Cd binding glucuronidase, under the control of several promoters increased Cd tolerance of tobacco and other plants, but had little effect on Cd transport to shoots (Chaney *et al*, 1997).

The group of Raskin also created transgenic plants to improve their ability to take up metals from soil. They have added the gene of metallothionein to several plants that have yet to be tested in field trials. Raskin's group has found that even though Indian mustard is a terrestrial plant, it can remove heavy metals from water (Harvey B., 1995).

A number of researchers have introduced metallothioneins from animal sources into plants using a transgenic approach, to reduce metal accumulation in shoots by trapping the metal in the roots (Table I-3). Expression of a mammalian MT in *N. tabacum* L. under the control of a constitutive promoter was able to reduce the translocation of cadmium into the shoot (Krämer *et al.*, 2001).

Table I-3: Expression of metallothionein in transgenic plants (adopted from Majáre *et al.*, 2001).

MT Source	Growth conditions	Cd tolerance/accumulation	References
Human MT-II	MS-media	Growth of root and shoot unaffected up to 100µM	Misra <i>et al.</i> , (1989)
Twelve repeated a-domains of human liver MT-I _A	MS-media	Growth of root and shoot unaffected up to 100µM	Pan <i>et al.</i> , (1994a)
Nicotiana glutinosa MT-like gene	MS-media selected on 200µM Cd	Cd resistance up to 200µM Cd. Transgenic seeds	Suh <i>et al.</i> , (1998)
Mouse MT-I	In vitro grown seedlings exposed to 0.02µM ¹⁰⁹ Cd (field-like. conc.)	Cd accumulation in transgenes 25% lower in leaves and 5% higher in roots than controls	Maiti <i>et al.</i> , (1989)
Mouse MT-I	Field grown	14 % lower Cd accumulation in leaves of transgenes than in controls	Yeargan <i>et al.</i> , (1992)
Chinese hamster MT-β-glucuronidase fusion	In vitro grown seedlings	70% less ¹⁰⁹ Cd accumulation in leaves of transgenes than in controls	Hattori <i>et al.</i> , (1994)
Human-MT- β-glucuronidase fusion	In vitro grown seedlings	60-70% lower Cd accumulation in shoots of transgenes than controls	Elmayan and Tepfer, (1994)
Human MT-II	Field grown	73% less Cd in leaf lamina than in controls. Overall Cd accumulation not affected compared with controls	de Borne <i>et al.</i> , (1998)

The use of Chinese hamster metallothionein II gene (ChMTII) to increase the capacity of plants to bind and detoxify Cd was first proposed by Lefebvre *et al.* (1987). Their experiments showed that the ChMTII gene expressed in *Brassica campestris* L. with a cauliflower mosaic virus vector dramatically reduced the free Cd in transfected leaves. Maiti *et al.* (1989) demonstrated that constitutive expression of a mouse metallothionein gene driven by the 35S promoter in transgenic burley tobacco seedlings (*N. tabacum* cv. Ky 14) yielded leaf Cd concentrations 15-day-old seedlings that were up to 20% lower than that of untransformed controls. The authors concluded that Cd sequestration in the roots of transgenic tobacco seedlings was responsible for reduced Cd concentrations in the leaves (Brandle *et al.*, 1992). Yeargen *et al.* (1992), introduced the same MT gene into different tobacco varieties, such as Petit Havana, but no decrease in leaf Cd was observed either in vitro or in the field (Elmayan and Tepfer, 1994).

Arabidopsis thaliana seedlings expressing pea MT (PsMTA) under the control of a constitutive promoter accumulated up to eight-fold higher copper concentrations than untransformed control seedlings (Evans *et al.* 1992).

Hattori *et al.* (1994) constructed Chinese hamster metallothionein II and β -glucuronidase coding regions. The fusion protein expressed in *Escherichia coli* retained cadmium-binding capacity and β -glucuronidase activity. When expressed under the constitutive 35S promoter in transgenic tobacco, the levels of ^{109}Cd accumulation in leaves were reduced to about 70% of those in untransformed control plants. Metallothionein-beta-glucuronidase did not sequester a significant proportion of the leaf ^{109}Cd taken up through the roots in vitro and therefore a sink for Cd was not created.

Brandle *et al.* (1992) showed that expression of Chinese hamster MT II, as a fusion protein with β -glucuronidase (GUS) does not significantly alter pattern of Cd accumulation in field-grown transgenic tobacco. Gene expression measured by β -glucuronidase activity showed that all of the transgenic lines expressed the MT II gene in the upper portion of the plant. One line did not express the MT II gene in the roots. Cd levels in the leaf tissue of transformed lines were not significantly different from the untransformed control (Brandle *et al.*, 1992).

Elmayan and Tepfer (1994) introduced chimeric genes under the control of a CaMV 35S promoter with a doubled enhancer (35SS) that encoded a mammalian metallothionein (hMTII), or an hMTII/GUS fusion protein into the genome of tobacco (*Nicotiana tabacum* cv. PBD6). Transcripts and Cd-binding proteins of the expected size were observed in plants expressing either the 35SS-hMTII or the 35SShMTII/GUS gene, and in the latter plants a protein with GUS activity that was larger than the native GUS enzyme was observed. Thus,

plants expressing the hMTII-GUS gene synthesize a bifunctional protein, with both GUS and Cd-binding activity. Using an *in vitro* assay the same authors also showed that five lines of transformed tobacco seedlings expressing either the 35S2hMTII or the 35S2hMTII/ GUS genes had greatly reduced Cd in their shoots compared to controls, due to reduced Cd translocation to the shoot system.

de Borne *et al.* (1998) described the pattern of Cd accumulation observed during a period of two years in greenhouse tests and one year in field trials in tobacco plants expressing a metallothionein gene. In all three tests, leaf Cd levels were markedly decreased. For instance, in the field trial, Cd levels in the leaf lamina tissue of the transformed line were decreased by 73% compared to controls. The decrease in leaf Cd was correlated with an increase in Cd in the roots and stems. The plants had normal growth characteristics, and the distribution of other ions was not affected by the expression of the metallothionein gene.

A wound and pathogen inducible MT cDNA was previously isolated from *Nicotiana glutinosa* while cloning plant disease resistance-response genes by subtractive hybridization (Choi *et al.*, 1996). Suh *et al.*, (1998) and Liu *et al.* (2000) introduced the *Nicotiana glutinosa* MT cDNA into tobacco plants via agrobacterium-mediated transformation. Overexpression of the MT gene conferred Cd tolerance on transgenic tobacco plants. Suh *et al.*, (1998) report was the first report the possibility of bioremediation of heavy metal contaminated soil using MT genes of plant origin.

Pan *et al.* (1994b) and Zhu *et al.*, (1999) transformed mouse MT cDNA and *E. coli gshII* encoding glutathione synthetase into tobacco and Indian mustard (*B. juncea*), respectively. The overexpression of the MT gene or glutathione synthetase conferred cadmium resistance to the transgenic plants.

Because of their metal-binding activity and inducibility by heavy-metal ions, plant MTs are thought to play a role in metal metabolism and detoxification. Recently, several MT cDNA clones have been characterized from soybean (Kawashima *et al.*, 1991), *Arabidopsis* (Zhou and Goldsbrough, 1994), *Brassica napus* (Buchanan-Wollaston), *Vicia faba* (Foley and Singh, 1994), *Sambucus nigra* (Coupe *et al.*, 1995), rice (Hsieh *et al.*, 1995), and cotton (Hudspeth *et al.*, 1996). MT genomic sequences from pea (Evans *et al.*, 1990), maize (de Framond, 1991), wheat (Kawashima *et al.*, 1992) and *Arabidopsis* (Zhou and Goldsbrough, 1994), have also been reported. Plant MT genes that exist in plant genomes as a multigene family are expressed in specific organ tissues such as the root, leaves, and embryos. They are also differentially induced by various environmental stresses, such as heavy metals, heat-shock,

plant hormones, wounding, senescence, and viral infection. However, little is known about the biochemical mechanisms involved in such responses (Suh *et al.*, 1998).

In phytoextraction, the aim is to prevent vacuolar compartmentation in roots and enhance it in shoots. Therefore, the use of tissue-specific promoters is likely to be of key importance in advanced transgenic phytoremediation approaches. Since the shoot is the most easily harvestable part of the plant, controlling the translocation from roots to shoots to favour high concentrations of trace elements in the aboveground biomass, represent an effective phytoextraction approach. In transgenic plants, the overexpression of several tonoplast metal transporters increased metal concentrations in the roots and, so far, never drastically reduced metal concentrations in shoots (Hirschi 1999; Van der Zaal *et al.*, 1999; Hirschi *et al.*, 2000). Similar observations were made in plants overproducing a number of metal chelators. In phytoextraction, however, it is desirable to maximize root-to-shoot transport of trace elements (Krämer *et al.*, 2001).

Furthermore, a number of transgenic plants have been engineered to contain large amounts of recombinant proteins with a possible role in chelation, assimilation or membrane transport of trace elements. To assess trace element accumulation or volatilization rates, these plants were grown in hydroponic or agar-based media. Virtually no data are yet available on the performance of these transgenic plants on soil substrates or under field conditions, where trace element bioavailability is substantially lower.

I.4 Aim of this thesis

Biotechnology has already been successfully employed to manipulate metal uptake and tolerance properties in several species. For example, in tobacco (*Nicotiana tabacum*) increased metal tolerance has been obtained by expressing genes encoding mammalian metallothionein, metal-binding proteins (Lasat, 2000).

Genetic engineering will make feasible to manipulate the capacity of plants to tolerate, accumulate, and metabolize pollutants and thus to create the ideal plant for containment of environmental pollution.

The goals of this thesis are to elucidate the mechanisms and factors involved in plant heavy metal tolerance and accumulation and to identify transgenic tobacco plants with improved capacities to clean up heavy metals from contaminated soils.

In this thesis, we introduced ChMTII and ScMTII genes into the tobacco plants via *Agrobacterium* mediated gene transfer. Transient expression and stable transformation of

tobacco plants was tested by immunoblot, and microscopic analysis. We selected tobacco lines with the highest expression MT II protein level. T1 generation of p-S-ChMTII and p-S-ScMTII plants seeds and T2 generation of p-cV-ChMTII GFP plant seeds were used for hydroponic and soil experiments. The objective of these studies was to modify levels of MTII expression by using genetic engineering in transgenic tobacco plants to increase metal accumulation and tolerance levels. Transgenic tobacco plants were compared to wild type tobacco (*Nicotiana tabaccum* Petit Havana, SR-1) plants with respect to their heavy metal tolerance and accumulation. These plants were analyzed, physiologically and biochemically to investigate the biological factors controlling heavy metal tolerance and accumulation. A schematic overview of this Ph.D thesis is presented in (Figure I-6).

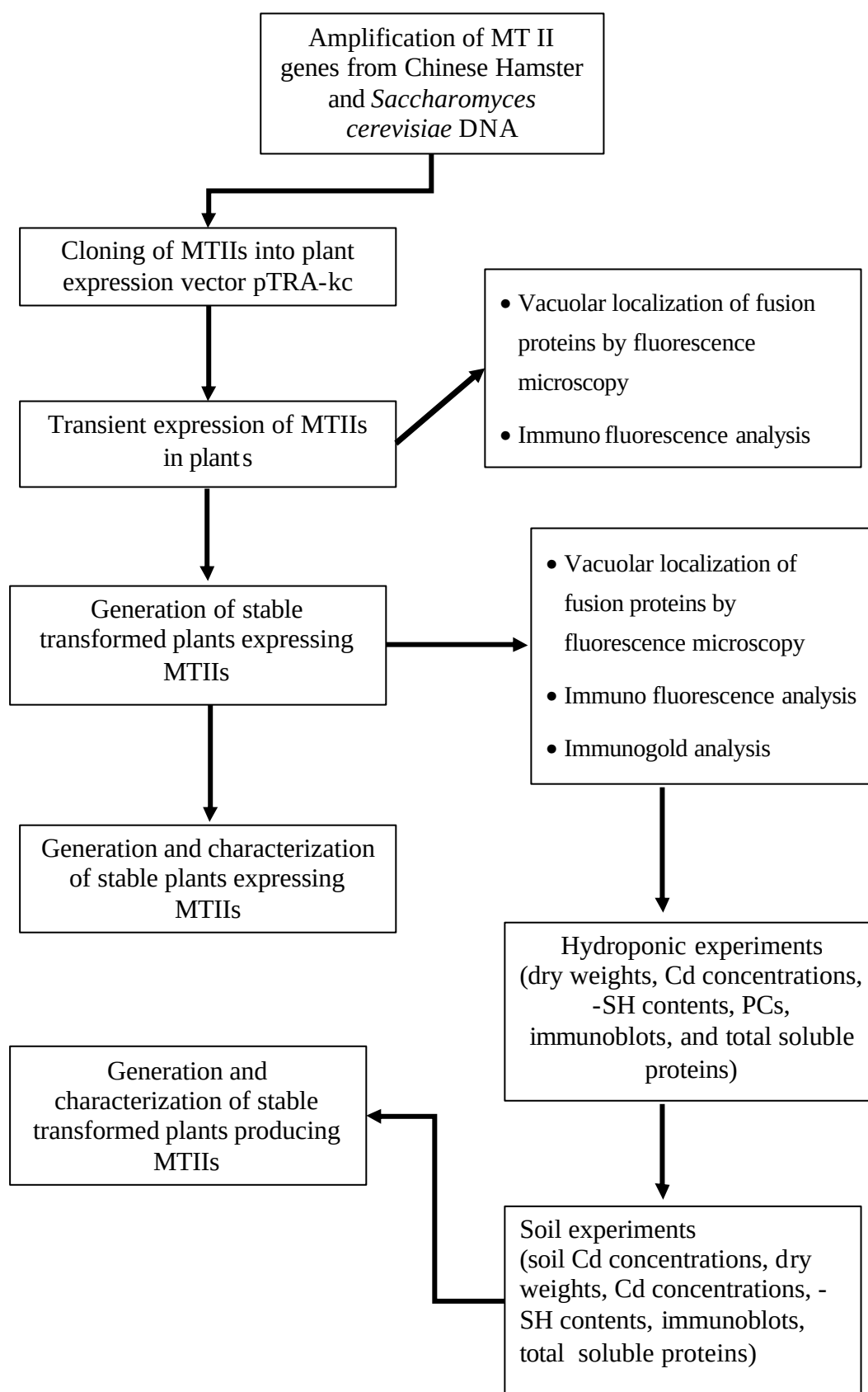


Figure I-6: Schematic overview of the project.

II MATERIALS AND METHODS

II.1 Materials

II.1.1 Chemicals and consumables

The chemicals used throughout the work were purchased from the following companies: Agros Organics (Geel, Belgium), Amersham Pharmacia Biotech (Freiburg), Bio-Rad (München), BD Biosciences (Heidelberg), Fluka (Neu-Ulm), Gibco BRL (Eggenstein), Invitrogen (Karlsruhe), Roche (Mannheim), Roth (Karlsruhe), Merck (Darmstadt), Serva (Heidelberg), Sigma-Aldrich (München). The consumables were from: Amicon (Witten), Biozym (Hess. Oldendorf), Eppendorf (Hamburg), Greiner (Solingen), Kodak (Stuttgart), Millipore (Eschborn), Nunc (Bieberach), Schott Glaswerke (Mainz), Serva (Heidelberg), USB/Amersham (Braunschweig), Whatman (Bender & Hobein, Bruchsal) and Zeiss (Oberkochen).

II.1.2 Enzymes and reaction kits

Restriction enzymes either from New England Biolabs (Schwalbach) or GibcoBRL (Eggenstein) were used for DNA digestion. ExpandTM high fidelity *Taq* DNA polymerase from Gibco BRL was used for SOE-PCR (II.2.1.16). Metallothionein II fragments were amplified with *Taq* DNA polymerase from GibcoBRL (II 1.15). The same enzyme was also used for identification of recombinant DNA by PCR. The following kits were used:

Plasmid isolation kits (Mini)	Qiagen (Hilden)
QIAquick gel extraction kit	Qiagen
QIAquick PCR purification kit	Qiagen
Bradford protein assay kit	Bio-Rad
PCR 2.1 Topo Cloning Kit	Invitrogen

II.1.3 Primary antibodies, secondary antibodies and substrates

Mouse anti-*c-myc* tag monoclonal antibody (9E10) (Evan *et al.*, 1985, ATCC clone number CRL-1729) and mouse anti-his6 (Qiagen) were used for detection of metallothionein fragments by immunoblot (II.2.4.3). Rabbit anti GFP polyclonal antibody (Clontech, Palo

Alto, USA) was used for detection of metallothionein GFP fusion protein by immunoblot (II.2.4.3). Alkaline phosphatase (AP) conjugated to goat anti-mouse IgG (H+L, Fc) (Dianova) and goat anti-rabbit IgG was used as secondary antibody in immunoblot analysis (II.2.4.3). NBT/BCIP (Bio-Rad) was used as substrate for detection of immobilized proteins in Immunoblot (II.2.4.3) respectively.

Rabbit anti metallothionein polyclonal antibody (Metallothionein (FL-61): sc-11377, Santa Cruz Biotechnology) was used for immuno localisation of metallothioneins in plant cell vacuoles.

II.1.4 Bacterial strains

II.1.4.1 *Escherichia coli* Strains

E. coli strains DH5 α , SCS 110, TG1 and XL1-Blue were used as host cells for all intermediate cloning constructs;

Table II-1 Names, suppliers and genotypes of *Escherichia coli* strains used throughout the work.

Strain	Source	Genotype
DH5a	Ausubel <i>et al.</i> , 1995	F ⁻ (<i>f80d Lac</i> 2 ⁺ M15) ? (<i>LacZYA-argF</i>) U169 <i>end A1 rec1 hsdR17</i> (<i>r_k⁻ m_k⁺</i>) <i>deoR thi-1 supE44 gyrA96 relA1</i> ?
XL1-Blue	Stratagene	<i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac</i> [F ⁺ <i>proAB lacI^f ZΔM15 Tn10</i> (Tet ^r)]
TG1	Stratagene	<i>supE thi-1 Δ(lac-proAB) Δ(mcrB-hsdSM) 5(r_K⁻ m_K⁻)</i> [F ⁺ <i>traD36 proAB lacI^f ZΔM15</i>]
SCS 110	Stratagene	<i>rpsL</i> (^{Str}) <i>thr leu endA thi-1 lacY galK galT ara tonA tsx dam dcm supE44 Δ(lac-proAB)</i> [F ⁺ <i>traD36 proAB lacIq ZΔ^{M15}</i>]

II.1.4.2 *Agrobacteria* strains:

Agrobacterium tumefaciens GV 3101 (pMP90RK Gm^R, Km^R, Rif^R (Koncz and Schell, 1986) was used for agrobacterium-mediated gene transfer (II.2.1.6 and II.2.1.8).

II.1.5 Vectors

pUC18-*NdeI**scFv24-VTSchi (Verena Hoppmann, RWTH-Aachen, Biologie VII, Hoppmann, 2000) containing 5' untranslated region of Chalcon Synthase (CHS5'UTR), N-terminal codon optimised vacuole targeting signal of Strictosidin Synthase from *Catharanthus roseus* (VTS), the TMV specific scFv 24 and the carboxylterminal *c-myc* and His6 sequences was used for subcloning of ScMTII and ChMTII genes.

- pUC18-*NdeI**GFP-S65C-VTS (Verena Hoppmann, RWTH-Aachen, Biologie VII, Hoppmann, 2000) plasmid containing the modified mGFP4 gene (Reichel *et al.*, 1996) was used for subcloning of ScMTII and ChMTII genes downstream CHS5'UTR and VTS and upstream of mGFP4 via *EcoRI/HindIII* restriction sides.
- PCR 2.1 Topo from Invitrogen was used for cloning of PCR amplified of ScMTII and ChMTII fragments.
- pTRA plant expression vector (Thomas Rademacher, RWTH-Aachen, Biologie VII) was used for transformation of *A. tumefaciens* and expression of metallothionein II into the plant cell. This vector was used as an optimized plant expression vector containing the 35S promoter and the pA35S untranslated region from CaMV.

II.1.6 Oligonucleotides

Oligonucleotides used for sequence analysis and amplification of DNA are listed below. All oligonucleotides were synthesized by MWG (Ebersberg, Germany). Degeneracy codes: K= G or T; M = A or C; S = C or G; R = A or G; W = A or T

1. Primers used for PCR amplification of recombinant DNA (*E. coli* and *A. tumefaciens*) from:

- pTRA

Forward pSS 5':3' - GAC CCT TCC TCT ATA TAA GG (20-mer)

Reverse pSS 3':5' - C AC ACA TTA TTC TGG AGA AA (20-mer)

- pUC18 and pCR 2.1 Topo:

M13 universe5': 3' - GTT GTA AAA CGA CGG CCA GT

M13 reverse 3': 5' - ACA CAG GAA ACA GCT ATG AC

2. Primers used for PCR amplification of recombinant ChMTII and ScMTII fragments for Topo cloning:

ChMTII 5' N	CAT GCC ATG GAC CCC AAC TGC TCC TGT GC
ChMTII 3' N	CAT GCC ATG GCG CAG CAG CTG CAC TTG TCC
ChMTII 3' S	CTA GTC GAC GCC GCA GCA GCT GCA CTT GTC C
ScMTII 5' N	CAT GCC ATG GTC AGC GAA TTA ATT AAC TTC C
ScMTII 3' N	CAT GCC ATG GCT TTC CCA AGA GCA GCA TGA C
ScMTII 3' S	CTA GTC GAC TTT CCC AGA GCA GCA TGA C

3. Primers used for SOE-PCR to optimize codon usage of ChMTII and ScMTII genes.

(P1) Eco-CHS 5'	GAA TTC ACA ACA CAA ATC AGA TTT
(P2) Eco-CHS 3'	TTGTTTTTTTTTTTTTTTATAAATCTCTCTATAAATCTGATT TGTGTTGTGAATT
(P3) CHS-SPA	AAA AAA AAC AAT GCA TTC CAG GTT CAA TCC C
(P4) SPA internal	GGT TCG TGT GTG GTG GGG AGG CGG ATG GGA TTG AAC CTG GA
(P5) SPA-NcoI	CCA CCA CAC ACG AAC CCG CCA TGG ACC CCA ACT GCT CCT GTG CT
ChMTII 3' S	CTA GTC GAC GCC GCA GCA GCT GCA CTT GTC C
ScMTII 3' S	CTA GTC GAC TTT CCC AGA GCA GCA TGA C

II.1.7 Buffers, media and solutions

All standard solutions, buffers, and media were prepared according to Sambrook *et al.* (1989), Ausubel *et al.* (1995) and Coligan *et al.* (1995). Compositions of non-standard solutions or buffers are listed at the end of the respective method section. Media for cultivating bacteria were sterilized by autoclaving (121°C/1-2 bar); all other solutions were sterile filtered (0.2 µm). Thermo labile components such as antibiotics were sterile filtered and added to the media after autoclaving and cooling to 50°C.

II.1.8 Matrices and membranes

ImmobilonTM-P transfer's membrane (PVDF) (0.45µm) from Millipore, HybondTM-C nitrocellulose membrane (0.45µm) from Amersham Life Science and Whatman no.1 paper from Whatman (Maidstone, England) was used in immunoblot analysis (II.2.4.3). Filters: 589/3, 110mm, blue ribbon, was used for plant (II.2.5.4) and soil extractions (II.7).

II.1.9 Equipment and applications

AAS: 1100B, (Perkin Elmer, California)

AAS-lamp Cd: M-2631, (Perkin Elmer, California)

AFS: Leica AFS (automatic freeze substitution), (Leica Wien)

Agfa Arcus II Scanner: (Leverkusen)

Biochrom 20 amino acid analyser: (Amersham Pharmacia Biotech, Freiburg) and EZchrom V 6.7 data system software (Amersham Pharmacia Biotech, Freiburg)

Balance: Sartorius BP 610, 1202 MP and BP 121S (Sartorius, Göttingen)

Cameras: MP4 (Polaroid, Cambridge, MA, USA). E.A.S.Y 429K camera (Herolab, Wiesloch)

Centrifuges: AvantiTM 30 and AvantiTMJ-25 (Beckman, California, USA), Biofuge A (Heraeus, Hanau), Sigma 3-10 and Sigma 4-10 (Sigma, St. Louis, Missouri, USA), RC5C and RC5B plus (Sorval instruments, Du Pont, Bad Homburg)

DNA gel electrophoresis apparatus: wide mini and mini cells for DNA agarose electrophoresis and power supplies (Bio-Rad, München)

DNA Sequencer Apparatus: Wavelength: 365 nm, ABI Prism 3700 Sequencer (Perkin-Elmer, Applied Biosystems, Foster City, USA)

Digital Camera: Nikon Cool Pix 4500, 4.0 Mega Pixels, 4x zoom

Electroporation apparatus: "Gene pulserTM", "Pulse controller" unit, Extender unit (BioRad) and 0.2 cm cuvettes (Bio-Rad)

Electroblotting Chamber: (electro tank Blotting and Semidry Blotting apparatus) (Bio Rad, München)

Electron Microscope: Philips EM 400T

Fluorescence microscopy: (Confocal Imaging, Leica, Heilderberg, Germany)

HPLC : Beckman System Gold (Krefeld, Germany)

InnovaTM 4340 incubator shaker: (New Brunswick Scientific, Nürtingen)

Incubator: WTB Binder (Tutlingen, Germany)

LI-COR IR2-4200 Sequencer: LI-COR MWG-Biotech and Base Image IRTM 4.0 software (LI-COR)

Membrane Vacuum Pump (air pump): WISA (ASF Thomas GmbH&Co.KG, Wuppertal)

Microwave: Micromat (AEG, Frankfurt)

Milling Machine: Type: MM2, (Retsch GmbH&Co.KG, Haan, Germany)

PCR Thermocyclers: Primus and Primus 96 plus (MWG-Biotech, Germany)

pH-Electrode: SE 103 Knick (Berlin, Germany)

Photometers: Spectrophotometer Uvikon 930 (Kontron, Neufahrn) and multi-channel spectrophotometer Spectromax 340 (Molecular Devices, Sunnyvale, California), Biophotometer (Eppendorf, Hamburg)

Protein gel electrophoresis equipment: Mini PROTEAN IITM and gel air dryer (Bio-Rad, München)

Plastic Pots: 3 L pots for hydroponic experiments and 1.5 kg pots for soil experiments (Obi, Aachen)

Plastic Vials: 20 mL plastic vials for laboratory and diagnostic, (Zinsser Analytic GmbH, Frankfurt)

Oven: Naber Industrieofenbau, Type 2804 (Lilienthal, Bremen)

Rotors: F0650, F2402H, JLA 10.500 and JA 25.50 (Beckman), #1140 and #11222 (Sigma), RLA-300, SS-34 and GS-3 (Du Pont)

Sterile Bench: Babcock-BSH Type THL 1.33.1 (Bad Hersfeld)

Shaker: Innova 4430 (New Brunswick Scientific GmbH, Nürtingen)

Software: Windows NT 4.0 operating system (Microsoft); Microsoft Office 2000 (Microsoft); Adobe Photoshop 6.0 (Adobe); Chromas; Origin 6.0 (Data analysis and technical graphics, Microcal Software, Inc.); GCG (Wisconsin Package TM of Genetic Computer Group)

Ultramicrotome: Leica Ultra Cut VCT (Leica, Wien)

UV-Transilluminators: wavelength 302 nm and UVT-20M (Herolab)

UV-chamber: (Bio-Rad, München)

Vacuum exicator (Glaswerk Wertheim)

Vortex: K-550-GE (Bender and Hobein AG, Zürich)

Waste Tank: Ask 540, 500 L, (Edelhoff Polytechnick GmbH&Co, Iserlohn, RWTH-Aachen)

II.2 Methods

All experiments related to the genetic engineering were performed according to the regulations of “S1-Richtlinien” and were officially approved by the “Regierungspräsidium des Landes NRW” (RP-Nr.: 23.203.2 AC 12, 21/95) and “BGA” [AZ 521-K-1-8/98:AI3-04/1/0866/88 (S1) and 55.8867/-4/93 (greenhouse)].

General recombinant DNA techniques, i.e. PCI (phenol/chloroform/isoamyl alcohol) and CI (chloroform/isoamyl/alcohol) extraction, DNA precipitation, restriction enzyme digestion, DNA ligation, DNA agarose gel electrophoresis, were according to the standard protocols described in Sambrook *et al.* (1989) and Ausubel *et al.* (1995).

II.2.1 Recombinant DNA technologies

II.2.1.1 Preparation of heat-shock competent *E.coli* cells

E. coli strain DH5 α competent cells were prepared for RbCl-mediated transformation by heat-shock as described by Hanahan (1985). A single bacterial colony was inoculated in 5 ml of LB broth and cultured at 37°C overnight (o/n). 0.5 ml of the o/n culture was transferred into 50 ml of LB broth containing 20 mM MgSO₄ and 10 mM KCl. The cells were cultured at 37°C for 3-4 hours until the OD_{600nm} reached 0.4-0.5 and then transferred to an ice-cold tube. After incubation on ice for 10 min, the cells were recovered by centrifugation (2000g/4°C/10 min). The pellets were resuspended in 15 ml ice-cold Tfb-I solution by gentle vortexing and stored on ice for 10 min. The cells were recovered by centrifugation as described above and resuspended in 2 ml ice-cold Tfb-II. 200 μ l-aliquots of the suspension were dispensed into prechilled micro centrifugation tubes, frozen immediately in liquid nitrogen and stored at -80°C.

Tfb-I pH 5.8:

Potassium acetate	30 mM
MnCl ₂	50 mM
CaCl ₂	10 mM
Glycerol	15% (v/v)

Tfb-II pH 6.8:

MOPS	30 mM
CaCl ₂	75 mM
RbCl	10 mM
Glycerol	15% (v/v)

II.2.1.2 Transformation of *E. coli* by heat-shock

As soon as the competent cells (II.2.1.1) were thawed, plasmid DNA (up to 100 ng) (II.2.1.12) or ligation products (Sambrook *et al.*, 1989) were mixed gently with the competent cells and incubated on ice for 30 min. The cells were then exposed to 42°C for 90 seconds and placed on ice for 2 min. 800 µl of LB medium were added to the tubes and incubated at 37°C for 45 min. 200 µl of cells were plated onto a LB-agar plate supplemented with appropriate antibiotics and incubated at 37°C overnight.

II.2.1.3 Preparation of electrocompetent *E. coli* cells

Electrocompetent *E. coli* were prepared from the following strains, DH5α, SCS110, XL1-blue, and TG1 as described by Dower *et al.* (1988). A single bacterial colony from an LB plate was inoculated in 5 ml LB-broth and cultured at 37°C o/n. Three ml of fresh o/n culture was transferred into 500 ml of LB broth. The cells were cultured at 37°C for 3-4 hours until the mid-log phase ($OD_{600nm} = 0.5-0.8$). Then the cells were placed on ice for 15-20 min and harvested by centrifugation (3000g/4°C/10 min). Cells were washed three times with sterile water and resuspended in ice-cold 10% (v/v) glycerol to a 300-fold concentration from the original culture volume (at $>10^{10}$ cells/ml). 40 µl aliquots were stored at -80°C.

II.2.1.4 Transformation of *E. coli* by electroporation

Electrocompetent cells (II.2.1.3) were thawed on ice and mixed with 1 pg to 300 ng of DNA in sterile dH₂O. The cell/DNA mixture was transferred into a prechilled electroporation cuvette (0.2 cm) and assembled into a safety chamber. After application of the pulse (25 µF, 2.5 kV, 200 Ω), the cells were diluted in 1 ml of SOC medium and incubated at 37°C with shaking for 1 h. Finally; 100 µl of the cells were plated onto LB-agar containing appropriate antibiotics and incubated at 37°C o/n.

II.2.1.5 Preparation of electrocompetent *Agrobacterium* cells

A single colony of *Agrobacterium tumefaciens* strain GV3101 grown on YEB-agar plate containing 100 µg/ml rifampicin (Rif) and 25 µg/ml kanamycin (Km) (YEB-Rif-Km) was inoculated in 5 ml of YEB-Rif-Km medium in a 100 ml Erlenmeyer flask and incubated at 28°C for two days with shaking (250 rpm). 1 ml of the culture was transferred into 100 ml of

YEB-Rif-Km medium and cultivated at 28°C for 15-20 h with shaking (250 rpm) until the OD_{600nm} reached 1-1.5. The cells were chilled on ice for 15 min and spun down by centrifugation (4,000g/4°C/5 min). The culture medium was decanted and the cells were washed three times with 10 ml of dH₂O by centrifugation and resuspended in 500 µl of sterile 10% (v/v) glycerol. 45 µl-aliquots of the suspension were dispensed into prechilled micro centrifugation tubes, frozen immediately in liquid nitrogen and stored at -80°C.

YEB-Rif-Km medium:

Nutrient Broth	0.5% (w/v)
Yeast Extract	0.1% (w/v)
Peptone	0.5% (w/v)
Sucrose	0.5% (w/v)

2 mM MgSO₄, 100 µg/ml rifampicin, 25 µg/ml kanamycin were added after autoclaving and cooling.

II.2.1.6 Transformation of *Agrobacterium* by electroporation

0.2-1.0 µg of plasmid DNA (II.2.1.12) in sterile dH₂O was added to a thawed aliquot of electrocompetent *Agrobacterium* cells (II.2.1.5) and incubated on ice for 3 min. The cell/DNA mixture was transferred into a prechilled electroporation cuvette (0.2 cm) and assembled into a safety chamber. After application of the pulse (25 µF, 2.5 kV, 200 Ω), the cells were diluted in 1 ml of SOC medium in a 4.0-ml tube and incubated at 28°C with shaking (250 rpm) for 1 h. Finally, 1-10 µl of the cells were plated on YEB-agar containing 100 µg/ml rifampicin (Rif), 25 µg/ml kanamycin (Km) and 100 µg/ml carbenicillin (Carb) (YEB-Rif-Km-Carb) and incubated at 28°C for 2-3 days. As a control transformation of *Agrobacterium* cells with H₂O was performed.

II.2.1.7 Preparation of heat-shock competent *Agrobacterium* cells

A single colony of *Agrobacterium tumefaciens* strain GV3101 grown on YEB-agar plate containing 100 µg/ml rifampicin (Rif) and 25 µg/ml kanamycin (Km) (YEB-Rif-Km) was inoculated in 5 ml of YEB-Rif-Km medium in a 100 ml Erlenmeyer flask and incubated at 28°C for two days with shaking (250 rpm). 2 ml of the culture was transferred into 100 ml of YEB-Rif-Km medium and cultivated at 28°C for 15-20 h with shaking (250 rpm) until the OD_{600nm} reached 1.0. The cells were spun down by centrifugation (4,500rpm/4°C/15 min). The cells were resuspended in 1ml of ice cold sterile 10 mM CaCl₂. 100 µl aliquots of the

suspension were dispensed into prechilled micro centrifugation tubes, frozen immediately in liquid nitrogen and stored at -80°C (Walkerpeach *et al.*, 1994).

II.2.1.8 Transformation of *Agrobacterium* by heat-shock

Agrobacterium tumefaciens strain GV3101 (II.2.1.7) was thawed, 5µl (0.2-1.0 µg) of plasmid DNA was mixed gently with the competent cells and incubated in liquid Nitrogen for 5 min. The cells were then exposed to 37°C for 5 min. and placed on ice and 1 ml YEB-MgSO₄ (2mM) medium added. Finally, 10-100 µl of the cells were plated on YEB-agar containing 100 µg/ml rifampicin (Rif), 25 µg/ml kanamycin (Km) and 100 µg/ml carbenicillin (Carb) (YEB-Rif-Km-Carb) and incubated at 28°C for 2-3 days (Cindy *et. al.*, 1994). As a control transformation of *Agrobacterium* cells with H₂O was performed.

II.2.1.9 Determination of the efficiency of recombinant bacteria transformation

Efficiency of transformation of each new batch of competent cells was measured by test transformations with known concentrations of super coiled pUC18 plasmid for *E. coli* and pTRA for *A. tumefaciens* cells. The following transformation rates were obtained; RbCl >10⁷/µg pUC18, electrocompetent *E. coli* >10⁸/µg pUC18 and *A. tumefaciens* >10³/µg pTRA.

II.2.1.10 Culturing of *E. coli* and glycerol stock preparation

Individual colonies of all strains were obtained by plating the pertaining strain on LB agar plates. Strains carrying an F' factor were spread on M9 plates. Incubation was performed at 37°C. The plates were stored at 4°C for short periods (less than 2 weeks). LB medium containing the suitable antibiotics and 2 % (w/v) glucose was inoculated with a single recombinant colony of *E. coli* and grown o/n at 37°C with vigorous shaking (225 rpm). Glycerol stocks were prepared by mixing 600 µl of a fresh overnight culture with 600 µl of 40 % (v/v) sterile glycerol. Bacteria glycerol stocks were stored at -80°C.

II.2.1.11 Growth of recombinant *A. tumefaciens* and preparation of glycerol stocks

Single colonies of *A. tumefaciens* were examined for the presence of plasmids by control PCR (II.2.1.15). Positive colonies were inoculated in 10 ml of YEB-Rif-Km-Carb medium and

cultivated at 28°C for 2-3 days with vigorous shaking at 250 rpm. The culture was transferred to Falcon tubes and *Agrobacteria* cells were pellet by centrifugation at 4000g for 10 min at 15°C. The cells were resuspended in a 1:1 volume of YEB Rif-Km-Carb medium and glycerol stock media (GSM). The suspension was aliquoted (100µl) and stored at -80°C for further experiments.

Glycerol stock media (GSM):

Glycerol	50% (v/v)
MgSO ₄	100 mM
Tris, pH 7.4	25 mM

II.2.1.12 Isolation of plasmid-DNA from *E.coli*

Recombinant plasmid DNA was purified with the Qiagen plasmid DNA Mini prep kits according to the manufacturers instructions based on the alkaline lysis method (Sambrook *et al.*, 1989). Quality and yield of plasmid DNA was examined by reading the absorbance at 260 nm and 280 nm in a spectrophotometer according to Müller *et al.*, (1993) and Sambrook *et al.* (1996). The integrity of DNA was verified by a control restriction digest followed by agarose gel electrophoresis (II.2.1.13). Isolated plasmid DNA was stored at -20°C.

II.2.1.13 Agarose gel electrophoresis of DNA

Plasmid DNA and PCR-fragments were separated in 1.0-2.0 % (w/v) agarose gels. Preparation of agarose gels and electrophoresis of the samples were carried out as described by Sambrook *et al.*, (1989). Ethidium bromide was added to the gel solution and TBE electrophoreses running buffer prior to the experiment. Known amounts of DNA molecular markers such as 1 Kb ladder, 100 bp ladder and λ -digested with *Pst*I were used for evaluation and determination of DNA concentration and size. The DNA bands were visualised directly upon illumination with a UV transilluminator at 302 nm. Documentation of the DNA gels was performed by using a black and white E.A.S.Y 429K camera (Herolab) and a photo printer (Mitsubishi).

II.2.1.14 Preparative agarose gel electrophoresis

Preparative gel electrophoresis was used for the isolation of PCR amplified DNA fragment after digestion with restriction enzyme. The agarose containing the DNA fragment of interest was excised from the gel on an UV transilluminator with a sterile scalpel. The DNA extraction was performed with QIAquick Gel extraction kit according to the manufacturer's guidelines. The concentration of recovered DNA was measured by spectrophotometer or determined by agarose gel electrophoresis and was used in further experiments.

II.2.1.15 PCR amplification

Polymerase chain reaction (PCR) was used for amplification of metallothionein II gene from a Chinese Hamster (ChMTII) and *Saccharomyces cerevisiae* (ScMTII) using synthetic oligonucleotides based on the Metallothionein II database sequence (VII.2-3). DNA was amplified, based on the protocol of Sambrook *et al.* (1989) with *Taq*- or high fidelity DNA-polymerase and DNA polymerase buffer from Roche. The reactions were performed in 0.2 ml PCR reaction tubes (Biozym Diagnostik GmbH, Hessisch Oldendorf), using a DNA thermal Cycler (MWG). The cycler contained a heated lid to avoid the use of mineral oil.

For rapid identification of recombinant *E. coli* and *Agrobacteria* control-PCR was carried out to detect plasmids as described by Jesnowski *et al.* (1995).

The optimal annealing temperature (T_p) of the primers was experimentally optimised or calculated based on the empiric formula (Wu *et al.*, 1991):

$$T_p = \{22 + 1, 46 [2 * (G + C) + (A + T)]\}$$

PCR reactions were carried out in a total volume of 50 μ l as described below:

Components	Volume	Final concentration
10X PCR buffer	5 μ l	1X
50 mM MgCl ₂	1.5 μ l	1.5 mM
10 mM dNTPs	1 μ l	0.2 mM each
10 pmol forward Primer	0.5-1 μ l	0.2 pmol
10 pmol backward primer	0.5-1 μ l	0.2 pmol
Template DNA	0.5-5 μ l	10-100 ng
<i>Taq</i> DNA polymerase (5U/ μ l)	0.25 μ l	1.25 units
dd H ₂ O	to 50 μ l	

For rapid identification of recombinant *E. coli* colonies amplification was carried out under the following conditions:

x 35	{	5min	95°C
		45sec	95°C
		0.3 °C/sec	52°C
		1min 30sec	52°C
		45sec	72°C

For rapid identification of recombinant *Agrobacteria* amplification was carried out under the following conditions:

x 25-35	{	5min	95°C
		1 min	94°C
		1 min	55°C
		1 min	72°C
		5 min	72°C

The annealing temperature and the time for denaturation were changed according to T_p value of primers and the length of the target gene. The performance of each PCR reaction was checked by running 5µl of each reaction on 1.2 and 1.5 % (w/v) agarose gels (II.2.1.13), with appropriate DNA markers.

II.2.1.16 Splice overlap extension PCR

Splice overlap extension (SOE) PCR is a modified PCR method in which the primers are designed in a way that the coding region of the 3' end of one primer is complementary to the 5' region of the next primer (Horton *et al.*, 1989).

The potato Sporamin A vacuolar targeting signal (S) (VII.4) and metallothionein II (ChMTII and ScMTII with c-mycHis6 tag) fragments were constructed by SOE PCR. The S and MTII regions were amplified as three separate reactions with specific primers (II.1.6) using 0.2 units ExpandTM high fidelity Taq DNA polymerase under the following conditions:

SOE I

x 7	{	5min	95°C
		2min	58°C
		10min	72°C

SOE II

x 20	{	15min	95°C
		2 min	58°C
		3 min	72°C
		1 min	94°C
		2min	58°C
		10 min	72°C

SOE III

x 25	{	5min	95°C
		30 sec	95°C
		30 sec	58°C
		40 sec	72°C
		5 min	72°C

The product of the SOE PCR was analysed on a 1.2% (w/v) agarose gel (II.2.1.13).

II.2.1.17 DNA sequencing by LICOR and ABI Method

Fluorescently labelled primers were used for sequence analysis by chain terminating inhibitors (Sanger *et al.*, 1977) using the “Thermosequenase sequencing kit” and the LI-COR 4200 IR2 automated DNA sequencer. For evaluation of sequencing data the Base Image IR 4.0 software package was used. Further more the automated fluorescent sequencing using the ABI Prism 3700 Big Dye Terminator chemistry was the method of choice for high throughput DNA sequence determination. Sequence ladders were generated by the Sanger (1977) method using fluorescently labelled Big Dye™ chain terminator nucleotides (Prober *et al.*, 1987). Automated sequencers separate the labelled sequence extension products using a molecular sieve matrix and identify the terminal nucleotide by its specific emission wavelength.

Universal and reverse primers were employed for sequencing genes in pUC and PCR 2.1 Topo vectors. pSS 5’ and pSS 3’ were used for sequence analysis of genes in pTRA-kc vector.

II.2.1.18 Sequence analysis

Chromas software package was used for displaying the chromatogram files from LI-COR and ABI and automated DNA sequencer. The sequences were edited and exported for further analysis with the Wisconsin Package™ of Genetic Computer Group (GCG).

II.2.2 Generation and characterisation of transgenic plants

II.2.2.1 Transient assay in tobacco leaves by vacuum infiltration

Growth of recombinant *Agrobacterium* (II.2.2.11) and vacuum infiltration of tobacco leaves was performed as described by Kapila *et al.* (1996) and Vaquero *et al.* (1999).

II.2.2.2 Preparation of recombinant *Agrobacteria*

100 ml of YEB-Km-Rif-Carb medium was inoculated with 100 μ l of glycerol stock (II.2.1.11) of the selected recombinant *Agrobacteria* carrying with a plant expression vector. The culture was grown at 28°C o/n with shaking at 250 rpm. Next day the cells were pelleted by centrifugation at 5000 g for 10 min at 15°C and transferred into 250 ml of induction medium and cultivated at 28°C o/n with shaking at 250 rpm. *Agrobacteria* cells were centrifuged (4000g/15-25°C/15 min) and resuspended in 50 ml of MMA solution and kept at RT for 2 h. The OD_{600nm} was measured after 1:10 dilution and the cell suspension was adjusted to an OD_{600nm} of 1. 100 ml of the diluted cell suspension was used for vacuum infiltration of plant leaves (II.2.2.3).

Induction medium:

YEB medium pH 5.6
MES 10mM

2 mM MgSO₄, 25 μ g/ml kanamycin, 100 μ g/ml rifampicin, 100 μ g/ml carbenicillin, 20 μ M acetosyringone were added after autoclaving and cooling.

MMA buffer:

MS-salts (Murashige & Skoog, basic salt mixture)	0.43% (w/v)
MES; pH 5.6	10 mM
Sucrose	2% (w/v)
Acetosyringone was added directly before use	200 μ M

II.2.2.3 Vacuum infiltration of intact leaves

Young *N. tabacum* cv. Petite Havana SR1 leaves (4 leaves for each construct) were placed in 100 ml of *agrobacteria* suspension in a “Weck” glass and a continuous vacuum (60-80 mbar) was applied for 15-20 min. The applied vacuum was released rapidly; the leaves were briefly rinsed in tap water and kept on wet Whatman paper no. 1 with adaxial side upwards. The plastic tray was sealed with saran wrap and placed at 22°C with a 16 h photoperiod for 60 h.

Leaves were weighed, frozen in liquid nitrogen and stored at -80°C until analysis. As control, leaves were infiltrated with agrobacteria suspension, which did not contain the pTRA plasmid.

II.2.2.4 Recombinant agrobacterium-mediated stable transformation of tobacco plants

Stable transformation of *N. tabacum* was performed with the help of Dr. Flora Schuster (RWTH Aachen, Institute für Biology VII). Transgenic *N. tabacum* cv. Petite Havana SR1 was generated by leaf disc transformation using recombinant Agrobacteria transformed with pTRA plasmid carrying vacuolar metallothionein II gene (II.2.1.6 and II.2.1.8). Transgenic T₀ plants were regenerated from transformed callus (Fraley *et al.*, 1983; Horsch *et al.*, 1985). Briefly, wild type plants were grown on MS medium in “Weck” glasses and the youngest leaves (length up to 4 cm) were used for transformation. The agrobacteria suspension was prepared as described above and the OD_{600nm} was adjusted to at least 1.0 after dilution in MMA buffer. The leaves were cut into 8-10 pieces and transferred into “Weck” glasses containing 50-100 ml of agrobacteria suspension and incubated at RT for 30 min. The leaf pieces were then transferred onto sterile pre-wetted Whatman filters in petri dishes closed with saran wrap and incubated at 26-28°C in the dark for two days. Following washing with distilled water containing 100 µg/ml kanamycin, 200 µg/ml claforan and 200 µg/ml Betabactyl (Ticarcillin/Clavulanic acid, 25:1), leaf pieces were transferred onto MS II-plates and incubated at 25°C in the dark for one week and with a 16 h photoperiod for 2-3 weeks. After shooting, the shoots were removed and transferred onto MS-III-plates and incubated at 25°C with a 16 h photoperiod for 10-14 days until roots developed. The small plants were transferred into “Weck” glasses containing MS-III medium and incubated at 25°C in 16 h light rhythm for 2 weeks until transferred into soil. The young leaves from regenerated transgenic plants were used for immunoblot analysis of expressed Metallothionein II.

MS medium:

MS-salts	0.43% (w/v)
Myo-Inosite (SERVA)	0.1% (w/v)
Sucrose	2% (w/v)
Thiamin-HCl	0.4 mg/l
A. bidest	add to 1000 ml

The pH was adjusted to 5.8 with 1 N NaOH (for preparation of solid medium, 0.8% (w/v) agar were added), autoclaved and 500 µl of vitamin solution I were added upon cooling to 55°C.

MS-II medium:

MS medium supplemented with:	
BAP (in DMSO, from Sigma)	1 mg/l
NAA (from Sigma)	0.1 mg/l
Kanamycin	100 mg/l
Claforan	200 mg/l
Betabactyl	200 mg/l

MS-III medium:

MS medium supplemented with:	
Kanamycin	100 mg/l
Claforan	200-250 mg/l
Betabactyl	200-250 mg/l

Vitamin solution I:

Glycin	0.4% (w/v)
Nicotinic acid	0.1% (w/v)
Pyridoxin	0.1% (w/v)

Filter sterilized and stored at 4°C.

II.2.2.5 Growth of *N. tabacum* cv. Petite Havana SR1

Tobacco plants were grown in ED73 standard soil (Patzner, Sinntal-Jossa) with 0-30 % (v/v) sand under the following conditions: 16 h artificial light, 25°C (or higher depending on the outside temperature), 10 000 Lux (plus the sun light) and 70-90% humidity. To prevent pollination from other plants flowers were covered with plastic bags with micro pores. Mature, dried seeds were stored in paper bags at RT.

II.2.2.6 Preparation of total soluble proteins from plant leaves

For the extraction of transiently expressed metallothionein II in vacuum infiltrated tobacco leaves (II.2.2.3) or in stable transformed tobacco plant (II.2.2.4), frozen leaves were ground in liquid nitrogen to a fine powder with a mortar and pestle. Total soluble proteins were extracted using 2 ml of extraction buffer per gram leaf material. Cell debris removed by two rounds of centrifugation (16000g/4°C/30 min) and the supernatant was used for expression analyses by immunoblot (II.2.3.3).

1) Extraction buffer:

	200 mM
EDTA	5 mM
DTT	0.1 mM
Tween 20	0.1% (v/v)

2) Extraction Buffer

Tris-HCl pH=6, 8	75 mM
Urea	9 M
SDS	4.5 %
Mercaptoethanol	7.5 %
PMSF	5 mM

II.2.3 Protein analysis**II.2.3.1 Quantification of proteins**

The concentration of purified protein determined by Bradford (Bradford, 1976) or BCA (Pierce) assays. For Bradford assay the protein solution of interest was serially diluted. BSA was also serially diluted and used as standard. 10 µl of each dilution was transferred into the wells of a low binding microtiter plate (Greiner, Solingen, Germany). 10 µl of the buffer was used as a blank. 200 µl of Bradford reagent were added to each well, mixed with the proteins and incubated at RT for 10 min followed by the measurement of OD_{595nm}. For each dilution, measurements were performed in duplicate and the average was taken for the calculation of the protein concentration.

The Pierce BCA protein assay is a detergent-compatible formulation based on bicinchoninic acid (BCA) for the colorimetric detection and quantification of proteins (Smith *et al.*, 1985; Sorensen and Brodbeck, 1986). This method combines reduction of Cu⁺² to Cu⁺¹ by proteins in an alkaline medium with the sensitive and selective colorimetric detection of the cuprous cation (Cu⁺¹) using bicinchoninic acid (Wiechelman *et al.*, 1988). Concentration measurements were done according to the manufacturer's instructions, using BSA as standard.

II.2.3.2 SDS-PAA gel electrophoresis and Coomassie brilliant blue staining

Discontinuous SDS-polyacrylamide gels (for the stacking gel: T = 4 %, C = 2.6 %, pH 6.8; for the separating gel: T = 12 %, C = 2.6 %, pH 8.8) (Ausubel *et al.*, 1995) were used for separation of protein samples. Before loading onto the gel, protein samples were denatured in the presence of SDS and β-mercaptoethanol. The proteins were separated

electrophoretically with 20V/cm for 1 hour. Protein bands were revealed by staining with Coomassie brilliant blue or transfer to nitrocellulose membrane for immunoblot analysis (Ausubel *et al.*, 1995). Proteins were detected after incubating the gel for 30 min in Coomassie staining solution at RT under constant rocking. Coomassie staining was removed by destaining solution until the protein bands were clearly visible.

SDS-PAGE running buffer (pH 8.3):

Tris	125 mM (w/v)
Glycine	960 mM (v/v)
SDS	0.5% (w/v)

Coomassie staining solution:

Coomassie brilliant blue G-250	0.25% (w/v)
Methanol	50% (v/v)
Glacial acetic acid	9% (v/v)

Coomassie destaining solution:

Methanol	10% (v/v)
Glacial acetic acid	10% (v/v)

II.2.3.3 Immunoblot analysis

Separated proteins (II.2.3.2) were transferred from an SDS-PAA gel to PVDF or HybondTM-C nitrocellulose membrane (0.45 μ m). After blotting the membrane was blocked with PBS buffer containing 3 % (w/v) skim milk powder (MPBS). As primary antibody both anti *cmyc*, anti His6 were used in a dilution of 1:5000, anti GFP and anti metallothionein were used in a dilution of 1:1000 in 1xPBS. Attachment of the primary antibody was detected by addition of the secondary polyclonal antibody coupled to alkaline phosphatase (AP). Both, primary and secondary antibodies were diluted in blocking buffer. The target protein was finally revealed by addition of substrate BCIP/NBT.

PBS buffer (pH 7.3):

NaCl	137 mM
KCl	2.7 mM
Na ₂ HPO ₄ x2H ₂ O	8.1 mM
KH ₂ PO ₄	1.5 mM

Transfer buffer (pH 8.3):

Tris-HCl, pH 8.3	25 mM
Glycine	92 mM
Methanol	20% (v/v)

AP buffer (pH 9.6):

Tris-HCl, pH 9.6	100 mM
NaCl	100 mM
MgCl ₂	5 mM

II.2.4 Immuno Localisation

Transiently expressed metallothionein II in vacuum infiltrated tobacco leaves (II.2.2.3) or in stable transformed tobacco plant (II.2.2.4) were cut in 1 mm² square pieces and fixed o/n at 4°C in the fixative solution. This solution contained 4 % (v/v) paraformaldehyde and 0.2 % (v/v) glutaraldehyde in 0.1 M PBS. Fixing solution was carefully removed and leaf samples were washed several times with 0.1 M PBS at 4°C. They were then dehydrated through ethanol series at -20°C in AFS (Automatic Freeze Substitution, Leica).

<u>Ethanol (%)</u>	<u>Time (min)</u>	<u>Temperature (°C)</u>
50	30	4
70	30	-20
96	30	-20
100	30	-20

Leaf samples were embedded in LR White resin according to the following steps at -20°C.

<u>Resin/Solvent (v/v)</u>	<u>Time (h)</u>
1/3	1
2/2	1
3/1	1
Pure resin	o/n

The polymerisation of the resin was done by UV irradiation for 24h at -20°C.

Samples were cut in 90 nm (immunogold labelling) and 1200 nm (immunofluorescence labelling) thin sections using Leica Ultracut ultramicrotome and collected on 400 mesh grids.

II.2.4.1 Immunogold Labelling

Sections were floated into 5 % (v/v) bovine serum albumin (in 0.1 M PBS pH 7.4) drops for 15 min at room temperature to block the non-specific binding sites. Sections were then incubated o/n at 4°C with rabbit anti Metallothionein polyclonal antibody applied at dilution 1:50 in PBS o/n. After 3 washes with 0.25 % (v/v) Tween 20 in 0.1 M PBS, sections were transferred to the goat anti-rabbit antibody conjugated to 10 nm-diameter gold particles at the dilution 1:30 in PBS. After 1 hour of incubation at room temperature, the sections were

washed 3 times 10 min with 0.1 M PBS pH 7.4 and then with distilled H₂O. After immunogold labelling, Philips EM 400 carried out electron microscopy at 80kV.

II.2.4.2 Immunofluorescence Labelling

Sections were floated in to 5 % (v/v) bovine serum albumin (in 0.1M PBS pH 7.4) drops for 15 min at room temperature to block the non-specific binding sites. Sections were then incubated with rabbit anti Metallothionein polyclonal antibody applied at dilution 1:50 in PBS o/n at 4°C. After 3 washes with 0.25 % (v/v) Tween 20 in 0.1 M PBS, sections were transferred to the goat anti rabbit Alexa Fluor 594 at dilution 1/100 in PBS. After 1 hour of incubation at room temperature, the sections were washed 3 times 10 min with 0.1 M PBS, pH 7.4 and then with distilled H₂O. After Immunofluorescence labelling, electron microscopy was carried out using a Philips EM 400 at 80kV.

II.2.4.3 Fluorescence Microscopy

Transiently expressed metallothionein II in vacuum infiltrated tobacco leaf (II.2.3.3) was imaged with a TCS-SP spectral confocal microscope equipped with argon ion, krypton, and helium-neon lasers (Leica, Heidelberg, Germany). Images were acquired with a 1.2 numerical aperture x 63 oil immersions PLAN-APO objective. GFP fluorescence was excited with 488 nm Ar laser line and confocal sections were collected using a 510-550 nm emission setting.

II.2.5 Hydroponic Experiments

II.2.5.1 Plants Growth

Seeds of tobacco plants (*Nicotiana tabaccum* SR-1) were germinated in a mixture of peat and sand. After 3-4 weeks (depending on the temperature and the greenhouse conditions), the seedlings were transferred into the pots. Seeds of transgenic tobacco plants were germinated in MS III (II.2.2.4) plates for about 4 weeks. The seedlings were firstly transferred to mixture of peat-sand for 2 weeks and then to plastic vessels (2 seedlings per vessel) containing aerated nutrient solution, changed every 2-3 days. The composition of nutrient solution consisted of macro and microelements as indicated below:

Macro Elements

K ₂ SO ₄	0.88 mM
Ca (NO ₃) ₂	2.0 mM
KH ₂ PO ₄	0.25 mM
MgSO ₄	1 mM
KCl	0.1 mM

Micro Elements

Fe-EDTA	100 µM
H ₃ BO ₃	1 µM
MnSO ₄	0.5 µM
ZnSO ₄	1 µM
CuSO ₄	0.2 µM
(NH ₄) ₆ Mo ₇ O ₂₄	0.02 µM

Deionized water was used for preparation of the nutrient solution. Plants were grown in growth chamber under controlled environmental conditions (light/dark regimes of 16/8 h, temperature 25/20 °C, and humidity 60 % and photosynthetic photon flux 320µmol/m²/s at plant height). After 6 days of growth in nutrient solution, plants were supplied with increasing concentrations of 3CdSO₄.7 H₂O (e.g., 0-50-100 µM) for a week. Plants were harvested when the effects of increasing Cd supply on growth of plants became severe (e.g. after 14 days of growth with Cd supply under given conditions). Before the harvest, plants were assessed for severity of Cd toxicity on leaves and roots.

II.2.5.2 Tissue preparation

Plant samples (shoots and roots) were washed in deionized water and dried between Kleenex tissues. For measurement of protein analysis (II.2.3), non-protein SH-groups (II.2.5.6), and PCs (II.2.5.7) in fresh tissues, separate samples of roots and whole shoots were taken and stored in liquid nitrogen until analysis. At harvest, roots and shoots were separated and dried at 70°C for determination of dry weight and Cd concentration. Samples were oven-dried at 70°C for 48 h and dry weight was determined. Subsequently, the plant tissues were ground in a ball mill (Jeffery *et al.* 1990).

II.2.5.3 Dry digestion

After milling, 200 ± 5 mg of dried plant tissue (11.2.5.2) were weighed into porcelain crucible. The plant tissue was ashed at 500°C for 5 h in a muffle furnace. The crucible was removed and cooled down at room temperature. 2 ml of 15 % (v/v) HCl were added and sample was dried at 60°C . After cooling down at RT, 2 ml of 15 % (v/v) HCl were added and the ash was dissolved with the assistance of a plastic stick, and subsequently filtered through a blue ribbon filter. The volume was filled up to 20 ml with millipore water. Cd was measured by atomic absorption spectrometer (AAS).

II.2.5.4 Atomic absorption spectrometry (AAS)

AAS measurement was conducted with a Perkin Elmer 1100B spectrometer. Tab. 2-2 shows the conditions under which the measurements were performed.

Table II-2 Measurement-conditions.

Character	Value
Energy	66
Gap (H)	0.7
Current (mA)	5
Wavelength (nm)	288.8
Time (s)	1
Repetition	3

II.2.5.5 Analysis of SH-Groups

Measurement of total non-protein SH-groups has been carried out as described by Cakmak and Marschner (1992). Approximately 0.5 g fresh leaf and 1.0 g fresh root samples were extracted with 5 ml of 5 % (v/v) *meta*-phosphoric acid, and centrifuged at $15\,000\,g$ for 15 min. For the assay of SH-groups, 2.5 ml of 150 mM phosphate buffer (pH 7.4) containing 5 mM EDTA and 0.5 ml 6 mM 5-5'-dithiobis-(2-nitro-benzoic acid) were added to 0.5 ml of plant extract. Following incubation at room temperature, the colour produced was measured at 412 nm with spectrophotometer (Hitachi U-2000, Japan). Glutathione was used as a standard in the range of 0 to $100\,\mu\text{g ml}^{-1}$.

II.2.5.6 Analysis of phytochelatins

0.5 ml of plant extract (II.2.5.5) was taken and 150 μ l NaBH₄ (solved in 2.5M NaOH) were added. Then was incubated about 5 min. at room temperature and then 200 μ l of 3.5 M HCl were added to the mixture and on ice for 10 minutes. Sample was centrifuged for 10 min with maximum rpm (13000rpm), and then the supernatant was filtered through 0.2 μ m sterile filters. 1 ml of filtrate was analysed by HPLC at 412 nm under the following conditions:

Injection volume	0.05 ml
<u>Chromatography</u>	
Column	Li Chrosorb RP 18, 250*4mm with pre-column
Solvent A	0.05 % (v/v) phosphoric acid
Solvent B	100 % (v/v) acetonitrile
Flow	1 ml/min
Gradient	8 min 100% A, then within 20 min to 20% B, then constant 20% B for 6 min, then within 6 min to 100% A and 100% A for 10 min.
Detection	Ellmanns-Reagent used for next measurement with post-column derivatisation by 412nm
Ellmanns-Reagent	0.1 mg/ml in 50mM potassium phosphate, pH 8.0, flow:1 ml/min

Retention Times of Peaks:

Cysteine: in 2-3 min
 Glu-Cyc: in 3-4 min
 GHS: in 5-6 min
 Phytochelatines: in 18-25 min

II.2.6 Soil Experiments

II.2.6.1 Soil properties

The soil used for the pot experiments was collected from a field in Melaten, RWTH-Aachen (BioV) at a depth of 0 to 30 cm. The soil was air-dried and sieved through a 2 mm sieve. Some of the soils physical and chemical properties are listed in Table II-3.

Table II-3 Properties of the soil used in the experiment.

Chemical Characters	Content
Organic matter (%)	4.3
pH (CaCl ₂)	7.2
Salt (%)	0.073
CaCO ₃ (%)	2.6
Total Cd (ppm)*	2.33
Bioavailable Cd (ppm)**	0.62

* Extracted with aqua regia

** Extracted with DTPA

Physical Characters		Content (%)
Texture	Sand	49.41
	Clay	8.49
	Silt	42.10

Soil texture Class is Loam.

Soil texture, pH, CaCO₃, salt, and organic matter, were determined with standard methods (II.2.6.3).

II.2.6.2 Soil preparation

Soil samples were analysed before the seedlings were planted into the pots, and after the harvesting. The soil was air dried and then passed through a sieve of 2 mm to remove the roots, which could influence the subsequent soil analysis.

II.2.6.3 Soil analysis

Soil texture: sand, clay, and silt fractions of soil samples were determined by Bouyoucos hydrometer methods (Bouyoucos, 1952)

pH: pH was determined with the CaCl₂-method (Lewandowski *et al.*, 1997)

CaCO₃: was determined with gravimetric method for loss of carbon dioxide (Loeppert *et al.*, 1996).

Salt: Salt content was determined through electrical conductivity method (Rhoades *et al.*, 1990)

Organic matter: according to Walkley, Black method (Nelson *et al.*, 1996).

II.2.6.4 DTPA-method

The DTPA-method was used to determine the bioavailable Cd and other metals such as Pb, Zn, Cu, and Ni in the soil (Risser *et al.* 1990). Therefore 20 ml of the DTPA solution containing 0.005 M DTPA, 0.01 M CaCl_2 , and 0.1 M TEA was mixed with 10 g of air-dried soil (II.2.6.1) and was shaken at 2 cycle's s^{-1} for 2 h. The suspension was gravity filtered through blue ribbon quantitative analytical filter paper. Cadmium analysis in the soil extract was performed by AAS. Cadmium standards were prepared by addition of 0.2-5 ppm $3\text{CdSO}_4 \cdot 7\text{H}_2\text{O}$ (Merck) into the DTPA solution and analysed under identical conditions.

II.2.6.5 Aqua regia-method

With the aqua regia method it is possible to determine the total amount of Cd and other metals in the soil (DIN 38414 Teil 7:1983-01). Three gram of air-dried soil was boiled with aqua regia (21 ml 32% HCl, 7 ml 63% HNO_3) for 120 min under reflux. Then deionised water was added to the suspension for a total volume of 100 ml. The suspension was gravity filtered through blue ribbon quantitative analytical filter paper. Cadmium analysis in the soil extract was performed by AAS. Cadmium standards were prepared by addition of 0.2-5 ppm $3\text{CdSO}_4 \cdot 7\text{H}_2\text{O}$ (Merck) into the aqua regia solution (21 ml 32% HCl, 7 ml 63% HNO_3) and analysed under identical conditions.

II.2.6.6 Pot Experiments

One kg of soil was filled in 1.5 L plastic pots. To each pot the following amounts of fertilizer were applied: 200 ppm $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, 100 ppm KH_2PO_4 and 2.5 ppm FeEDTA. Serial Cd concentrations ranging from 0 to 40 ppm were applied directly to the soil. Each treatment was replicated three times.

Nicotiana tabaccum Petit Havana SR-1 and transgenic tobacco plants used for the pot experiments were grown under controlled environmental conditions with a 16 h light period (light intensity of $320\mu\text{mol}/\text{m}^2/\text{s}$), a 25/20°C light/dark temperature regime, and 60 % relative humidity. Plants were harvested after 6 weeks of growth.

II.2.6.7 Tissue preparation

Plant samples (stem, leaves and bloom) were rinsed briefly in Millipore water and dried between Kleenex tissues. Samples were oven-dried at 70°C for 48 h and dry weight was determined. Subsequently, the plant tissues were ground in a ball mill (Jeffery A, *et al.* 1990).

II.2.6.8 Plant Analysis

For measurement of non-protein SH-groups (II.2.5.6) and protein analysis (II.2.3) in fresh tissues, samples were collected from leaf, stem and bloom dried at 70°C for 48 h. After measuring the dry weight, samples were subjected to Cd measurement studies (Jeffery *et al.*, 1990). Non-protein SH-group analysis (II.2.5.5), dry weight and cadmium concentration analysis (II.2.5.3) were determined like in hydroponic experiments.

III RESULTS

III.1 Cloning of Metallothionein II gene

III.1.1 Cloning of the Chinese Hamster Metallothionein II vacuolar targeting cassette

The Chinese hamster Metallothionein II (ChMTII) gene was amplified from the p35SMG plasmid (Brandle, 1992) using the combination of primers ChMTII N 5' and ChMTII N 3' as described in (II.1.6/2). The 200 bps PCR product (Figure III.1) showed the size calculated. The ChMTII PCR product was digested via *NcoI* and ligated into the *NcoI* digested pUC18-*NdeI**GFP-S65C-VTS vector (Dr. Verena Hoppman, RWTH Aachen) to generate the pUC18-cV ChMTII GFP gene fusion cassette.

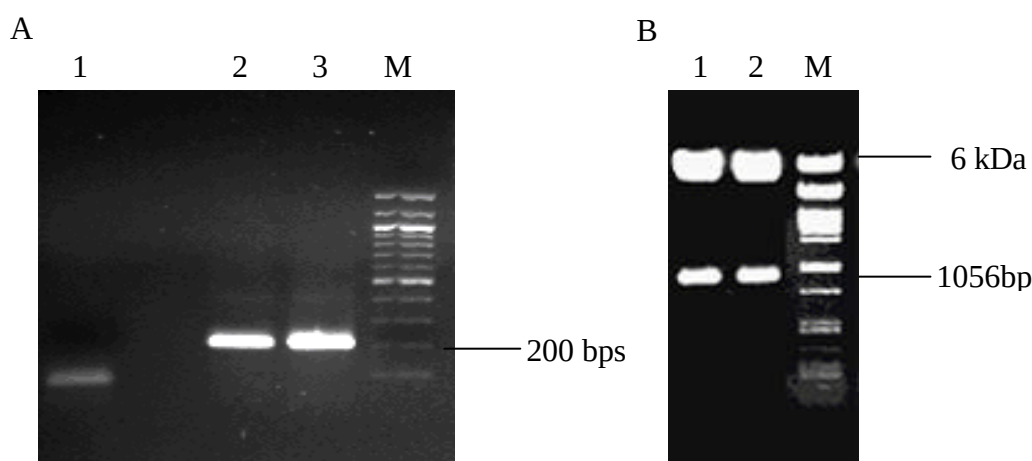


Figure III-1 PCR amplification and cloning of the ChMTII gene.

PCR products were separated on a 1.2% (w/v) agarose gel (II.2.1.13). A: (1) negative control without template; (2-3) 5 μ l of PCR amplified ChMTII gene from p35SMG plasmid; M: 100 bp. B: Double digestion of the pcV-ChMTII-GFP plasmid with *EcoRI/XbaI* restriction enzymes (1 and 2); M: ? *PstI*.

This cassette contained the 5' untranslated region of *chalcone synthase* (CHS5'UTR), the vacuolar targeting signal from the *Catharantus roseus strictosidine synthase* (cV), the Chinese Hamster metallothionein II (ChMTII), and in translational fusion a gene encoding the GFP4-S65C mutant of the Green Fluorescent Protein (GFP) as a marker for visual detection. The mGFP4-S65C gene contains a mutation encoding a Cystein in place of a Serine at 65 bp positions. The cV ChMTII GFP cassette was excised via *EcoRI/XbaI* and ligated into the *EcoRI/XbaI* digested pTRA-kc plant expression vector to generate the p-cV-ChMTII-GFP construct (Figure III-2). The construct was sequenced (II.1.6.1) using the primers pSS5' and pSS3'. The sequence analysis of six randomly selected colonies confirmed the presence of ChMTII gene (VII.2).

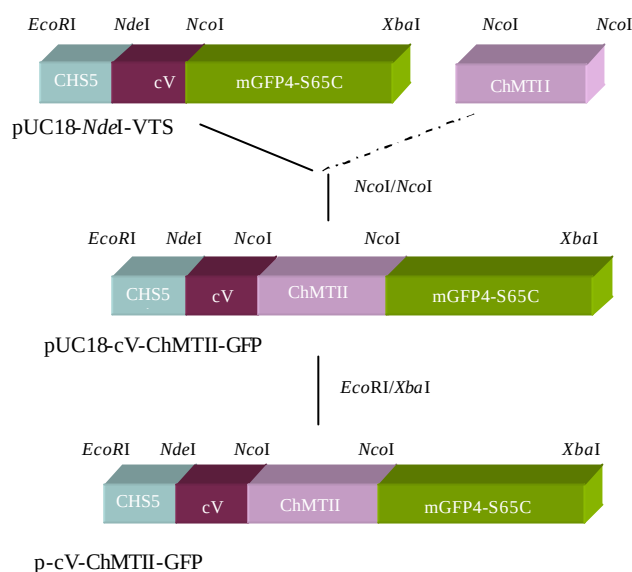


Figure III-2 Strategy for cloning of *Chinese hamster Metallothionein II* into pTRA-kc plant expression vector for vacuolar targeting.

CHS 5: 5' untranslated region of *chalcone synthase*; cV: Vacuolar targeting signal from the *C. roseus* strictosidine synthase; ChMTII: Chinese Hamster Metallothionein II; GFP: green florescent protein (mGFP4-S65C).

III.1.2 Cloning of *Saccharomyces cerevisiae* and *Chinese hamster Metallothioneins*

The *Saccharomyces cerevisiae* metallothionein II (ScMTII) gene (kindly provided by Dr. Martin Zimmermann, RWTH-Aachen, Biology IV) was amplified by PCR using the primers ScMTII S 5' and ScMTII S 3' (II.1.6.2) (Figure III-3/A).

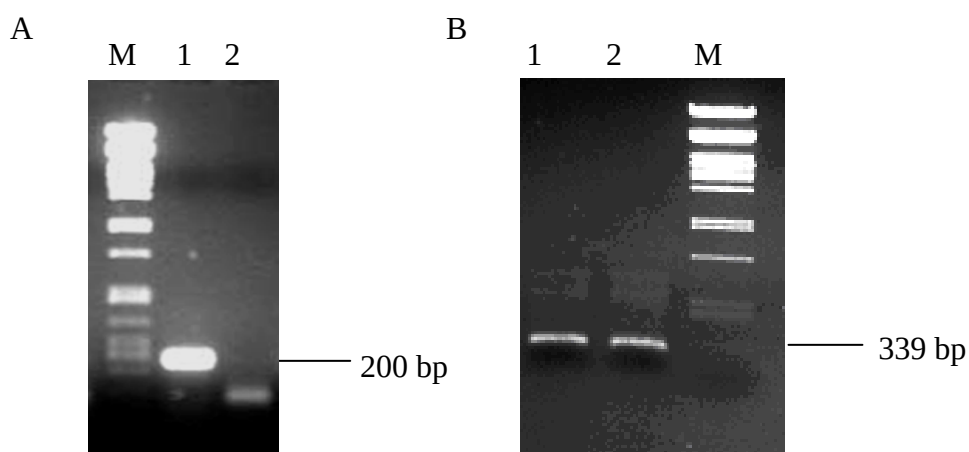


Figure III-3: PCR amplification of the ScMTII gene from yeast.

PCR products were separated on a 1.2% (w/v) agarose gel (II.2.1.13). A: Line 1: 5 µl of PCR amplified ScMTII gene; Line 2: negative control; M: ? Pst. B: Double digestion of SOE PCR product of S ChMTII (line 1) and S ScMTII (line 2) genes with *EcoRI/NcoI* restriction enzymes. M: ? Pst

The 200 bp PCR products encoding the *ScMTII* (Figure III.2/A) or the *ChMTII* (Figure III.1/A) were subcloned into the pUC18 vector downstream of vacuolar targeting signal from *Catarantus roseus* (cV) and upstream of c-myc and his6 tags to generate the CHS-cV-MTII cassette.

Since previous studies have indicated higher expression levels of recombinant proteins (Matsuoka, *et al.*, 1990; Koide, *et al.*, 1997) into the vacuole when potato Sporamine A vacuolar targeting signal (S) was used, a new cassette containing the potato Sporamine A vacuolar targeting signal instead of existing *Catarantus roseus* vts was constructed. The gene encoding Sporamin A was amplified by three step splice overlap extension (SOE) PCR using P2, P4 and P5 primers (II.1.6/2) based on the sequence of potato Sporamine A mRNA sequence (Gen-bank accession number X15091).

In the first step the primers P5 and MTII back were used to amplify the *ScMTII* and/or *ChMTII* in order to incorporate a 5' complementary region (II.1.6/2) that was used as the template-overlapping region in the final SOE PCR (Figure III-4). In the second step four primers (II.1.6/2) with complementary regions of 11-21bp (P1, P2, P3 and P4) were designed to synthetically fuse the CHS 5'UTR to the S signal (Figure III-4). The four primer SOE-PCR was performed to synthesize this DNA fragment. In the third step, 10 µl of the second SOE-PCR as well as 10 µL of the first PCR were used to assemble the complete CHS-S-MTII cassette with the outer P1 primer and the MTII backward primer.

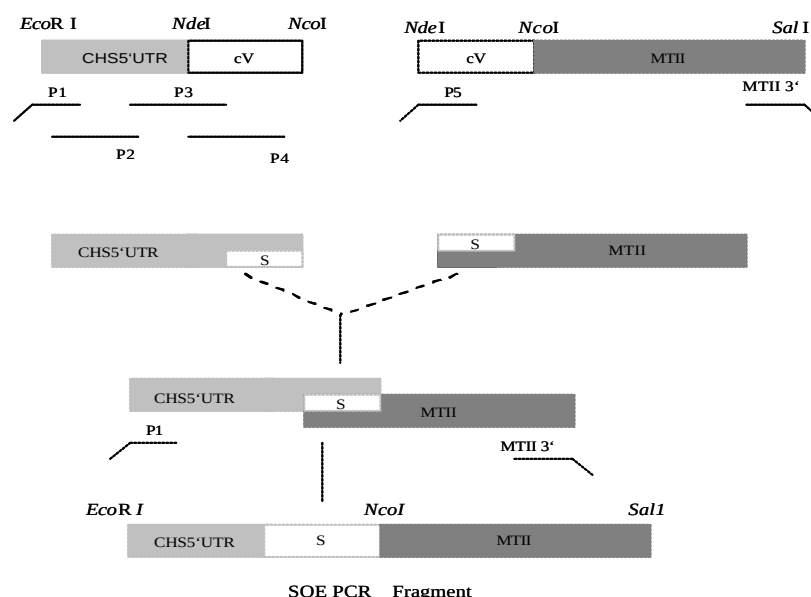


Figure III-4 Strategy for cloning of *ChMTII* and *ScMTII* genes into pUC18 vector containing the S vacuolar targeting signal by SOE PCR.

CHS5'UTR: 5' untranslated region of chalcon synthase; cV: Vacuolar targeting signal from the *C. roseus* strictosidine synthase; S: vacuolar targeting signal from potato Sporamin A; *ChMTII*: *Chinese hamster* Metallothionein II; *ScMTII*: *Saccharomyces cerevisiae* Metallothionein II; c-myc: myc epitope for detection; his 6: his-6 tag for detection.

The final SOE-PCR cassettes (S-ChMTII and S-ScMTII) were digested with *EcoRI*/*SalI* restriction enzymes and ligated into the linearized pUC18 plasmid (kindly provided by Dr. Verena Hoppman, RWTH-Aachen) upstream of the c-myc and his6 tags (Figure III-5).

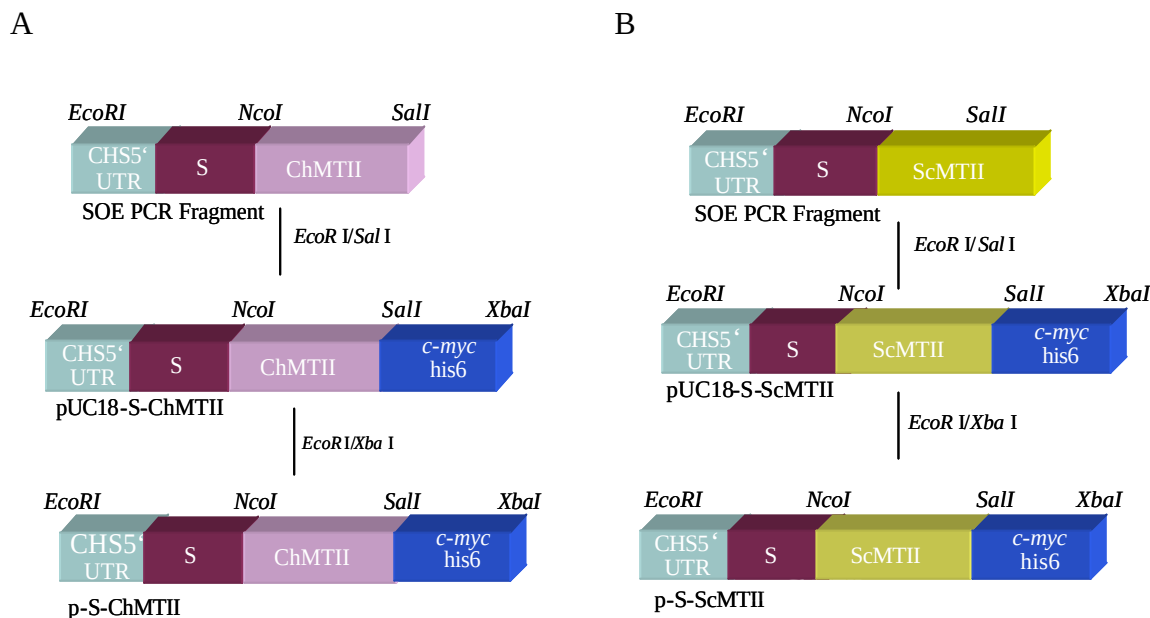


Figure III-5 Strategy for cloning of ChMTII (A) and ScMTII (B) genes into pTRA-kc plant expression vector.

CHS: 5' untranslated region of chalcon synthase; S: vacuolar targeting signal from potato Sporamin A; ChMTII: *Chinese hamster* Metallothionein, ScMTII: *Saccharomyces cerevisiae* Metallothionein II; c-myc: myc epitope for detection; his 6: his-6 tag for detection.

For generation of pS-ChMTII and pS-ScMTII plant expression constructs, cassettes containing the 5' UTR of CHS, the S vacuolar targeting signal, the ChMTII or ScMTII genes and c-myc and his6 detection tags were excised with *EcoRI*/*XbaI* from pUC18-S-ChMTII and pUC18-S-ScMTII vectors, respectively and cloned downstream of the enhanced 35S promoter and upstream of the 3' UTR of CaMV 35S polyadenylation signal (pA35SS) of the pTRA-kc plant expression vector. Seven independent recombinant colonies from each transformation were screened for the presence of S-ChMTII and S-ScMTII cassettes by double digestion with *EcoRI* and *XbaI* restriction enzymes (Figure III-6). In order to prevent blocking of the *XbaI* restriction site by dam methylation the p-S-ChMTII and p-S-ScMTII plasmids were previously transformed into *E. coli* SCS110. Agarose gel analysis indicated that the *EcoRI*/*XbaI* digested insert had the expected size of S-ChMTII and S-ScMTII cassettes (339 bp). Furthermore, sequencing results (VII.2-3-4) of five independent clones confirmed the presence of both cassettes into the pTRA-kc plant expression vector.

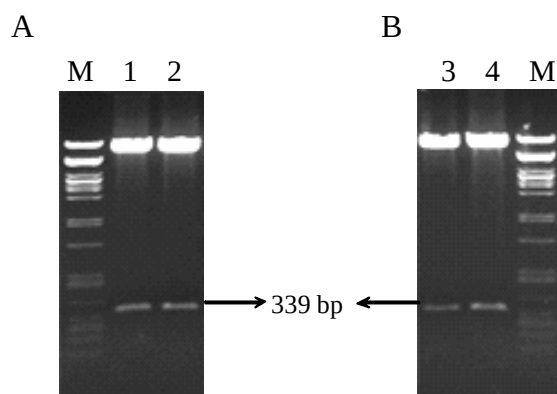


Figure III-6 Cloning of S-ChMTII and S-ScMTII cassettes into pTRA-kc plant expression vector.

DNA was separated on a 1.2 % (w/v) agarose gel (II.2.1.13). A: Double digestion of the p-S-ChMTII plasmid with *EcoRI/XbaI* restriction enzymes (line 1-2). B: Double digestion of the pS-ScMTII plasmid with *EcoRI/XbaI* restriction enzymes (line 3-4). M: ? Pst.

III.2 Generation and characterization of transgenic plants

The biological function of ChMTII and ScMTII was assessed by transient and stable expression in tobacco plants via *Agrobacterium*-mediated transformation. The expression constructs containing cV-ChMTII-GFP, S-ChMTII and S-ScMTII in the pTRA-kc vector were first transformed into *Agrobacterium tumefaciens* GV3101 by either electroporation or heat shock transformation (II.2.1.6 and II.2.1.8). Seven independent recombinant colonies from each transformation were screened by colony PCR (II.2.1.15) for the presence of the recombinant cassettes. Then cultures of the recombinant *agrobacteria* were prepared from single colonies for each construct (II.2.1.11) and used for transient expression (II.2.2.1) and stable transformation in *N. tabacum* (II.2.2.2).

III.2.1 Transient expression of Metallothioneins

Transient expression experiments were performed as preliminary assays because the procedure is fast and not affected by positional effects (Kapila *et al.*, 1996). Four leaves per each construct were infiltrated with recombinant *Agrobacteria*. After three days of incubation total soluble proteins were extracted from the leaves and subjected to immunoblot analysis to verify expression rate and integrity of the recombinant vacuolar proteins. Time-course analyses showed that maximum accumulation of ChMTII-GFP, ScMTII and ChMTII were reached at 50-60 h post infiltration (data not shown). Immunoblot analyses of the crude protein extracts at 50-60 hours post infiltration with *agrobacteria* transformed with the p-cV-ChMTII-GFP construct showed a distinct band of approximately 37 kDa that corresponded to

the estimated molecular weight of the ChMTII-GFP fusion protein upon processing of the vacuolar signal (Figure III-7/A). The two metallothioneins fused to the S targeting signal (ChMTII and ScMTII) should show a molecular weight of approximately 12kDa. However, the immunoblot analysis revealed the presence of distinct band at 17 kDa for ChMTII and ScMTII recombinant proteins (Figure III-7/B).

The level of expression of the recombinant proteins varied considerably between the constructs. The immunoblot analysis indicated that higher level of accumulation of the recombinant proteins was achieved in leaves expressing the ChMTII-GFP fusion.

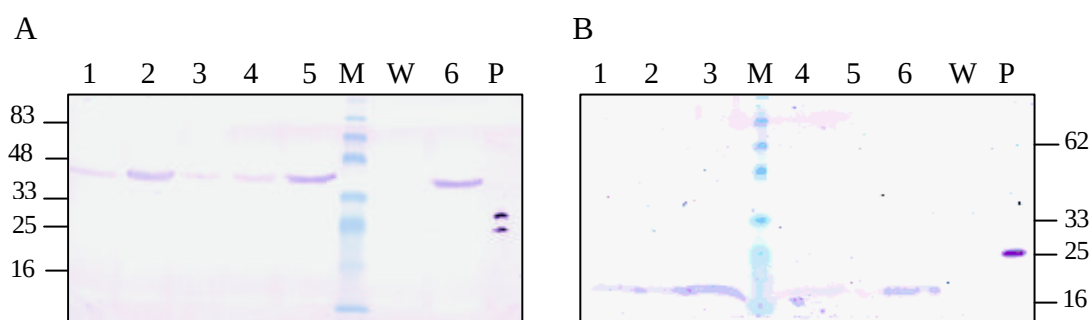


Figure III-7 Transient expression of MTIIs in the plant cell vacuole.

Total soluble leaf proteins (TSP) (II.2.2.6) were extracted by grinding tobacco leaf tissue in two volumes extraction buffer (II.2.2.6). 10 μ l of total soluble protein from each sample was separated by 12% SDS-PAGE (II.2.3.2) and blotted onto a nitrocellulose membrane (II.2.3.3). Immunodetection was carried out with anti-GFP antibody (1:1000) for (A) and anti-his tag antibody (1:2000) for (B), followed by 1 hr incubation with polyclonal anti rabbit (1:5000) and goat anti-mouse antibody conjugated to alkaline phosphatase (1:5000) and NBT/BCIP detection for 5 min at RT.

A: 1-6= 10 μ l of TSP extracted from individual tobacco leaves expressing ChMTII-GFP fusion protein in the vacuole under the *Chatarantus roseus* vacuolar targeting signal; W: 10 μ l of TSP from wild type leaves; M: prestained protein marker. P: 200 ng GFP used as positive control.

B: 10 μ l of TSP extracted from individual tobacco leaves expressing ChMTII (Lines: 1-3) and ScMTII (Lines 4-6) in the vacuole under the Sporamin A vacuolar targeting signal; M: prestained protein marker; W: 10 μ l of TSP from wild type fresh tobacco leaves; P: 200 ng of scFv4813 used as positive control.

III.2.2 Fluorescence microscopy analysis

III.2.2.1 GFP analysis

Fluorescence microscopy analysis was performed (II.2.4.1) to confirm expression and correct targeting of the recombinant ChMTII-GFP fusion protein to the plant vacuole. Wild type (SR-1) tobacco leaves were used as negative control. Florescence analysis of tobacco leaves transiently producing the ChMTII-GFP indicated a very intensive GFP fluorescence in the

nucleus and ER (Figure III-8), however no fluorescence signal was observed in the vacuole. The negative control samples did not show any fluorescence.

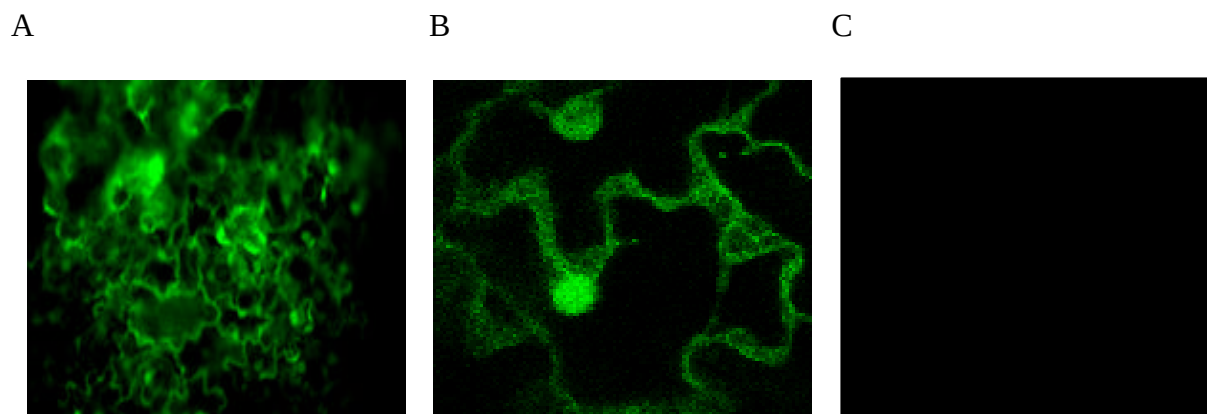


Figure III-8 Fluorescence analysis of transiently expressed vacuolar ChMTII-GFP by TSC-SP spectral confocal microscope.

A-B: Different infiltrated tobacco leaves with recombinant agrobacteria transformed with p-cV-ChMTII-GFP construct. C: wild type SR-1 tobacco leaves.

GFP fluorescence was excited with 488 nm. Ar laser line and confocal sections were collected using a 510-550 nm emission setting.

III.2.3 Immunofluorescence analysis

To further characterize protein expression and localization, immunofluorescence analysis was carried out on histological preparation from leaves transiently expressing the recombinant ChMTII or ScMTII into the plant vacuole. Wild type (SR-1) tobacco leaves were used as negative control. A polyclonal rabbit α -MTII was used as primary antibody whereas Alexa Fluor 594 Goat α -Rabbit was used as the secondary antibody for labelling. The samples were analyzed by confocal microscopy as described in (II.2.4.2) for detecting the subcellular localization of the fusion proteins. However, because of the high background of the chlorophyll, localization of the MTII proteins couldn't be detected (data not shown).

III.2.4 Generation and screening of transgenic tobacco plants expressing

Metallothioneins II

The p-cV-ChMTII-GFP, p-S-ChMTII and p-S-ScMTII constructs were stably transformed into tobacco plants by *Agrobacterium*-mediated transformation (II.2.2.2). Twenty-five primary transformant plants were screened for accumulation of the recombinant proteins. None of the transformed plants exhibited altered morphology and all set seed normally upon self-fertilization.

Accumulation of the recombinant proteins in tobacco leaves was assayed by immunoblot analysis using crude extracts of the total soluble leaf proteins. The recombinant proteins migrated according to their predicted molecular weight of 37 kDa for ChMTII-GFP fusion protein (Figure III-9/A-C) and 17 kDa for ChMTII (Figure III.10/A-B) and ScMTII (Figure III.10/C-D), respectively. Higher accumulation of the recombinant protein was observed in tobacco plants transformed with the cV-ChMTII-GFP construct, whereas the lower protein accumulation was observed for the S-ScMTII. The lines with the highest level of accumulation of recombinant metallothioneins for each of the three targeting cassettes the cV-ChMTII-GFP (line 6 and 13), S-ChMTII (line 18 and 21); and S-ScMTII (line 11 and 24) were selected and self-pollinated for establishment of homozygous lines. These T_0 plants segregated in a Mendelian manner into seedlings with the MTII transgene and those without it.

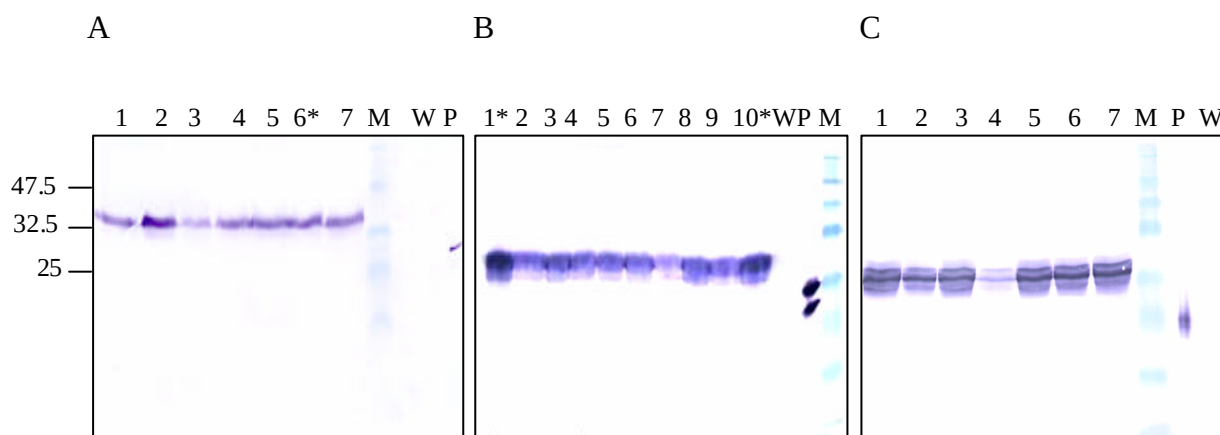


Figure III.9 Screening of transgenic tobacco plants expressing cV vacuolar ChMTII.

Total soluble leaf proteins were extracted by grinding tobacco leaf tissue in two volumes extraction buffer (II.2.2.6). 10 μ l of total soluble protein from each sample was separated by 12% SDS-PAGE (II.2.3.2) and blotted onto a nitrocellulose membrane (II.2.3.3). Immuno detection was carried out with anti-GFP antibody (1:1000), followed by 1 hr incubation with polyclonal anti-rabbit (1:5000) and goat anti-mouse antibody conjugated to alkaline phosphatase (1:5000) and NBT/BCIP detection for 5 min at RT.

A: 1-7: T_0 transgenic lines from the transformation experiments expressing ChMTII-GFP fusion protein in the vacuole under the *Chatarantus roseus* vacuolar targeting signal. B: 1-10: T_1 transgenic lines generated from the line 13 expressing vacuolar ChMTII-GFP C: 1-8: T_2 transgenic lines generated from the T_1 generation of cV-ChMTII-GFP line 13 indicated in the Figure III-9/B. W: total soluble protein extracted from wild type fresh leaves; M: prestained protein marker, P: 200 ng GFP as a positive control. Tobacco lines selected for establishment of homozygous lines are indicated by *.

The accumulation levels of the vacuolar ChMTII-GFP fusion protein in the T_1 generation were similar to the parental T_0 cV-ChMTII-GFP line 6 and line 13 (data not shown). The latter was chosen for further experiments. The T_2 generation of line 13 (Figure III-9/B) showed higher level of protein accumulation than the T_0 and T_1 generation as estimated by immunoblot analysis.

The T_1 generation of S-ChMTII line 18 and 21 accumulated comparable levels of recombinant ChMTII protein when compared to the T_0 lines (data not shown).

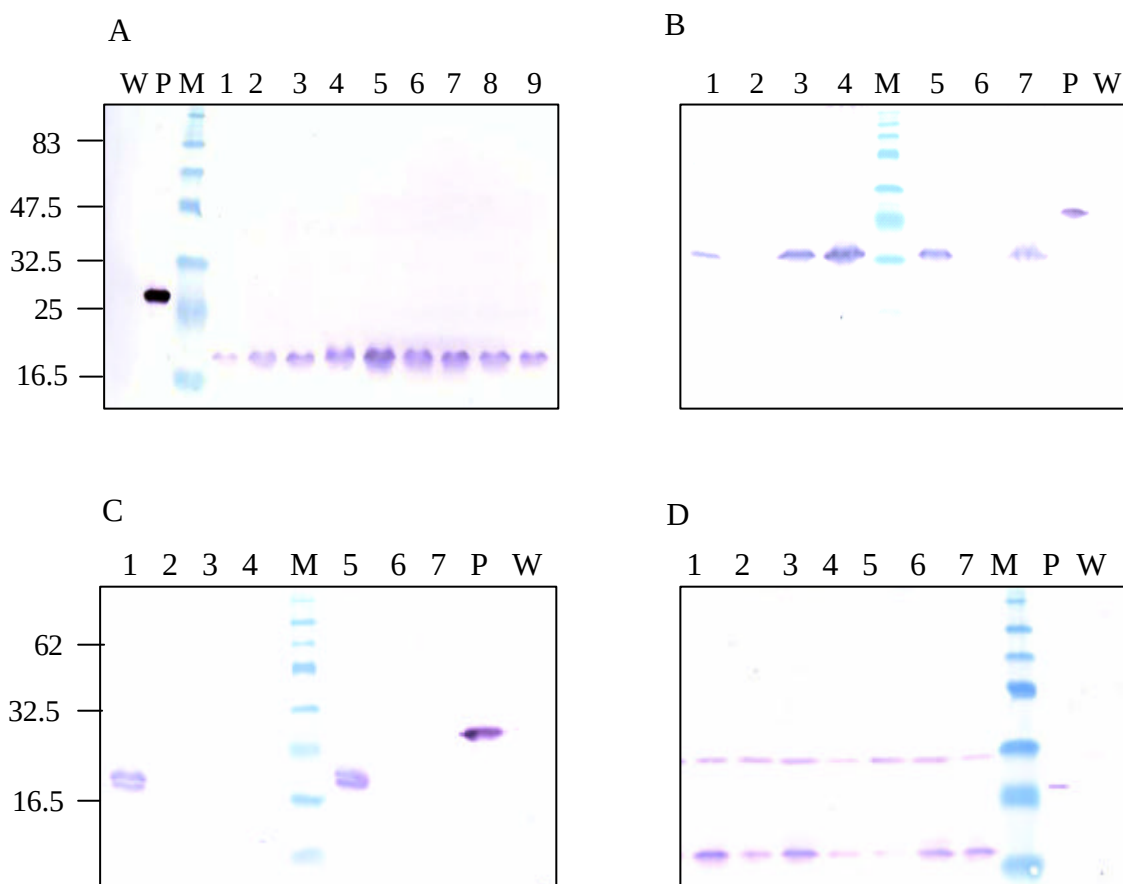


Figure III-10 Screening of stable transgenic tobacco plants expressing S vacuolar ChMTII and ScMTII.

Total soluble leaf proteins were extracted by grinding tobacco leaf tissue in two volumes extraction buffer (II.2.2.6). 10 μ l of total soluble protein from each sample was separated by 12% SDS-PAGE (II.2.3.2) and blotted onto a nitrocellulose membrane (II.2.3.3). Immunodetection was carried out with anti-his tag antibody (1:2000), followed by 1 hr incubation with goat anti-mouse antibody conjugated to alkaline phosphatase (1:5000) and NBT/BCIP detection for 5 min at RT.

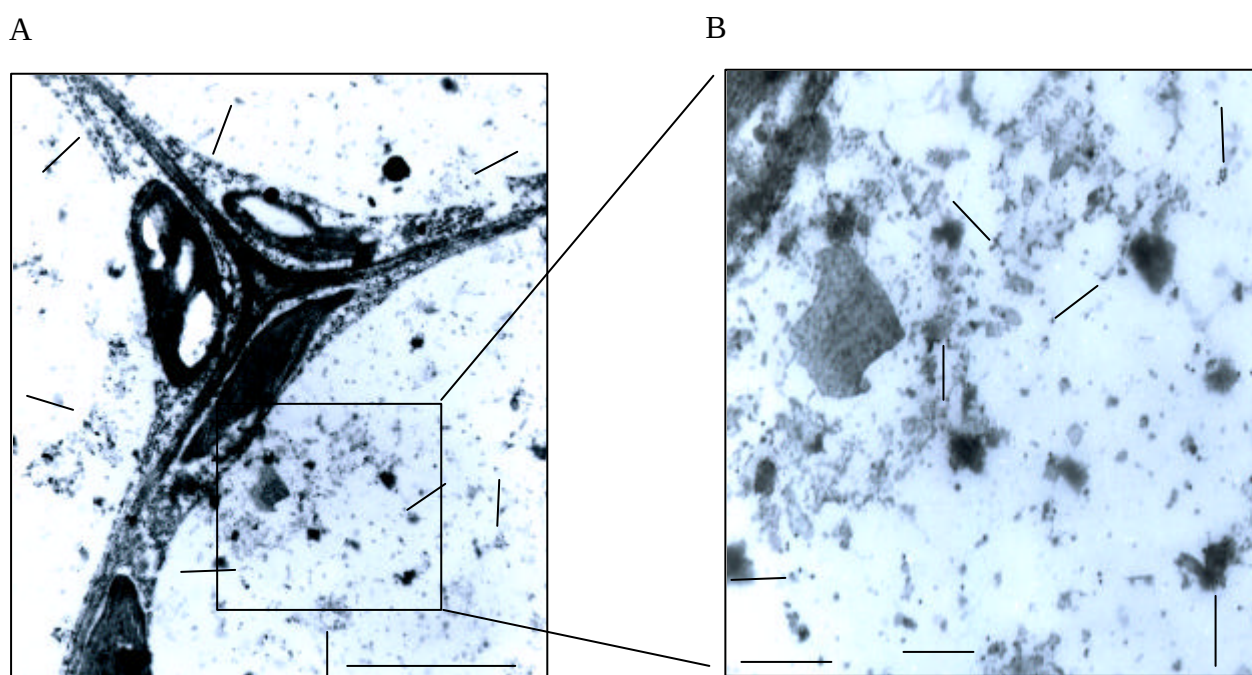
A: 1-9 T_0 transgenic lines expressing ChMTII in the vacuole under the Sporamycin A vacuolar targeting signal. B: 1-7: T_1 transgenic lines generated from the T_0 generation of S-ChMTII line 18. C: 1-7 T_0 transgenic lines expressing vacuolar ScMTII. D: 1-7: T_1 transgenic lines generated from the T_0 generation of S-ScMTII line 24 expressing vacuolar ScMTII. W: total soluble protein extracted from wild type fresh leaves; M: prestained protein marker; P: 300 ng of scFv4813 used as positive control.

The T_1 generation of S-ScMTII (lines 11 and 24) also showed comparable levels of recombinant ScMTII protein when compared to the T_0 lines.

The T_2 generation of tobacco lines expressing recombinant cV-ChMTII-GFP (line 13/3) as well as the T_1 generation of the S-ChMTII (line 18) and S-ScMTII (line 24) were used for evaluating *in vivo* protein function in two cultivation system: hydroponic culture and soil.

III.2.5 Immunogold labelling

Immunogold labelling experiments were carried out to further investigate protein localization. Leaves from T_1 transgenic plants expressing the recombinant ChMTII (line 18/4), ScMTII (line 24/3), the ChMTII-GFP (line 13/2) and untransformed tobacco SR-1 plants were used for recombinant protein localization studies. Rabbit polyclonal anti-MTII antibody was used to investigate localization of the ChMTII and ScMTII recombinant proteins, whereas rabbit anti-GFP antibody was used to immunolocalise ChMTII-GFP fusion protein. Goat anti-rabbit 10 nm gold particles labelled antibody was used as secondary antibody for immunogold labelling. All the samples were analyzed by electron microscopy (II.2.4.3). The vacuolar compartment of cross-sections from leaves transformed with the S or the cV targeting cassettes showed significant labelling for ChMTII (Figure III-11/A, B), ScMTII (Figure III-11/C) and ChMTII-GFP fusion protein (Figure III-11/E), as compared to sections from SR1 control plants (Figure III-11/D, F).



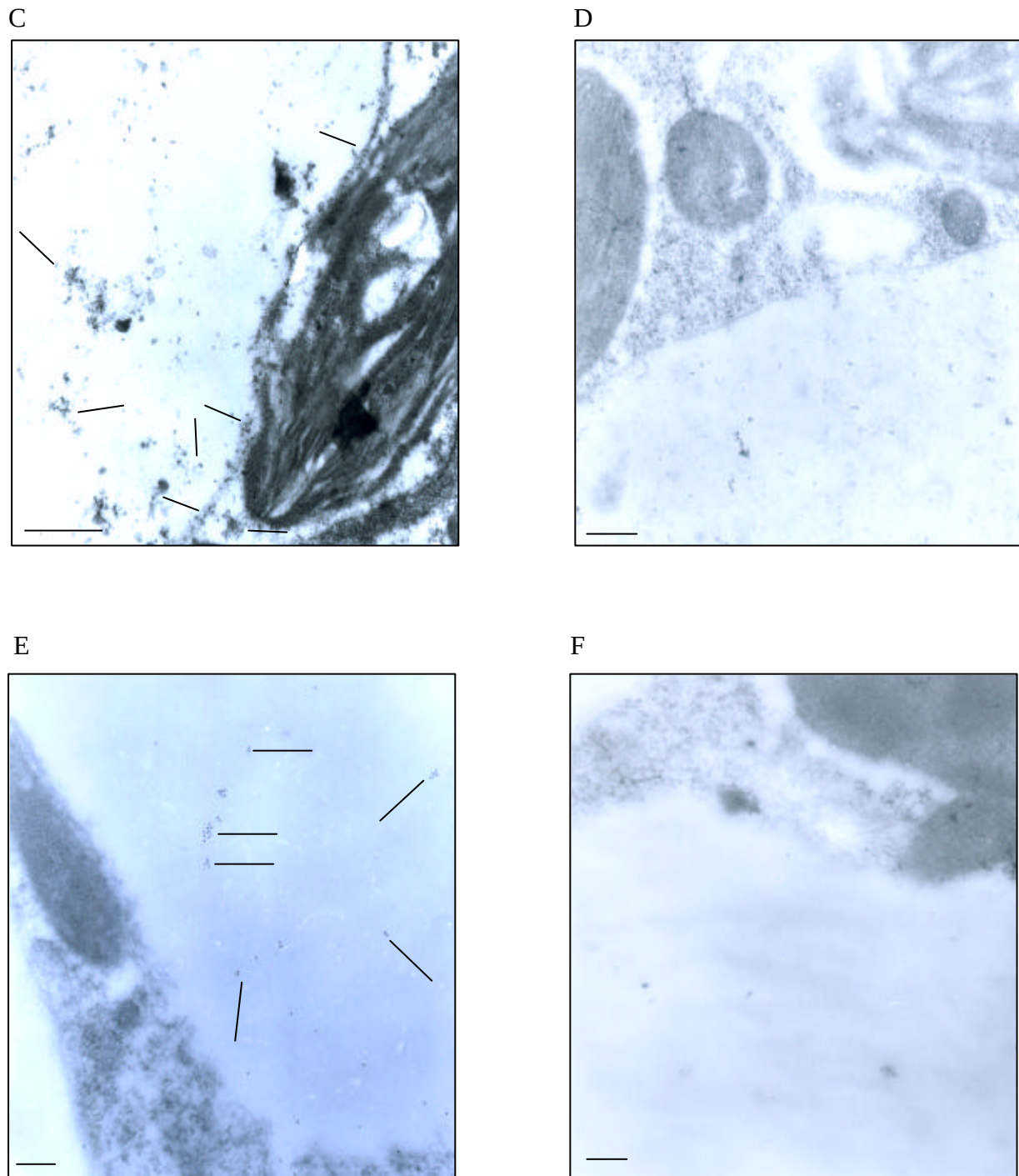


Figure III-11 Electron microscopy studies for ChMTII, ScMTII and ChMTII-GFP localisation. Cross-sections from transgenic T_1 tobacco plant line expressing recombinant ChMTII (A-B) and ScMTII (C) labelled with rabbit polyclonal anti-MTII antibody. E: Sections from transgenic T_1 line expressing ChMTII-GFP fusion protein labelled with rabbit α -GFP antibody. D, F: Sections from wild type SR1 tobacco plants. Goat anti-rabbit 10 nm gold particle labelled antibody was used for immuno detection. Leaf sections were analysed by electron microscopy for localisation of fusion protein in vacuole (II.2.4.3). Bar indicates 400 nm.

III.3 Hydroponic experiments

III.3.1 Symptoms of cadmium toxicity on shoots and roots

Tobacco plants from the selected T_2 or T_1 lines were cultivated in hydroponic culture as described in II.2.5.1. Throughout the experiment, the basal and middle fully expanded leaves were visually scored for Cd toxicity symptoms (Table III-1) as indicated below:

- 1- Healthy
- 2- Slight chlorosis
- 3- Marked chlorosis
- 4- Severe chlorosis with some necrosis or brown/silver spotting
- 5- Severe necrosis and presence of brown/silver spots

Table III-1 Cadmium toxicity symptom scale

Cd Treatment (μ M)	SR-1	p-cV-ChMTII- GFP	p-S-ChMTII	p-S-ScMTII
0	1	1	1	1
50	3	2	3	3
100	5	3-4	5	5

Transgenic tobacco plants transformed with the cV-ChMTII-GFP cassette showed a higher degree of tolerance to Cd than plants transformed with the S vacuolar targeting cassettes or than wild type plants used as control. Typical symptoms of Cd toxicity on wild type as well as susceptible transgenic tobacco plants appeared first with a diffuse leaf chlorosis, followed by browning and silver necrotic patches on basal and middle leaves (Figure III.12). Cd toxicity produced unequivocal diffused browning on the roots accompanied by dark brown areas at the root tips. The chlorotic symptoms were observed starting with 50 μ M Cd treatment (Figure III.12). Plants that were treated with lower concentrations (0-50 μ M) did not show any symptom (data not shown). Faint root browning was observed for plants treated with 50 μ M Cd; however severe browning and reduced growth of the shoots and roots became evident at 100 μ M Cd.

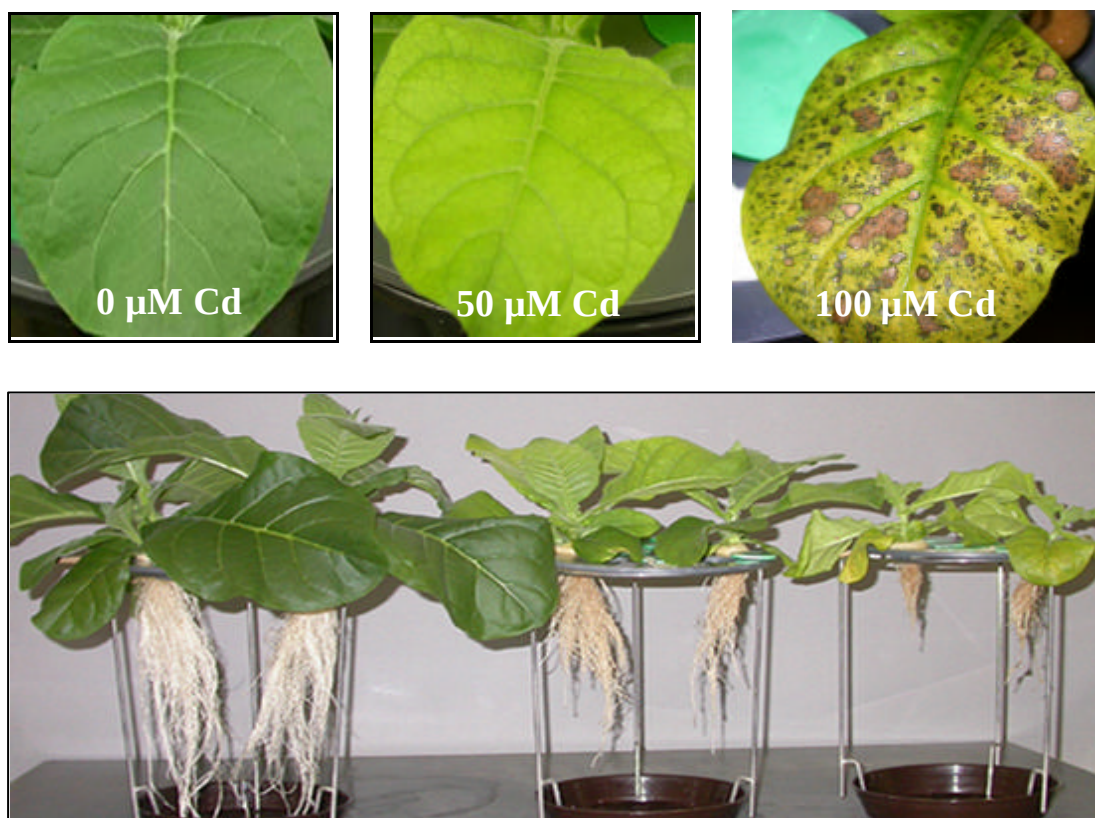


Figure III-12 Growth of wild type tobacco plants (II.2.5.2) as affected by increasing Cd supply in nutrient solution for 15 days (II.2.5.1).

Hydroponic cultures were treated with serial concentrations (0-100 μM) of cadmium.

III.3.2 Immunoblot analysis of Cd treated plants

Crude protein extracts from roots and shoots of transgenic plants of the hydroponic cultures that expressed the recombinant MTII with either the cV or the S vacuolar targeting signal were subjected to immunoblot analysis. The comparison of the accumulation levels of the recombinant MTIIs and Cd supplement indicated a significant correlation. The recombinant MTIIs accumulation levels of shoots and roots were decreasing with increasing concentration of Cd supplied in the nutrient solution. Transgenic plants that were transformed with the cV-ChMTII-GFP construct showed the highest expression of MTII protein in shoots and roots (Figure III-13/A-B and Figure III-15) as deduced by the protein signals on the western blots. The p-S-ScMTII transgenic plants accumulated the lowest level of recombinant protein whereas p-S-ChMTII transgenic plants showed intermediate protein levels (Figure III-13/E-F and C-D respectively). The expression level of the recombinant proteins in shoot and roots varied considerably between the constructs. In general, accumulations of the recombinant proteins in shoot were higher than in roots.

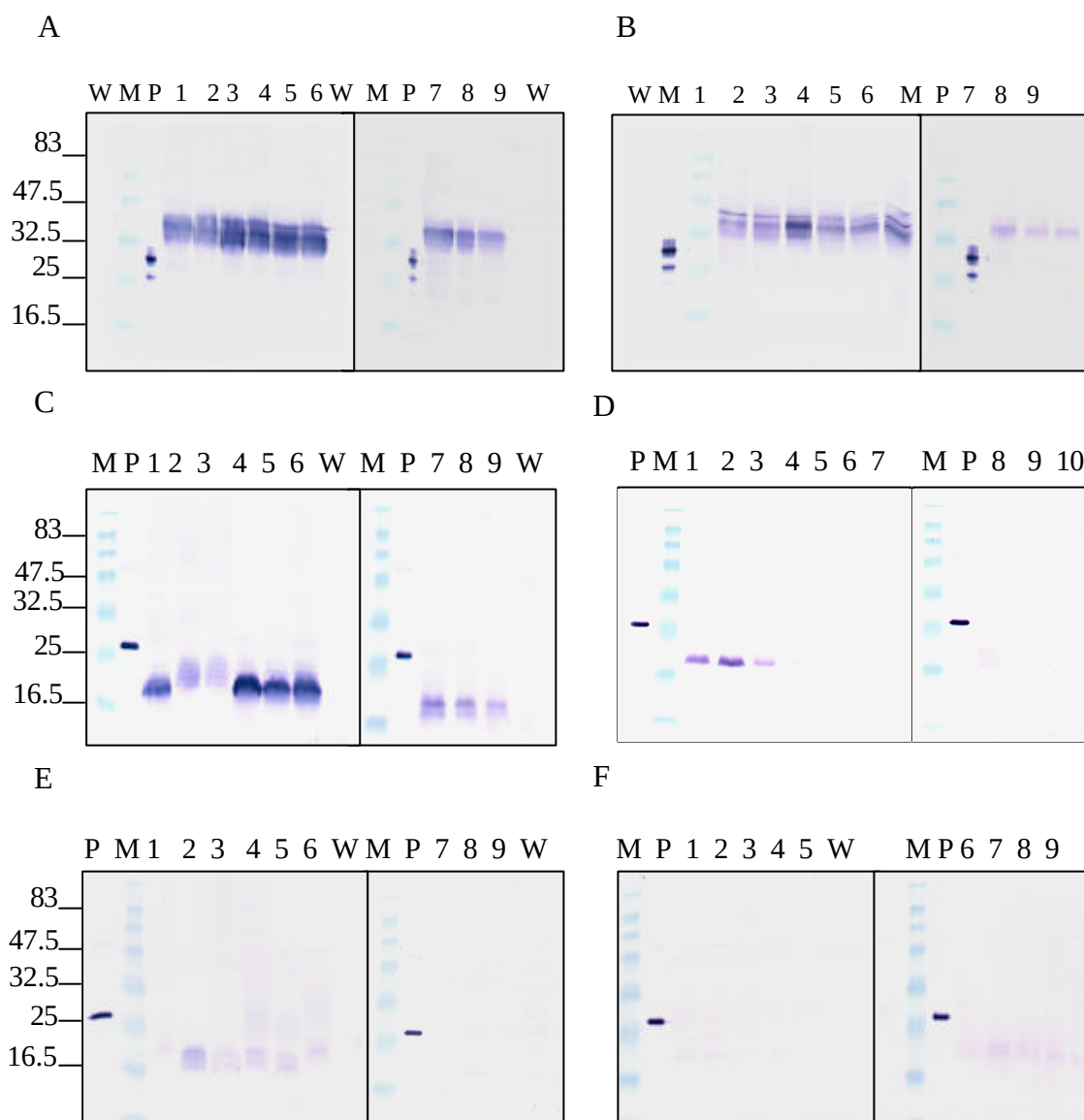


Figure III-13 Screening of transgenic tobacco plants expressing cV and S vacuolar MTIIs after Cd treatment.

Total soluble shoot and root proteins were extracted by grinding tobacco tissue in two volumes extraction buffer (II.2.2.6). 10 μ l of total soluble protein from each sample was separated by 12% SDS-PAGE (II.2.3.2) and blotted onto a nitrocellulose membrane (II.2.3.3). Immunodetection was carried out with anti-GFP antibody (1:1000) for (A) and anti-his tag antibody (1:2000) for (B and C), followed by 1 hr incubation with polyclonal anti-rabbit (1:5000) and goat anti-mouse antibody conjugated to alkaline phosphatase (1:5000) and NBT/BCIP detection for 5 min at RT.

A-B: 10 μ l of TSP extracted from shoots (A) and roots (B) of transgenic T_2 tobacco plants expressing vacuolar ChMTII-GFP fusion protein targeted by *C. roseus* vacuolar targeting signal. 1-3: 0 μ M Cd; 4-6: 50 μ M Cd; 7-9: 100 μ M Cd treatment. P: 200 ng GFP as positive control. W: TSP extracted from wild type SR1 leaves; M: prestained protein marker.

C-D: 10 μ l of TSP extracted from shoots (A) and roots (B) of transgenic T_1 tobacco plants (line 18) expressing vacuolar ChMTII targeted by Sporamin A vacuolar targeting signal. 1-3: 0 μ M Cd; 4-6: 50 μ M Cd; 7-9: 100 μ M Cd treatment.

E-F: 10 μ l of TSP extracted from shoots (A) and roots (B) of transgenic T_1 tobacco plants (line 24) expressing vacuolar ScMTII targeted by Sporamin A vacuolar targeting signal. 1-3: 0 μ M Cd; 4-6: 50 μ M Cd; 7-9: 100 μ M Cd treatment.

III.3.3 Analysis of the plant dry weight

The dry weight (dw) of the plants cultivated in hydroponic culture is shown in Figure III-14 A/B. The dw of wild type and transgenic tobacco plants decreased with increasing concentration of Cd supplied in the nutrient solution (Figure III-14 A/B). The highest shoot dw was observed for plants accumulating the ChMTII-GFP fusion protein whereas plants transformed with the S-ChMTII cassette showed the lowest dw. Wild type and ScMTII producing plants showed similar dw.

The analysis of the root dw showed similar results. Plants accumulating the ChMTII-GFP fusion protein showed the highest root dw followed by the wild type and the other transgenic plants.

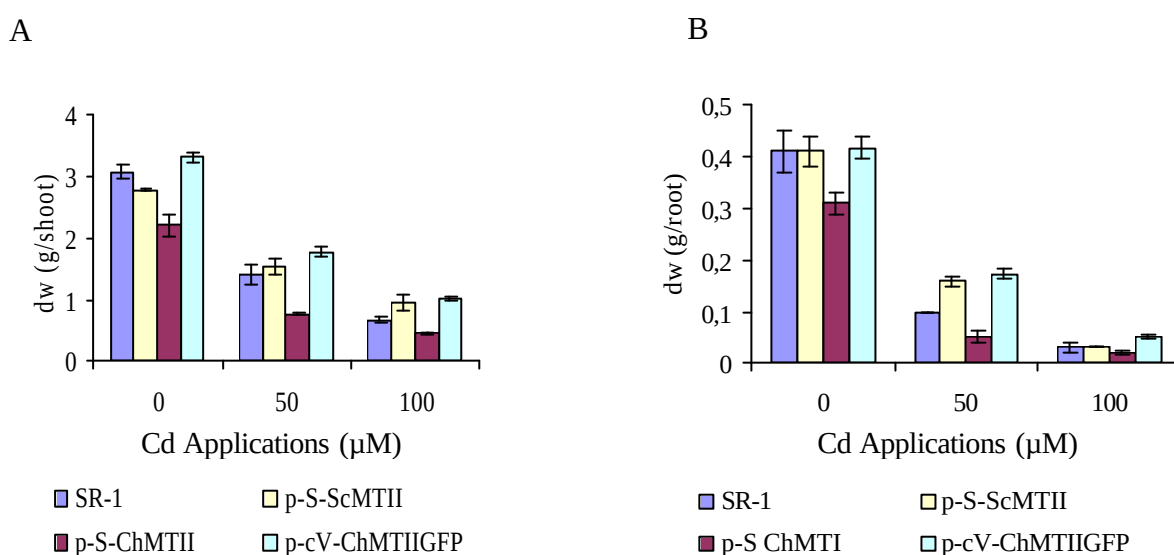


Figure III-14 Effect of increasing Cd supply (II.2.5.2) on shoots (A) and roots (B) (II.2.5.2) dry weight of transgenic and wild type (SR-1) tobacco.

All analysed plants were grown in nutrient solution for 15 days (II.2.5.1). The data represents means $\pm$ SD of three independent replications.

SR1: wild type SR1 tobacco plants; p-S-ScMTII: transgenic T₁ tobacco plants expressing vacuolar ScMTII targeted by Sporamin A vacuolar targeting signal; p-S-ChMTII: transgenic T₁ tobacco plants expressing vacuolar ChMTII targeted by Sporamin A vacuolar targeting signal; p-cV-ChMTII-GFP: transgenic T₂ tobacco plants expressing vacuolar ChMTII-GFP fusion protein targeted by cV vacuolar targeting signal

However, a treatment with 50 µM CdSO₄ induced a significant decrease in shoots and roots dw for either the control or the transgenic plants. Moreover, the symptoms of Cd toxicity started to appear with 50µM CdSO₄ treatment, whilst 0-50 µM CdSO₄ did not produce any symptoms (data not shown).

III.3.4 Analysis of the cadmium contents

In vivo function of the recombinant MTIIs was evaluated by the analysis of the level of Cd in the tissues of transgenic and wild type tobacco plants. The highest level of Cd in the shoots was detected in plants transformed with the cV-ChMTII-GFP cassette (1828 $\mu\text{g/g}$ shoot dw) whereas the lowest Cd content was found in plants transformed with the S-ChMTII cassette (794 $\mu\text{g/g}$ shoot dw). Plants transformed with the S-ScMTII cassette and wild type plants showed similar levels of Cd (1049 $\mu\text{g/g}$ and 1158 $\mu\text{g/g}$ shoot dw, respectively). The cV-ChMTII-GFP transgenic tobacco plants accumulated Cd 50-80 % more than control plants.

The content of Cd in the shoots was higher than in the roots in both transgenic and wild type plants (Figure III-15 A/B). The roots of wild type plants accumulated higher levels of Cd than the root of transgenic plants. A significant difference was detected especially between wild type and S-ScMTII transgenic plants. However, the level of Cd decreased with the increasing molarities of Cd in the nutrient solution. Due to a high dry matter production the highest amount of Cd accumulated in shoots. This result is one of the important aims of phytoextraction.

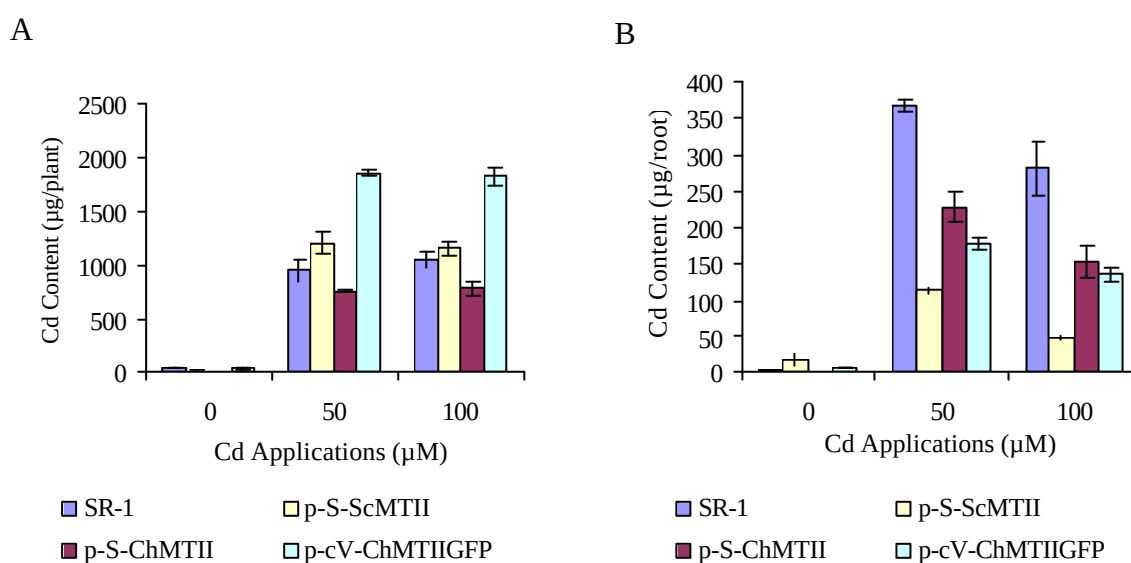


Figure III-15 Effect of increasing Cd supply (II.2.5.2) in nutrient solution on concentration of Cd in shoot (A) and root (B) of transgenic and wild type SR-1 tobacco plants.

All analysed plants were grown in nutrient solution for 15 days (II.2.5.1). The data represents means $\pm\text{SD}$ of three independent replications.

SR1: wild type SR1 tobacco plants; p-S-ScMTII: transgenic T_1 tobacco plants expressing vacuolar ScMTII targeted by Sporamin A vacuolar targeting signal; p-S-ChMTII: transgenic T_1 tobacco plants expressing vacuolar ChMTII targeted by Sporamin A vacuolar targeting signal; p-cV-ChMTII-GFP: transgenic T_2 tobacco plants expressing vacuolar ChMTII-GFP fusion protein targeted by cV vacuolar targeting signal.

III.3.5 Analysis of total soluble protein

The analysis of the total soluble protein level carried out on the crude protein extract of leaves and roots showed no significant correlation with the amount of Cd supplied with the nutrient solution either in transgenic or wild type plants (Figure III-16 A/B).

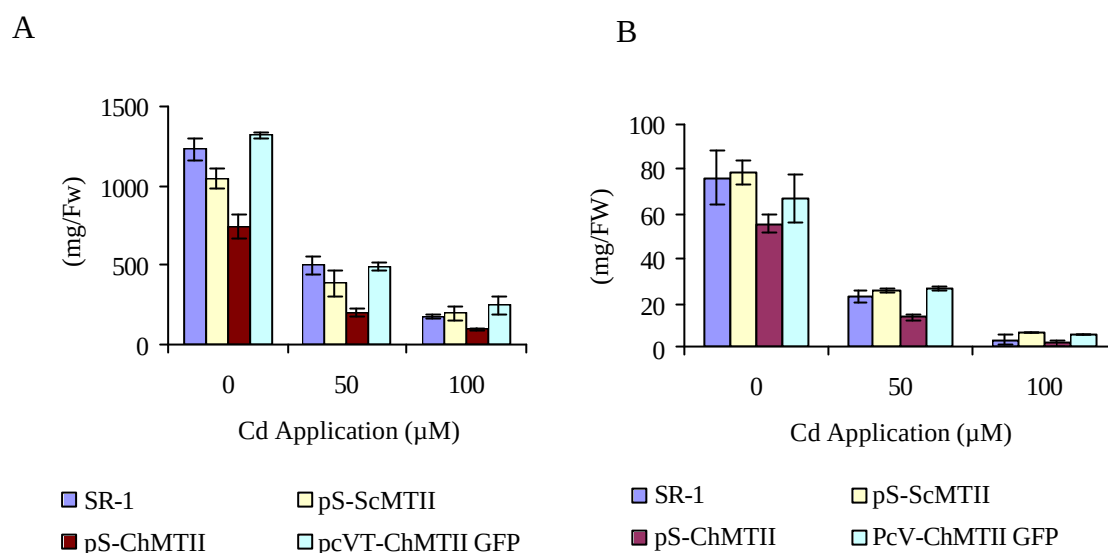


Figure III-16 Effect of increasing Cd supply (II.2.5.2) on concentration of TSP in shoots and roots of wild type (SR-1) and transgenic tobacco plants.

The data represent mean values of $\pm$ SD of three independent replications. All analysed plants were grown in nutrient solution for 15 days.

SR1: wild type SR1 tobacco plants; pS-ScMTII: transgenic T₁ tobacco plants expressing vacuolar ScMTII targeted by Sporamin A vacuolar targeting signal; pS-ChMTII: transgenic T₁ tobacco plants expressing vacuolar ChMTII targeted by Sporamin A vacuolar targeting signal; p-cV-ChMTII-GFP: transgenic T₂ tobacco plants expressing vacuolar ChMTII-GFP fusion protein targeted by cV vacuolar targeting signal.

III.3.6 Analysis of free sulfhydryl groups

The level of free non-protein SH-groups in the crude extracts of shoots and roots increased sharply with a 50µM Cd treatment (Figure III-17/A). However, in plants treated with a higher concentration of Cd the level of non-protein SH-group dropped again to values similar to those of untreated plants. Therefore, 50 µM Cd was the maximum Cd dosage for high rate production of non-protein SH-groups. Interestingly, when Cd was not supplied, shoot concentration of SH-groups were more than 10-fold higher than those found in roots. After treatment with 50 µM Cd supplements, only the transgenic plants expressing ChMTII-GFP fusion protein produced (synthesis) the highest concentration of non-protein SH-groups compared to other transgenic and wild type tobacco plants. Wild type tobacco (SR-1) plant,

had the highest non-protein SH-groups in shoots followed by S-ScMTII > S-ChMTII > cV-ChMTII-GFP transgenic plants.

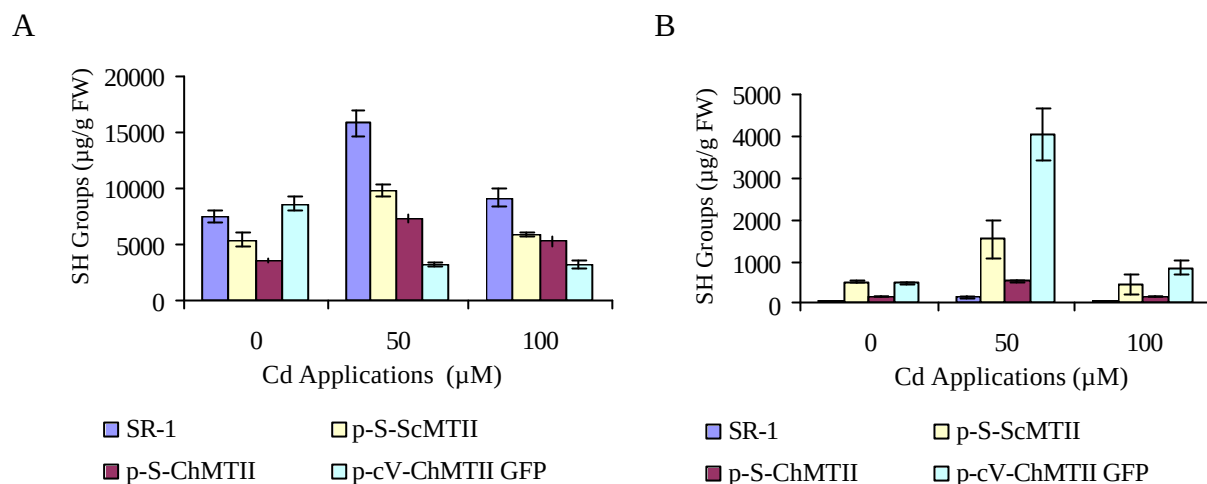


Figure III-17 Effect of increasing Cd supply (II.2.5.2) on concentration of non-protein SH-groups in shoots (A) and roots (B) of wild type (SR-1) and transgenic tobacco.

The data represent mean values of $\pm$ SD of three independent replications. All analysed plants were grown in nutrient solution for 15 days.

SR1: wild type SR1 tobacco plants; p-S-ScMTII: transgenic T₁ tobacco plants expressing vacuolar ScMTII targeted by Sporamin A vacuolar targeting signal; p-S-ChMTII: transgenic T₁ tobacco plants expressing vacuolar ChMTII targeted by Sporamin A vacuolar targeting signal; p-cV-ChMTII-GFP: transgenic T₂ tobacco plants expressing vacuolar ChMTII-GFP fusion protein targeted by cV vacuolar targeting signal.

III.3.7 Analysis of phytochelatins in shoots

Transgenic tobacco plants transformed with the cV-ChMTII-GFP cassette and wild type (SR-1) plants showed approximately the same amount of Phytochelatins (PCs) and, Glutathion (GSH) in the shoots either when untreated or after being treated with 100 µM Cd (Figure III-18). The synthesis of GSH was higher in untreated plants than in plants treated with 100µM Cd. In contrast, the synthesis of PCs increased significantly when the plants were treated with 100µM Cd.

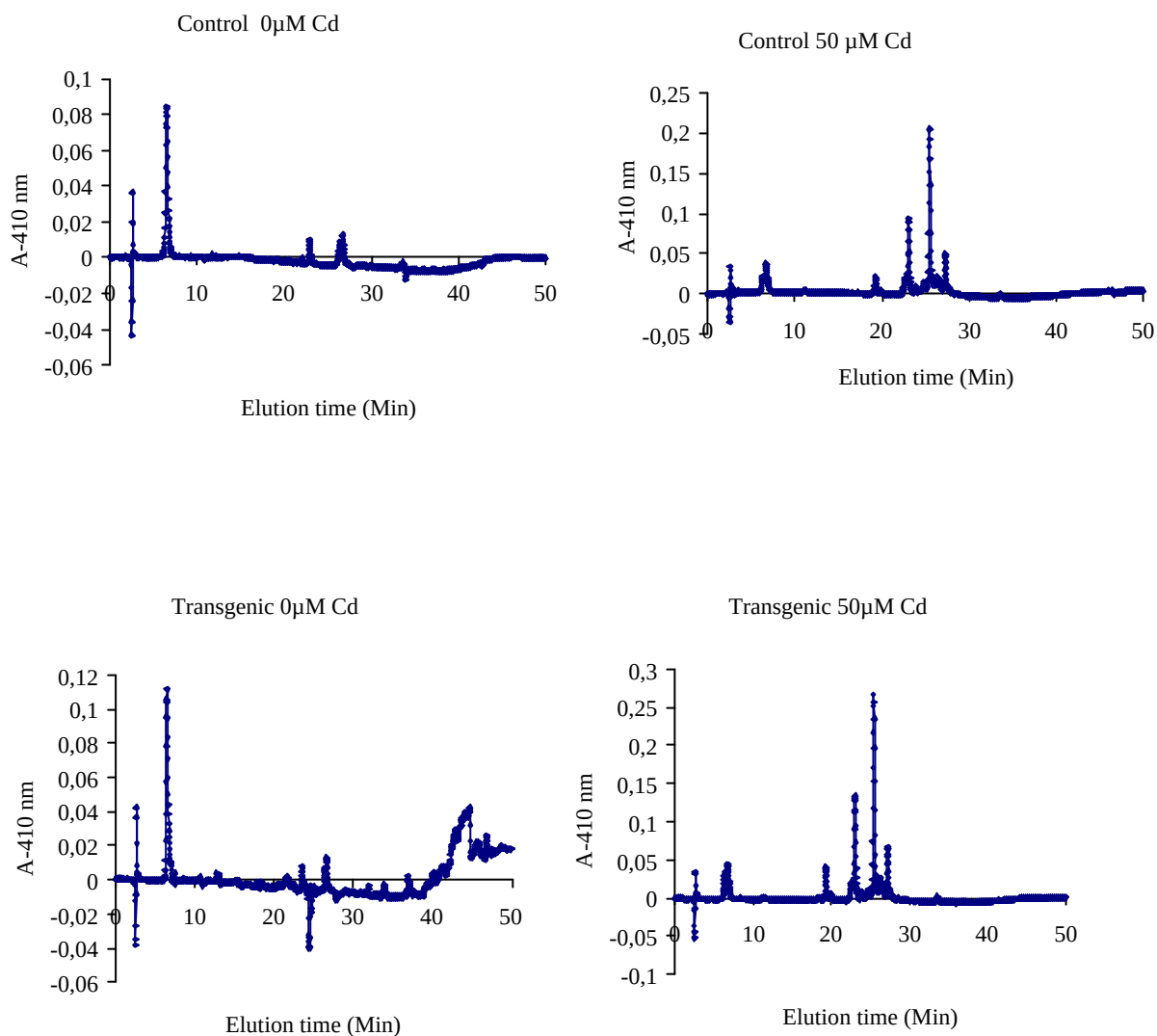


Figure III-18 Effect of increasing Cd supply (II.2.5.2) on PCs (II.2.5.6) concentrations in shoots of wild type (A-B) and T₂ transgenic plants expressing vacuolar ChMTII-GFP (C-D).

All analysed plants were grown in nutrient solution for 15 days (II.2.5.1). The data represent mean values of $\pm$ SD of three independent replications. GSH: 5-6 min, PCs: 18-25 min synthesis.

III.4 Soil experiments

III.4.1 Symptoms of Cd toxicity on leaves

Further studies were carried out to compare the effect of Cd on transgenic and wild type plants grown in the soil.

Throughout the experiment, the basal and middle fully expanded leaves were visually scored for symptoms of Cd toxicity:

- 1- Healthy
- 2- Slight chlorosis
- 3- Marked chlorosis
- 4- Severe chlorosis with some necrosis or brown/silver spotting
- 5- Severely necrotic tissue and brown/silver spotting

No symptoms were observed on transgenic or wild type plants treated with the highest concentration (30 ppm) of Cd (Table III-2, Figure III-19). Morphologically, the only difference was observed for plants transformed with the p-cV-ChMTII-GFP cassette that flowered earlier than wild type plants.

Table III-2 Cadmium toxicity symptoms scale.

Cd Treatment (ppm)	SR-1	p-cV-ChMTII GFP	p-S-ChMTII	p-S-ScMTII
0	1	1	1	1
10	1	1	1	1
20	1	1-2	1	1
30	1-2	1-2	1-2	1-2

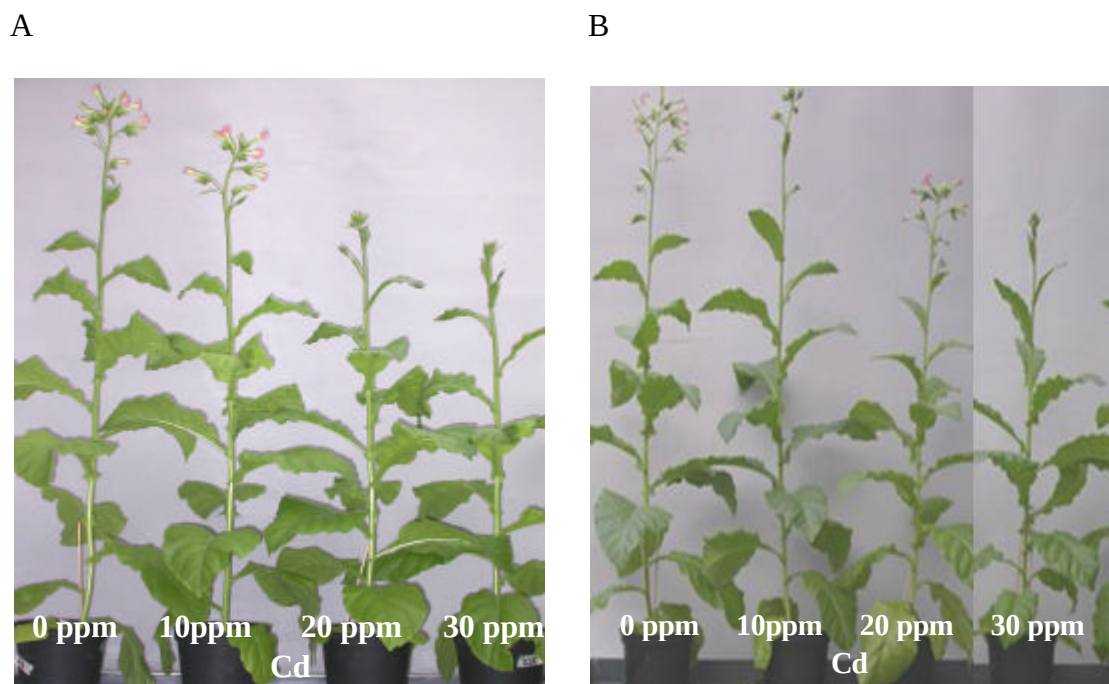
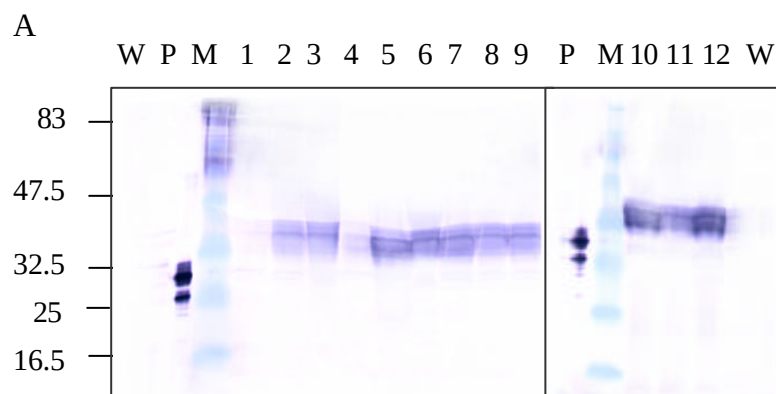


Figure III-19 Growth of tobacco plants as affected by increasing Cd supply (II.2.6.6) in soil for 45 days.

Soil experiments were treated with serial concentrations (0-10-20-30 ppm) of cadmium. A: SR-1 Control tobacco plants B: p-cV-ChMTII GFP tobacco plants.

III.4.2 Immunoblot Analysis

Immunoblot analysis of crude protein extracts showed that plants transformed with the cV-ChMTII-GFP construct accumulated the highest level of recombinant metallothionein whereas the lowest accumulation was detected in plants transformed with the S-ScMTII construct (Figure III-20/A-B-C).



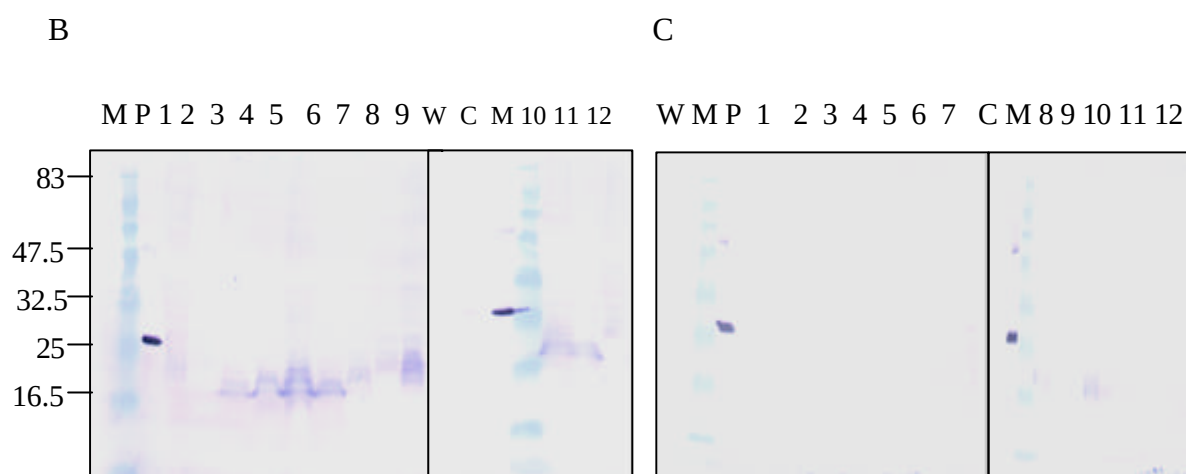


Figure III-20 Screening of transgenic tobacco plants expressing ChMTII-GFP, ChMTII and ScMTII after Cd treatment.

Total soluble shoot proteins were extracted by grinding tobacco tissue in two volumes extraction buffer (II.2.2.6). 10 μ l of total soluble protein from each sample was separated by 12% SDS-PAGE (II.2.3.2) and blotted onto a nitrocellulose membrane (II.2.3.3). Immunodetection was carried out with anti-GFP antibody (1:1000) for (A) and anti-his tag antibody (1:2000) for (B and C), followed by 1 hr incubation with polyclonal anti-rabbit (1:5000) and goat anti-mouse antibody conjugated to alkaline phosphatase (1:5000) and NBT/BCIP detection for 5 min at RT.

A: 10 μ l of TSP extracted from shoots of transgenic T₂ tobacco plants expressing ChMTII-GFP fusion protein. 1-3: 0 ppm Cd; 4-6: 10 ppm Cd; 7-9: 20 ppm Cd; 10-12: 30 ppm Cd treatment; P: 200 ng GFP as positive control. W: TSP extracted from wild type SR1 shoots; M: prestained protein marker.

B: 10 μ l of TSP extracted from shoots of transgenic T₁ tobacco plants expressing vacuolar ChMTII. 1-3: 0 ppm Cd; 4-6: 10 ppm Cd; 7-9: 20 ppm Cd; 10-12: 30 ppm Cd treatment; P: 200 ng GFP as positive control.

C: 10 μ l of TSP extracted from shoots of transgenic T₁ tobacco plants expressing vacuolar ScMTII. 1-3: 0 ppm Cd; 4-6: 10 ppm Cd; 7-9: 20 ppm Cd; 10-12: 30 ppm Cd treatment; P: 200 ng GFP as positive control.

III.4.3 Analysis of the plant dry weight

The production of dry matter of wild type and transgenic tobacco plants was only slightly affected by the treatment with Cd. Plants transformed with the cV-ChMTII-GFP cassette showed the highest dw compared to the other transgenic lines. However, wild type and transgenic plants showed no significant differences in the dw of leaves, stems and flowers (Figure III-21/A-B-C-D).

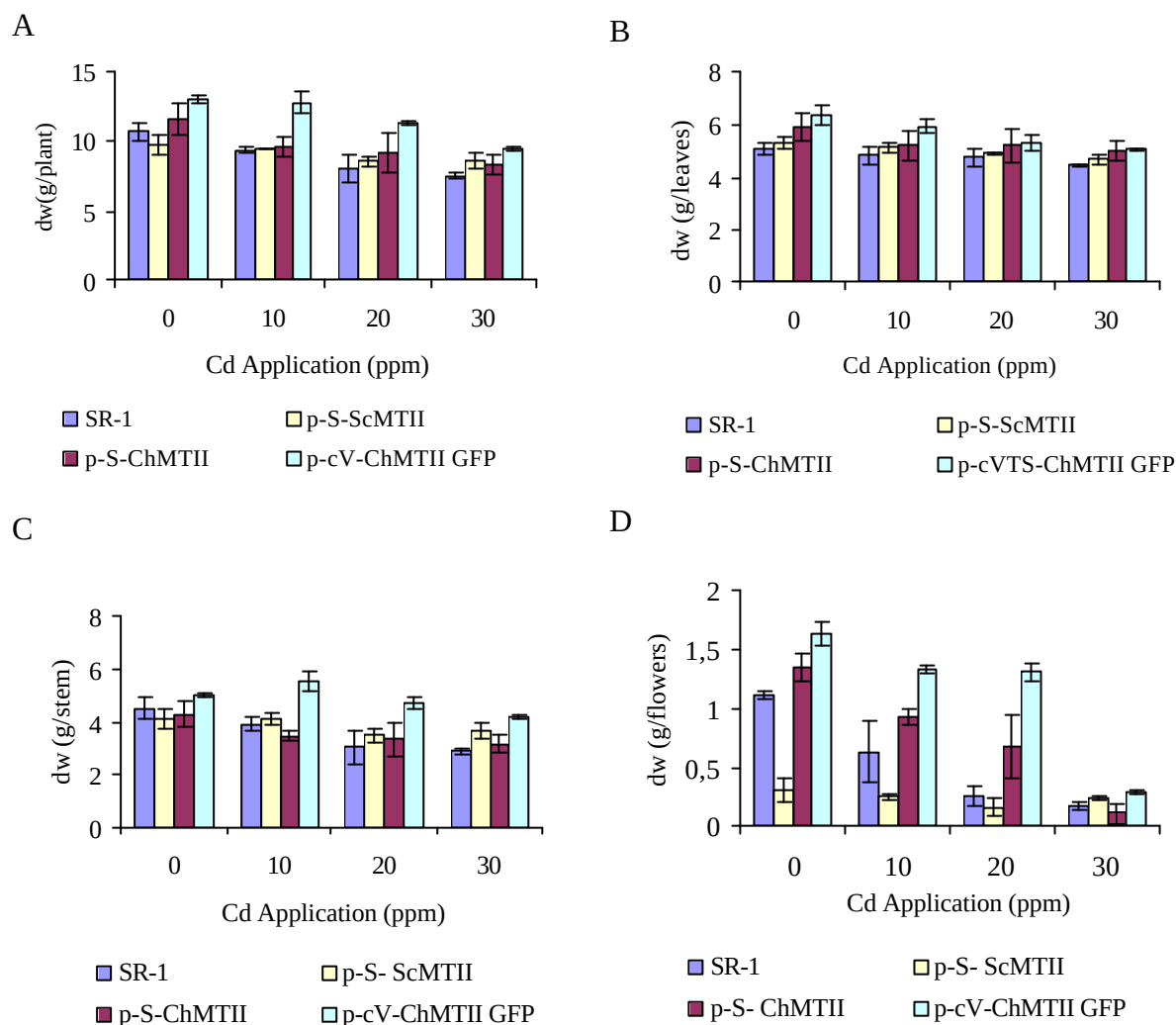


Figure III-21 Effect of Cd supply (II.2.6.6) on the dry weight of whole plant (A), leaf (B), stem (C) and flowers (D).

Wild type and transgenic tobacco plants were grown in soil for 45 days. The data represent means $\pm$ SD of three independent replications.

SR1: wild type tobacco plants; p-S-ScMTII: transgenic T₁ tobacco plants expressing vacuolar ScMTII targeted by Sporamin A vacuolar targeting signal; p-S-ChMTII: transgenic T₁ tobacco plants expressing vacuolar ChMTII targeted by Sporamin A vacuolar targeting signal; p-cV-ChMTII-GFP: transgenic T₂ tobacco plants expressing vacuolar ChMTII-GFP fusion protein targeted by cV vacuolar targeting signal.

III.4.4 Analysis of Cadmium Content

The level of cadmium in wild type and transgenic tobacco plants did not differ significantly (Figure III-22/A). The level of Cd in the plant tissues markedly increased with the dose of Cd in the soil. Moreover, the analysis of the level of Cd in different organs showed that the highest amount of Cd accumulated in leaves followed by stems and flowers (Figure III-22/B-C-D). Nevertheless, the differences in Cd levels between leaves of transgenic and wild type plants were relatively small compared to those observed for stems and flowers.

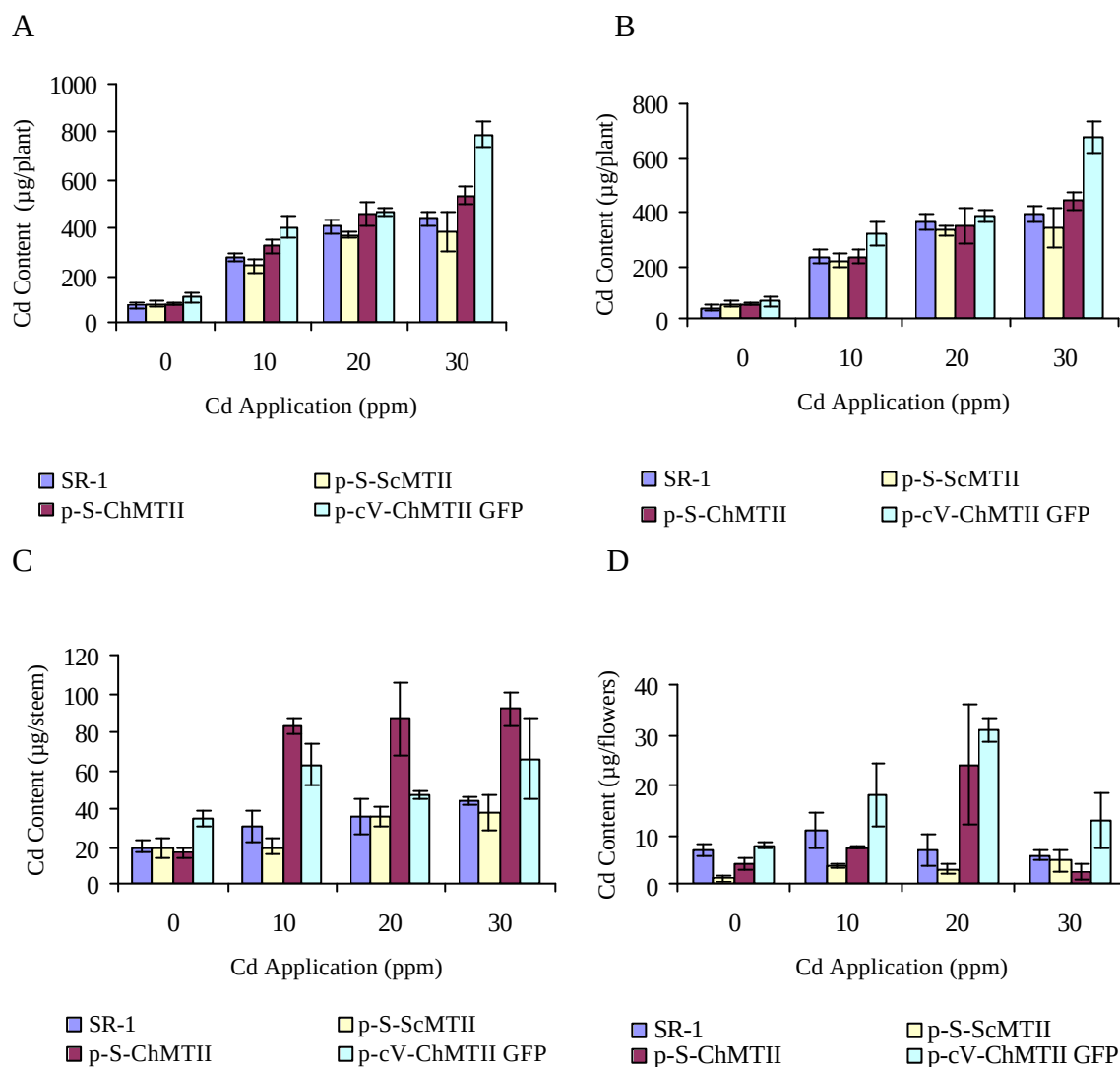


Figure III-22 Effect of increasing Cd supply (II.2.6.6) on the Cd concentration of the whole plant (A), leaf (B), stem (C) and flowers (D).

The wild type and transgenic tobacco plants were grown in soil for 45 days. The data represent mean values of $\pm$ SD of three independent replications.

SR1: wild type SR1 tobacco plants; p-S-ScMTII: transgenic T₁ tobacco plants expressing vacuolar ScMTII targeted by Sporamin A vacuolar targeting signal; p-S-ChMTII: transgenic T₁ tobacco plants expressing vacuolar ChMTII targeted by Sporamin A vacuolar targeting signal; p-cV-ChMTII-GFP: transgenic T₂ tobacco plants expressing vacuolar ChMTII-GFP fusion protein targeted by cV vacuolar targeting signal.

III.4.5 Analysis of free sulfhydryl groups

The concentration of free sulfhydryl groups in leaves of transgenic as well as wild type plants decreased with inverse proportion to the concentration of Cd supplied in the soil (Figure III-23). Transgenic tobacco plants transformed with the cV-ChMTII-GFP cassette showed a higher concentration of non-protein SH groups than other transgenic and control plants. But only without Cd supplement S-ChMTII construct produced more non-protein SH than other plants.

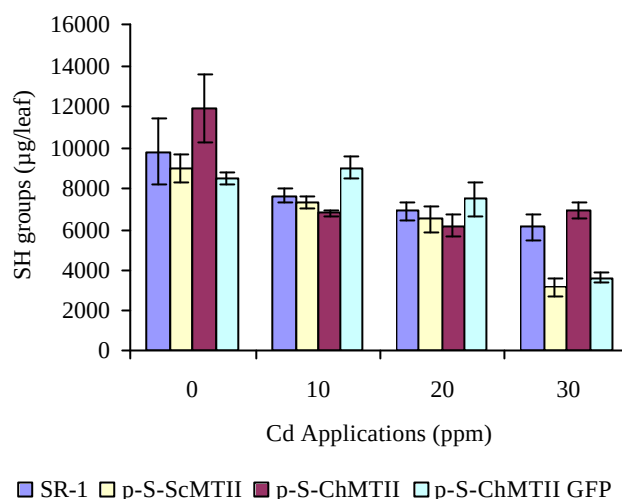


Figure III-23 Effect of increasing Cd supply (II.2.6.6) on concentration of non-protein SH-groups in leaves of wild type (SR-1) and transgenic tobacco.

The data represent means $\pm$ SD of three independent replications. All analysed plants were grown in soil for 45 days.

SR1: wild type SR1 tobacco plants; p-S-ScMTII: transgenic T₁ tobacco plants expressing vacuolar ScMTII targeted by Sporamin A vacuolar targeting signal; p-S-ChMTII: transgenic T₁ tobacco plants expressing vacuolar ChMTII targeted by Sporamin A vacuolar targeting signal; p-cV-ChMTII-GFP: transgenic T₂ tobacco plants expressing vacuolar ChMTII-GFP fusion protein targeted by cV vacuolar targeting signal.

IV DISCUSSION

Phytoremediation is the use of plants to clean up environmental pollution. Phytoextraction is one aspect of phytoremediation and compared to other methods, cheaper, easier, and environmental-friendly. Phytoextraction has been used successfully for removing environmental pollution. However, much of the underlying biological processes that plants use for uptaking and translocating the pollutants are still unknown.

The objectives of this thesis were;

- a) to generate and characterize transgenic tobacco plants which expressed vacuolar targeted metal binding proteins, such as the metallothionein II (MTII) from Chinese hamster and *Saccharomyces cerevisiae*,
- b) to test whether these transgenic plants would be more efficient than wild type plants in the uptake of heavy metals, such as Cd, from polluted soils.

The rationale of the present study was to constitutively express recombinant MTIIs in tobacco plants under control of the strong CaMV 35S promoter and to investigate metal uptake. At the subcellular level the distribution of heavy metals in plant cells is not homogenous. Cd for instance, accumulates mainly in the cytosol whereas in organelles such as the nucleus, mitochondria, and microsomes a significant reduction of this metal has been described (Klassen *et al.*, 1998). MTs are cytoplasmic proteins (Kägi *et al.*, 1985; Klassen *et al.*, 1999; Tapia *et al.*, 2004). In mature plant cells the cytosol occupies a smaller volume than the vacuole and cannot accumulate high concentration of metals such as Cd, Zn, Cu, and Hg. On the contrary, vacuoles are large subcellular compartments with dual function of storage and/or degradation and may represent the ideal subcellular compartment for detoxifying heavy metals. Therefore, the expression cassettes for recombinant MTIIs were engineered with two different vacuolar targeting signals derived from the *Catharanthus roseus*'s *Stricosidine synthase* and from the potato *Sporamin A*.

The recombinant MTIIs were first transiently expressed in tobacco leaves. However, the characterization of the recombinant proteins was carried out on stable transformed tobacco plants. The ability of these plants to uptake heavy metals was tested in hydroponic and soil experiments where Cd was used as the reference pollutant and was supplied at different concentrations to ascertain whether the transgenic plants had acquired tolerance to Cd toxicity.

IV.1 Generation and characterization of transgenic plants

As presented in the results section, the genes encoding for the ChMTII (III.1.1 and III.1.2) and ScMTII (III.1.2) were introduced into tobacco plants via *Agrobacterium*-mediated transformation. Transiently (II.2.1) and stably transformed (II.2.4) tobacco plants were tested by immunoblot analysis (II.2.1, II.2.4), immunofluorescence (II.2.2) and immunogold (II.2.5) labelling. Localization studies on ChMTII and ScMTII gene in tobacco leaves and transgenic tobacco plants by immunogold labelling demonstrated that the MTIIs were localized in the vacuole.

To acquire knowledge on the biological function of the recombinant MTIIs stably transformed tobacco plants were required. Preliminary characterization of the recombinant MTIIs was carried out in transient transformed tobacco leaves. Data from transient and stable transformation experiments of different recombinant proteins indicated that levels of protein accumulation observed in *Agrobacterium*-infiltrated leaves strongly correlated to those obtained with the best expressing transgenic lines. In addition, transient expression is not influenced by positional effects that can bias gene expression levels in stable transformed plants (Kapila *et al.*, 1996; Schalthof *et al.*, 1996). Therefore, transient gene expression seems particularly suited for rapidly verifying a gene construct and the gene product before moving onto the generation of transgenic plants (Kapila *et al.*, 1996; Fischer *et al.*, 1999c). In addition, transient gene expression can rapidly provide large amounts of recombinant proteins for detailed structural and functional characterization (Vaquero *et al.*, 1999). Our results showed that, higher accumulation level of the recombinant proteins was achieved in leaves expressing the cV-ChMTII-GFP.

Epifluorescence or immunofluorescence analysis was carried out on tobacco leaves that transiently expressed the recombinant MTIIs to evaluate if correct vacuolar targeting had occurred. Leaves transformed with the p-cV-ChMTII GFP construct were analysed by TSC-SP spectral confocal microscopy. Although immunoblot analysis showed the ChMTII-GFP fusion protein accumulated to the highest, GFP fluorescence could not be detected in the vacuole. Bright green fluorescence was observed in the ER and nucleus. These results were likely due to one of the following reasons:

- a) vacuoles are described as acidic compartments and it has been reported that GFP fluorescence is attenuated by protonation of its fluorophore core. According to Tsien (1998), Zimmer (2003) and Tamura *et al.* (2003); acidic pH can cause quenching of the GFP fluorescence rather than emission shifts toward shorter wavelengths as would be expected for protonated chromophores. These results suggest that for

vacuolar targeting of GFP fusion proteins it would be necessary to select pH sensitive GFP mutants.

- b) compared to the ER or the nucleus, vacuoles are large compartments. It is likely that the GFP fusion protein was diluted in the vacuole and could not produce visible signals.

In our experiments plants/leaves were usually grown under normal light conditions. Tamura *et al.*, (2003) indicated that in *Arabidopsis* plants exposure of GFP to light conditions does not influence transport of GFP fusion proteins to the vacuoles but activates rapid GFP degradation mediated by vacuolar resident proteases. Their results showed that absorption of blue light at the low vacuolar pH made GFP susceptible to proteinase attack, which lead to its complete degradation. Vacuolar localization studies for leaves transformed with the p-S-ChMTII and p-S-ScMTII constructs were carried out by immunofluorescence labelling. However, the high chlorophyll background signal did not allow detection of vacuolar localization of the recombinant MTIIs.

Stable transgenic tobacco plants were generated for all the different targeting constructs either for the ChMTII or the ScMTII genes. The accumulation levels of the best expressing lines correlated with the accumulation levels obtained in the transient agroinfiltration thereby corroborating the suitability of transient expression to predict the expression levels obtained in stable transformants. Western blot analysis showed that 18, 12 and 8 out of 25 independent transgenic lines per each construct expressed vacuolar targeted ChMTII-GFP fusion, ChMTII and ScMTII, respectively. Eight lines per construct with high accumulation levels of the recombinant MTIIs were selected and self pollinated for establishment of non segregating lines. Further screenings were carried out with T₁ and T₂ plants.

Immunoblot analysis showed that accumulation of ChMTII in the vacuole driven by the *strictosidine synthase* vacuolar targeting signal from *Catharanthus roseus* or by the *Sporamin A* targeting signal was higher than that of the vacuolar ScMTII targeted via the *Sporamin A* signal. This result was confirmed also by immuno labelling analysis and indicated that the ChMTII was likely more stable in the vacuole than the ScMTII counterpart. This was particularly the case when the ChMTII was fused to GFP indicating that the fusion partner exerted a stabilizing effect on the MTII.

Vacuolar localization of the recombinant MTIIs in transgenic plants was revealed by immunogold labeling. 10 nm immunogold particles were observed in vacuoles of stably transformed transgenic tobacco plants (p-cV-ChMTII GFP, p-S-ChMTII and p-S-ScMTII) but

not in wild type plants. Detection of the gold particles indicated correct vacuolar targeting of the recombinant MTIIs.

Primary transformants that showed the highest level of protein accumulation per targeting cassette were selected for further studies. T1 lines obtained from plants transformed with the p-S-ChMTII and p-S-ScMTII cassette and T2 lines of the p-cV-ChMTII GFP transformants were used for hydroponic (III.3) and soil (III.4) experiments. The degree of tolerance to Cd toxicity in transgenic plants was evaluated by several parameters such as: symptoms of Cd toxicity (III.3.1), immunoblotting (III.3.2), analysis of the shoots and roots dry weights (III.3.3), Cd content (III.3.4), total soluble protein (III.3.5), concentration of non-protein –SH groups (III.3.6) and PCs (III.3.7).

IV.2 Hydroponic Experiments

In a first set of assays transgenic plants were evaluated in hydroponic culture for their tolerance to Cd. The principal advantages of the hydroponic system compared to traditional cultivation in the soil are that plants can be grown in a controlled environment with a virtual indifference to temperature and seasonality. The controlled conditions allow more efficient disease and pest management, use of water and fertilizers. The T2 generation of tobacco plants transformed with the p-cV-ChMTII GFP cassette and the T1 generation of p-S-ChMTII and p-S-ScMTII transgenics were tested at different concentration of Cd supplied to the nutrient solution of the hydroponic system.

During the experiments, altered morphology and development of the plants were observed. Shoots and roots of wild type and transgenic tobacco plants were affected by Cd (Figure III.13 and Table III.1). Many researchers have reported toxic effects of Cd on plants (Gou, 1995, Kayser, 2000, Öztürk, *et al.*, 2003). The transgenic tobacco plants transformed with the cV-ChMTII-GFP cassette showed 2 fold more tolerance to Cd than plants transformed with the S vacuolar targeting cassette or than wild type plants used as control. Transgenic plants of the T2 generation transformed with this cassette showed even higher tolerance to Cd as compared to T1 plants. This well correlated with the level of protein accumulation as indicated by immunoblot analysis (III.3.2). Similar findings were described by Liu, *et al.* (2000) and Suh *et al.*, (1998). These authors reported that the level of accumulation of a recombinant MT in transgenic T2 tobacco seedlings strongly correlated with the degree of resistance to CdSO₄. PCR analysis of these plants confirmed that resistance to Cd was efficiently inherited from the T1 plants.

Transgenic plants that were transformed with the cV-ChMTII-GFP construct showed the highest accumulation of MTII as compared to other transgenic and wild type plants. However, in the cV-ChMTII-GFP plants, the levels of recombinant MTIIs in shoots and roots decreased when the concentration of Cd in the nutrient solution exceeded 50 μM . It was clearly visible that high Cd concentrations damaged the photosynthetic machinery and caused reduced plant growth and tolerance to Cd. This result indicated that high accumulation of recombinant MTII controlled the toxic effects of Cd within a concentration threshold beyond which key cellular processes were altered to the extent that protein synthesis and growth of the plants were drastically reduced.

It has been reported that Cd damages the light harvesting complex II and photosystems I, and II, which are crucial to photosynthesis. Cd treatment resulted in decreased content of total chlorophyll, in *B. napus* (Suziki *et al.*, 2001). In the present study transgenic and wild type plants in hydroponic culture developed chlorotic symptoms with Cd treatments above 50 μM . This result indicated that a sharp drop in chlorophyll content had occurred upon increasing concentration of Cd in the nutrient solution. These observations were consistent with those reported by Liu *et al.*, (2000) and Suh *et al.*, (1998). Liu *et al.*, showed that transgenic tobacco plants that expressed a metallothionein isolated from *N. glutinosa* under the control of the CaMV 35S promoter were able to grow in MS media supplemented with concentrations of up to 200 μM CdSO₄. Wild type control plants promptly underwent leaf chlorosis and their growth and development were inhibited with 50 μM CdSO₄ in the medium. Suh *et al.*, (1998) also introduced the *N. glutinosa* MT gene into *N. tabacum* plants via *Agrobacterium*-mediated transformation. Shoots that showed kanamycin resistance were subsequently rooted on medium containing 200 μM CdSO₄. Approximately 30 % of these primary transgenic plants developed normally. Non-transgenic plants promptly underwent leaf chlorosis, and their growth and development were inhibited on MS medium containing 50 μM CdSO₄.

According to the information reported in the literature, Cd affects the root growth more severely than the growth of the shoots. There are, however, contrasting reports showing that the effect of Cd on the growth of shoots and roots is similar (Öztürk *et al.*, 2003). We observed similar results. The highest shoot dw was observed for plants accumulating the ChMTII-GFP fusion protein whereas plants transformed with the S-ChMTII cassette showed the lowest dw. Wild type and S-ScMTII showed similar dw. The analysis of the root dw showed similar results. Plants accumulating the ChMTII-GFP fusion protein showed the highest root dw followed by the wild type and the other transgenic plants. However, a treatment with 50 μM CdSO₄ induced a significant decrease in shoots and roots dw for either

the control or the transgenic plants. This indicated that in hydroponic culture the tolerance level for Cd in the transgenic and wild type tobacco plants was 50 μ M.

Foliar Cd levels above 1 ppm usually are considered toxic. In the present study *in vivo* function of the recombinant MTII was evaluated by the analysis of the level of Cd in the tissues of transgenic and wild type tobacco plants. The highest level of Cd in the shoots was detected in plants transformed with the cV-ChMTII-GFP cassette whereas the lowest Cd content was found in plants transformed with the S-ChMTII cassette. Plants transformed with the p-S-ScMTII cassette and wild type plants showed similar levels of Cd. The roots of wild type plants accumulated higher levels of Cd than the roots of transgenic plants. A significant difference was detected especially between wild type and p-S-ScMTII transgenic plants. However, the level of Cd decreased with the increasing molarities of Cd in the nutrient solution. These results indicated that in hydroponic experiments, due to a high dry matter production and high protein expression, the highest amount of Cd accumulated in shoots.

The introduction of a metallothionein gene was considered to improve the ability of a plant to tolerate or accumulate heavy metal ions and has already been tested by many authors using MT genes from different sources (e.g. yeast MT or mammalian MT as reported by Macek *et al.*, 2002). For instance, the use of Chinese hamster metallothionein II gene (ChMTII) to increase the capacity of plants to bind and detoxify Cd was first proposed by Lefebvre *et al.* (1987). Their experiments showed that the ChMTII gene expressed in *B. campestris* L. under the control of the cauliflower mosaic virus promoter dramatically reduced the free Cd in transfected leaves. Plants overexpressing mammalian MTs were reported to be unaffected by concentrations of 100–200 μ M cadmium, whereas growth of *N. tabacum* control plants was severely inhibited at external cadmium concentrations of 10 μ M. Transformants of *B. oleracea* expressing the yeast metallothionein gene *CUP1* tolerated up to 400 μ M cadmium, whereas wild-type plants were unable to grow at concentrations above 25 μ M cadmium in a hydroponic medium. Transformants grown at 50 μ M cadmium accumulated 10–70% higher concentrations of cadmium in their upper leave than did non-transformed plants grown at 25 μ M cadmium. This indicates that the enhanced tolerance observed in the transgenic plants was unlikely to be a consequence of excluding cadmium from the leaves. *A. thaliana* seedlings expressing pea MT (PsMTA) under the control of a constitutive promoter accumulated up to eight-fold higher copper concentrations than untransformed control seedlings (Evans *et al.* 1992). Our results suggest that, stable transgenic tobacco plants expressing cV vacuolar ChMTII showed the highest accumulation of Cd in shoots and roots with increasing Cd concentration.

Macek, *et al.*, (2002) tested the effectiveness of genetically modified tobacco plants to accumulate heavy metals on sand media with the addition of KNOP's nutrient solution modified by the addition of cadmium (0.2 mg Cd/l as Cd (NO₃)₂ × 4 H₂O). After six weeks of growth there were no significant differences between the tested genetically modified tobacco lines in the dry weight of roots or above ground biomass at the Cd concentration used. The author concluded that in the case of plants expressing the HisCUP constructs, higher Cd translocation occurred than in the control plants.

The antioxidative defence mechanisms developed by plants are another important aspects of Cd tolerance. To understand the contribution of antioxidative defences to differential expression of Cd tolerance in the transgenic tobacco plants, we analysed the levels of glutathion (non-protein SH-groups), which is considered the major antioxidant in plant cells. This antioxidant is involved in detoxification of reactive O₂ species in plants (Öztürk, *et al.*, 2003). The results presented showed that the level of free non-protein SH-groups in the crude extracts of shoots and roots increased sharply with a 50µM Cd treatment (Figure III.18/A). However, in plants treated with a higher concentration of Cd the level of non-protein SH-group dropped again to values similar to those of untreated plants. Therefore, 50µM Cd was the maximum Cd dosage for high rate production of non-protein SH-groups. Analysis of the level of phytochelatin (PCs) and Glutathione supported this observation.

Most of the non-protein SH-groups in plants represent glutathione (Grill *et al.*, 1979). Glutathione is involved not only in detoxification of ROS (reactive oxygen species), but also essentially required for synthesis of Cd-binding peptides such as PCs which inactivate and sequester Cd by formation of stable Cd-complexes in the vacuole (Cobbett, 2000; Hall, 2002). The importance of Cd-binding proteins for the development of Cd tolerance in plants has been shown by several studies (Howden *et al.*, 1995; Clemens, 2001; Hall, 2002). PCs were induced by heavy metals such as Cd in many plants tested. Recent studies using yeast and *Arabidopsis* mutants with abolished PC production suggest that the role of PCs is correlated with metal tolerance. Therefore, PCs including the *N. glutinosa* MT might be useful proteins in the production of heavy metal tolerant plants (Liu *et al.*, 2000). Our transgenic tobacco plants transformed with the p-cV-ChMTII-GFP cassette as well as wild type (SR-1) plants were tested to determine the concentration of PCs. Both plants showed approximately the same amount of Phytochelatin (PCs) and, Glutathion (GSH) in the shoots either when untreated or after being treated with 100 µM Cd (Figure III.19). The synthesis of GSH was higher in untreated plants than in plants treated with 100µM Cd. In contrast, the synthesis of PCs increased significantly when the plants were treated with 100µM Cd. These results

resemble those described by Xiang *et al.*, (2001). They showed that plants with low levels of GHS were highly sensitive to low levels of Cd in the growth medium due to limited capacity of the plants to synthesize phytochelatins. Also effects of excess of other metals, such as Cu, on the internal Cd and PC-SH concentrations are sometimes different. These apparent discrepancies can be explained by the mechanism underlying induction of PC-SH synthesis in plants. Since PC-SH synthesis is assumed to be directly linked to the level of 'actual' metal stress (De Knecht, 1994; Keltjens and Van Beusichem, 1998), differences in total internal or external metal concentration between or within species do not necessarily induce different levels of stress and, consequently, different PC-SH levels. For example, De Knecht (1994) found higher Cd concentrations in roots of a Cd-tolerant *Silene vulgaris* cultivar, but significantly lower PC-SH levels than in a Cd-susceptible cultivar. Probably, in the Cd-tolerant cultivar a significant fraction of the total Cd was stored in non-metabolic cellular compartments, e.g. the vacuoles, where it did no longer contribute to the 'actual' stress. The lack of a further increase in shoot PC-SH with increasing shoot Cd levels at high internal Cd levels, as observed in this work, was probably due to storage of part of the Cd in the vacuoles, or to general high rates of PC synthesis in the plant tissues (Keltjens, *et al.*, 1998).

Interestingly, when Cd was not supplied, shoot concentration of SH-groups were more than 10-fold higher than those found in roots. 50 μ M Cd supplement in plants transformed with the cV-ChMTII-GFP construct produced the highest concentration of non-protein SH groups as compared to other transgenic and non-transgenic tobacco plants. Wild type tobacco (SR-1) plant had the highest non-protein SH-groups in shoots followed by S-ScMTI > S-hMTII > cV-ChMTII-GFP transgenic plants. Some explanations for these results can be found in the current literature. Cadmium-binding polypeptides are generally considered as a detoxification mechanism, but may also play a role in regulating Cd distribution between roots and shoots. In bean, however, the low translocation of Cd from roots to the shoot is a result of several factors including a) low production of PCs in the roots, b) low proportion of Cd in the soluble fraction of cell-free root extracts, c) binding of Cd in HMWs components in the soluble fraction; and d) retention of Cd in the cortex cells, the xylem vessels, and surrounding tissues during long distance transport from roots to the shoot (Guo, 1995).

The ability of plant genotypes to detoxify Cd by Cd-binding proteins can differ between and within plant species, and this plays a critical role in expression of high tolerance to Cd toxicity. Glutathione (or non-protein SH-groups) is directly involved in synthesis of Cd-binding proteins and thus in development of Cd tolerance in plants (Howden *et al.*, 1995; Hall, 2002). Despite similar Cd concentrations in leaf or shoot, plant genotypes can differ in

their tolerance to Cd toxicity. When taken up in the cells, cadmium can be detoxified by Cd-binding proteins such as phytochelatins or metallothionins (Grant *et al.*, 1998; Cobbett, 2000; Hall, 2002). However, the specific reason why plants developed PCs, but not MTs as a detoxification tool is not known (Kotrba, *et al.*, 1999).

According to Pan *et al.* (1994) and Zhu *et al.*, (1999), mouse MT cDNA and *E. coli* gshII encoding *glutathione synthetase* were transformed into tobacco and Indian mustard (*B. juncea*), respectively. The overexpression of the MT gene or *glutathione synthetase* conferred cadmium resistance to transgenic plants. Our results are in accordance with the ones reported by these authors.

IV.3 Soil experiments

In a second set of experiments tolerance to Cd toxicity was evaluated for transgenic plants grown in pots. The aim of these experiments was to quantify the genotypic variation in the uptake and distribution of Cd in transgenic and non-transgenic tobacco plants.

No symptoms were observed on transgenic or wild type plants treated with the highest concentration (30 ppm) of Cd (Table III.2). Morphologically, the only difference was observed for plants transformed with the p-cV-ChMTII-GFP cassette that flowered earlier than wild type plants. de Borne *et al.*, (1998) introduced a chimeric gene encoding a human metallothionein (hMTII) into a different tobacco variety from the one of the present study. These authors observed that morphologically, the hMTII plants were normal, except that they flowered earlier than controls, a feature that has been reported also when other unrelated genes were introduced in tobacco. Our results are in accordance with the ones described by de Borne *et al.*

We observed a significant correlation between levels of the recombinant proteins, plant dw, and Cd accumulation in transgenic and wild type tobacco plants. Immunoblot analysis of crude protein extracts showed that plants transformed with the cV-ChMTII-GFP construct accumulated the highest level of recombinant metallothionein whereas the lowest accumulation was detected in plants transformed with the S-ScMTII construct (Figure III.20 and Figure III.22).

The dw production of wild type and transgenic plants was not affected by application of Cd in the soil to levels that simulated the polluting concentrations for Cd in agricultural soils (Figure III.15 a/B). Sauerbeck (1982) indicated that a critical content of 5-10 mg Cd per g shoot dry matter might affect plant growth. In our experiments, the Cd contents in the shoot dry matter were never above these critical levels. Depressions in dry matter production or

toxicity symptoms were not observed. The production of dry matter of wild type and transgenic tobacco plants was only slightly affected by the treatment with Cd. Plants transformed with the p-cV-ChMTII-GFP cassette showed the highest dw compared to the other transgenic lines. However, wild type and transgenic plants showed no significant differences in dw of the leaves, stems and flowers.

The level of Cadmium in wild type and transgenic tobacco plants did not differ significantly (Figure III.22/A). Nevertheless, the level of Cd in the plant tissues markedly increased with the dose of Cd in the soil and the analysis of the level of Cd in different organs showed that the highest amount of Cd accumulated in leaves followed by stems and flowers (Figure III.22/B-C-D). The differences in Cd levels between leaves of transgenic and wild type plants were relatively smaller compared to those observed for stems and flowers. According to Brandle *et al.*, (1992) the concentration of available Cd in the soil solution might induce plant uptake rates that exceed the binding capacity of the MT produced by the plants. Levels of MT accumulation were likely not sufficiently high to allow the MT to compete with other, more mobile, Cd-binding molecules. Alternatively, expression of the MT gene may have been not sufficiently high in those cell types involved in Cd uptake and translocation. It is also possible that the greater mobility of Cd within the plants was a result of low stability of the Cd-MT complex despite the multiyear half-life of metallothionein-Cd complexes described for humans (Kägi and Schaeffer, 1988, Messerle, *et al.*, 1990).

Brandle *et al.* (1992) showed that expression of Chinese hamster MT II, as a fusion protein with β -glucuronidase (GUS) did not significantly alter pattern of Cd accumulation in field-grown transgenic tobacco. Gene expression measured by GUS activity showed that all of the transgenic lines expressed the MT II gene in the upper portion of the plant. One line did not express the MT II gene in the roots. Cd levels in the leaf tissue of transformed lines were not significantly different from the untransformed control. These results indicated that the MT protein was not immobilizing Cd in the root to a degree that would result in the exclusion of Cd from the leaf tissues.

Suh *et al.*, (1998), introduced the *N. glutinosa* MT cDNA into *N. tabacum* plants via *Agrobacterium*-mediated transformation. Overexpression of the MT gene conferred Cd tolerance to the transgenic plants. This was the first report suggesting the possibility of using MT genes of plant origin for the bioremediation of heavy metal contaminated soil. These authors reported reduced growth of T1 transgenic tobacco plants that was likely due to the exposure to Cd stress during root development. However, the flowering, seed development and germination processes of all transgenic tobacco plants showed increased Cd tolerance.

Large genotypic differences in Cd contents were found between plant species and even between cultivars and inbred lines of the same species (e.g. lettuce, wheat and barley, maize and tobacco (Guo, 1995). Uptake of Cd by plants differs between plant species and cultivars of the same species. In many of these cases however, it is not clear whether the observed differences are due to a different uptake or a different internal distribution of Cd between roots and shoots (Guo, 1995).

The concentration of free sulfhydryl groups in leaves of transgenic as well as wild type plants decreased with inverse proportion to the concentration of Cd supplied in the soil. Transgenic tobacco plants transformed with the cV-ChMTII-GFP cassette showed a higher concentration of non-protein SH groups than other transgenic and control plants. However only without Cd supplement the S-ChMTII transformed plants produced more non-protein SH than other plants. This indicated that –SH groups are possibly involved in increased Cd tolerance of stable transgenic cV-ChMTII-GFP tobacco plants by increasing antioxidative defence mechanism.

Pan *et al.* (1994) and Zhu *et al.*, (1999) transformed mouse MT cDNA and *E. coli gshII* encoding glutathione synthetase into tobacco and Indian mustard (*B. juncea*), respectively. The overexpression of the MT gene or glutathione synthetase conferred up to 200mg/l kanamycin and 100µM Cd resistance to the transgenic plants.

Although transgenic plants overexpressing MT genes or PCs are more tolerant to acute Cd toxicity, it remains to be determined whether they are practically useful for phytoremediation. When the pea MT gene was introduced into *A. thaliana*, the expression of the PsMTA gene caused enhanced Cu accumulation and a reduction of Fe availability. No significant effect on the accumulation of either Zn or Cd was detected. When the progenies of transgenic plants overexpressing *N. glutinosa* MT were investigated for tolerance to Cu and Zn, T2 seedlings showed increased tolerance to Cu and Zn. Overexpression of *N. glutinosa* MT confers enhanced resistance for heavy metals such as Cd, Cu and Zn on transgenic plants, indicating that the MT gene in plant cells is probably involved in detoxification of excess metals. By analogy with the MTs of animals and microorganisms, the *N. glutinosa* MT may serve as an intracellular „sink” for excess metals (Liu *et al.*, 2000). We did however not observe any differences of PCs concentration between transgenic cV-ChMTII-GFP plants and wild type SR-1 tobacco plants.

A wound and pathogen inducible MT cDNA was previously isolated from *N. glutinosa* while cloning plant disease resistance-response genes by subtractive hybridization (Choi *et al.*, 1996). Liu *et al.* (2000) introduced the same MT gene into tobacco plants via an

Agrobacterium mediated transformation. In this study the overexpression of the MT gene conferred up to 200 μ M CdSO₄ tolerance to transgenic tobacco plants.

It is often difficult to predict the effects of the expression of transgenes at the level of the whole plant. However, an improved understanding of metal homeostasis in plants will be vital for the development of successful phytoremediation technologies. Future strategies are likely to involve the introduction of several transgenes in tandem, the use of tissue-specific and inducible promoters, the manipulation of regulatory processes in plant metal homeostasis and the generation of hybrids between metal hyperaccumulators and related high-biomass crop plants (Krämer *et al.*, 2001).

IV.4 Conclusion and future prospects

Within this thesis, transgenic tobacco plants expressing vacuolar targeted ChMTII, ChMTII-GFP fusion or ScMTII were generated and characterized for their properties to accumulate and tolerate heavy metals such as Cd. The results of the present study showed that heterologous expression and subcellular targeting of MTIIs in tobacco plants was successfully achieved. Cd treatments on plants grown in hydroponic culture or in soil proved the efficacy of our approach and confirmed findings reported by other using a similar strategy. However, *in vivo* function of vacuolar targeted MTIIs and the stabilizing effects that fusion partners, such as GFP, exert on MTs represent novel findings not previously reported.

The results obtained with the hydroponic culture and the soil experiments indicated that the characterization of the transgenic plants might require further studies. Treating plants in the range between 50 and 100 μ M or above 30 ppm in hydroponic or soil experiments, respectively, might test for instance tolerance to Cd

To acquire further knowledge on the use of MTs for phytoremediation the following aspects might be worth further of investigations.

1. *In vitro* binding activities of the recombinant ChMTII and ScMTII of the present study should be confirmed by ELISA or by using binding assays based on cation exchange chromatography.
2. Analysis of T2 and/or T3 transgenic lines with the same set of assay described in the present thesis.
3. MTs can bind Zn, Cd, Ni, Pb, and Cu with affinities that depend on the concentration and bioavailability of the heavy metals (Vasak and Kägi, 1983). Based on this knowledge, it would be interesting to challenge the transgenic tobacco plants expressing the

recombinant ChMTII and/or ScMTII proteins with a broader set of heavy metals to determine the spectrum of tolerance and accumulation towards other pollutants.

4. Pilon-Smits and Pilon (2002) suggested that overexpression of phytochelatin synthase (PS), the enzyme mediating PC synthesis from GSH, may further enhance metal tolerance and accumulation. The overexpression of PS is possible, because the corresponding genes have been cloned (Clemens *et al.*, 1999; Ha *et al.*, 1999). Overexpression of the vacuolar transporter responsible for shuttling the PC-metal complex into the vacuole may represent another target for enhancing metal tolerance and accumulation in transgenic plants.
5. Expression of MTs under the control of root or shoot specific promoters might help to tailor MT overexpression according to the requirements of specific applications (Krämer, *e. al.*, 2001).

The long term goal of the phytoextraction approach here described is the use of transgenic plants with improved extraction properties as a novel tool to remove metal pollutants from contaminated soils. The overexpressions of MTs can increase plant tolerance to specific metals, for example Cd or Cu. However, these findings should be confirmed under field conditions. Only in a few reports did MT overexpression result in slightly increased accumulation of metals in shoots. Thus, the use of MTs in phytoremediation shows still limitations but offers great potentials for improvement.

V SUMMARY

The use of plants to clean-up soils contaminated with trace elements could provide a cheap and sustainable technology for phytoremediation. The introduction of novel traits into plants that produce high levels of biomass using a transgenic approach is a promising strategy for the development of effective phytoremediation technologies (Krämer, *et al.* 2001).

This thesis describes the use of recombinant Chinese hamster and *Saccharomyces cerevisiae* MTIIs for generating transgenic tobacco plants with improved extraction, accumulation and tolerance to heavy metals. The ChMTII and ScMTII were amplified from cDNA and subcloned into plant expression vectors for transient expression in the vacuole. Detailed characterization of recombinant vacuolar targeted ChMTII-GFP fusion; ChMTII and ScMTII were carried out on stably transformed tobacco plants obtained by *Agrobacterium*-mediated transformation. The MTIIs were cloned into the pTRA-kc plant expression vector and analyzed for their stability and accumulation levels in the plant vacuole. Lines with the highest accumulation levels were selected for establishing homozygous lines.

Functional expression of ChMTII and ScMTII in the vacuole of transgenic tobacco plants was confirmed by immunoblot and immunogold analyses. Immunoblot analysis indicated that high accumulation of the recombinant proteins was achieved in tobacco plants transformed with the cV-ChMTII-GFP construct, whereas the lower protein accumulation was observed for the S-ScMTII. Immunogold labelling experiments were carried out on transiently and stably transformed tobacco plants demonstrated that MTIIs localized to the vacuole.

The T₂ generation of tobacco lines expressing recombinant cV-ChMTII-GFP (line 13/3) as well as the T₁ generation of the S-ChMTII (line 18) and S-ScMTII (line 24) were used for evaluating *in vivo* protein function with two cultivation system: hydroponic culture and soil.

The levels of accumulation for the recombinant MTIIs in shoots and roots decreased with increasing concentration of Cd supplied with the nutrient solution. Transgenic plants that were transformed with the cV-ChMTII-GFP construct showed the highest expression of MTII protein in shoots and roots and a higher degree of tolerance to Cd than plants transformed with the S vacuolar targeting cassettes or than wild type plants used as control.

The cV-ChMTII-GFP transgenic plants which accumulated high levels of MTII proteins in shoots and roots showed high tolerance to Cd toxicity with high biomass production in hydroponic experiments. Cd tolerance level was up to 50µM Cd for transgenics and wild type tobacco plants. Above this Cd concentration, the production of plant biomass, the accumulation of Cd and the degree of tolerance to the metal were decreasing. To determine the exact concentration threshold for Cd tolerance in the transgenic plants as compared to

wild type tobacco plants additional experiment should be carried out. Ideally, the 50-100 μ M Cd range should be tested in hydroponic experiments.

The transgenic and wild type plants in soil experiments didn't show any toxicity symptoms within a treatment range from 0 to 30 ppm Cd. The dry matter of wild type and transgenic tobacco plants was only slightly affected by the treatment with Cd. Plants transformed with the cV-ChMTII-GFP cassette showed the highest dw compared to the other transgenic lines. However, wild type and transgenic plants showed no significant differences in the dw of leaves, stems and flowers. Further experiments should be carried out with higher cadmium dosage to identify those transgenic lines with improved ability to accumulate and tolerate heavy metals.

The data presented in this study demonstrated that heterologous expression and subcellular targeting of MTIIs in tobacco plants was successfully achieved. The results of the hydroponics and soil experiment showed that the characterization of the transgenic tobacco plants might require further studies. However these data indicate that compared to traditional extraction approaches; phytoremediation represents a promising and environmental friendly tool to clean heavy metal from contaminated soils at reduced costs.

VI References

- Alloway B.J. (1995). Heavy Metals in Soils. 2nd edn. Blackie, Glasgow: The University of Reading U.K.
- Andrade, J. C. M. and Mahler., C. F. (2002). Soil Phytoremediation. In *4th International Conference o Engineering Geotechnology*. Rio de Janeiro, Brazil.
- Arnon, D. I. (1949). Copper enzymes in isolated chloroplasts; polyphenol-oxidase in *Beta vulgaris*. *Plant Physiol* **24**, 1-15.
- Ausubel, F. M., Brent, R., Kingston, R. F., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (eds) (1995). Current protocols in molecular biology. New York: John Wiley & Sons, Inc.
- Baker, A. and Brooks, RR. (1989). Terrestrial higher plants which hyperaccumulate metallic elements - a review of their distribution, ecology and phytochemistry. *Biorecovery* **1**, 81-126.
- Bhat, S. R. and Srinivasan, S. (2002). Molecular and genetic analyses of transgenic plants: Considerations and approaches. *Plant Science* **163**, 673-681.
- Blayblock, M. J. (2000). Field demonstration of phytoremediation of lead-contaminated soils. In *Phytoremediation of contaminated soil and water*, pp. 1-12. Edited by B. G. Terry N: CRC Press, Boca Raton, Florida.
- Booyoucou, G. J. (1952). A recalibration of hydrometer for making mechanical analysis of soils. *Agron. J.* **43**, 434-438.
- Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**, 248-54.
- Brandle, J. E., Labbe, H., Hattori, J. & Miki, B. L. (1992). Field performance and heavy metal concentrations of transgenic flue-cured tobacco expressing a mammalian metallothionein-beta-glucuronidase gene fusion. *Genome* **36**, 255-60.
- Brady, N. C. and Well, R. R. Prentice Hall, (2002). The nature and properties of soils. New Jersey.
- Buchanan-Wollaston, V. (1994). Isolation of cDNA clones for genes that are expressed during leaf senescence in *Brassica napus*. Identification of a gene encoding a senescence-specific metallothionein-like protein. *Plant Physiol* **105**, 839-46.
- Cakmak I. and Marschner H. (1992). Magnesium deficiency and high light intensity enhance activities of superoxide dismutase, ascorbate peroxidase and glutathione reductase in bean leaves. *Plant Physiol.* **98**, 1222-1227.
- Chaney, R., Malik M, Li Y. M., Brown S. L., Brewer E. P., Angle J. S. and Baker A. J. M. (1997). Phytoremediation of soil metals. *Curr OpinBiotechnol Lett* **8**, 279-284.

- Chaney, R. L. (1983). Plant uptake of inorganic waste. In: Parr JE, Marsh P.B., Kla (eds) Land treatment of hazardous wastes. Noyes Data, Park Ridge, III. pp:56-76.
- Choi, D., Kim, H. M., Yun, H. K., Park, J. A., Kim, W. T. and Bok, S. H. (1996). Molecular cloning of a metallothionein-like gene from *Nicotiana glutinosa* L. and its induction by wounding and tobacco mosaic virus infection. *Plant Physiol* **112**, 353-9.
- Cobbett, C. S. (2000). Phytochelatins and their roles in heavy metal detoxification. *Plant Physiol* **123**, 825-32.
- Coligan, J. E., Dunn, B.M., Ploegh, H.L., Speicher, D.W. and Wingfield, P.T. (eds.) (1995). *Current protocols in protein science*. John Wiley & Sons, New York.
- Cox , S., (2000). Mechanisms and Strategies for Phytoremediation of Cadmium. <http://lamar.colostate.edu/~samcox/CONCLUSIONS.html>.
- Cunningham, S. D., Berti W.R. and Huang J.W. (1995). Phytoremediation of contaminated soils. *Trends Biotechnol.* **13**, 393-397.
- de Borne, F.D., Elmayan, T., de Roton, C., de Hys, L. and Tepfer, M. (1998). Cadmium partitioning in transgenic tobacco plants expressing a mammalian metallothionein gene. *Molecular Breeding* **4**, 83-90.
- de Framond, A. J. (1991). A metallothionein-like gene from maize (*Zea mays*): cloning and characterization. *FEBS Lett.* **290**, 103-106.
- de Knecht, J. A., van Dillen, M. Koevoets, P.L.M., Schat, H., Verkleij, J.A.C., and Ernst, W.H.O. (1994). Phytochelatins in cadmium-sensitive and cadmium tolerant *Silene vulgaris*. *Plant Physiol* **104**, 255-261.
- DIN (1983-01). Deutsches Institut für Normung e.V. Germany.
- Dower, W. J., Miller, J. F. and Ragsdale, C. W. (1988). High efficiency transformation of *E. coli* by high voltage electroporation. *Nucleic Acids Res* **16**, 6127-45.
- Elmayan, T. and Tepfer, M. (1994). Synthesis of a bifunctional metallothionein/ β -glucuronidase fusion protein in transgenic tobacco plants as a means of reducing leaf cadmium levels. *Plant J* **6**, 433-440.
- EPA (1996). Phytoremediation Resource Guide U.S. Washington, DC: Environmental Protection Agency Office of Solid Waste and Emergency Response Technology.
- EPA (1999). Phytoremediation Resource Guide U.S. Washington, DC: Environmental Protection Agency Office of Solid Waste and Emergency Response Technology Innovation Office.
- EPA (2000). Introduction to Phytoremediation. Environmental Protection Agency Office of Solid Waste and Emergency Response Technology.

- Evan, G. I., Lewis, G.K., Ramsay, G. and Bishop, J.M. (1985). Isolation of monoclonal antibodies specific for human c-myc proto-oncogene product. *Molecular and Cellular Biology*. **5**, 3610-3616.
- Evanko, C. R. and Dzombak, D. A., (1997). Remediation of Metals-Contaminated Soils and Groundwater: Ground Water Remediation Technology Analysis Center.
- Evans, I. M., Gatehouse, L. N., Gatehouse, J. A, Robinson, N. J., and Croy, R. R. (1990). A gene from pea (*Pisum sativum* L.) with homology to metallothionein genes. *FEBS Lett.* **262**, 29-32.
- Evans, K., Gatehouse, J. A, Lindsay, W. P, Shi, J., Tommey, A. M., and Robinson, N. J. (1992). Expression of metallothionein-like gene PsMT-alpha in *Escherichia coli* and *Arabidopsis thaliana* and analysis of trace metal ion accumulation: implications for PSMT-ALPHA function. *Plant Mol Biol* **20**, 1019-1028.
- Farr, D. G. (2001). Why Heavy Metals are a Hazard to Your Health? <http://www.becomehealthynow.com/article/productshair/122/>
- Fischer, R., Liao, Y. C., Hoffmann, K., Schillberg, S. and Emans, N. (1999). Molecular farming of recombinant antibodies in plants. *Biol. Chem.* **380**, 825-839.
- Florijn, P., Knecht, J. A and Van Beusichem, M.L. (1993). Phytochelatin concentrations and binding state of Cd in roots of maize genotypes differing in shoot/root Cd partitioning. *J Plant Physiol* **142**, 537-542.
- Fraley, R. T., Rogers, S. G., Horsch, R. B., Sanders, P. R., Flick, J. S., Adams, S. P., Bittner, M. L., Brand, L. A., Flink, C. L., Fry, J. S., Galluppi, G. R., Goldberg, S. B., Hoffmann, N. L. and Woo, S. C. (1983). Expression of bacterial genes in plant cells. *Proc. Natl. Acad. USA*. **80**, 4803-4807.
- Glass, D. (1999). Markets for phytoremediation 1999-2000. Glass Associates, Needham, Mass., <http://www.channel1.com/dglassassoc/index.htm>.
- Grant, C. A., Buckley, W.T., Bailey, L.D. and Selles, F. (1998). Cadmium accumulation in crops. *Can. J. Plant Sci* **78**, 1-17.
- Grill, D., Esterbauer, H. and Klösch, U. (1979). Effect of sulphur dioxide on glutathione in leaves of plants. *Environ. Pollut.* **19**, 187-194.
- Grill, E., Winnacker, E.L. and Zenk, MH. (1987). Phytochelatin, a class of heavy-metal-binding peptides from plants, are functionally analogous to metallothioneins. *Proc Natl Acad Sci U S A* **84**, 439-443.
- Grill E., Winnacker, E. L., and Zenk H.M. (1985). Phytochelatin: The principal heavy-metal complexing peptides of higher plants. *Science* **230**, 674-676.
- Guo, Y. (1995). Genotypic Differences in Uptake and Translocation of Cadmium and Nickel in Different Plant Species. PhD Thesis. In *Institute für Pflanzenernährung*. Stuttgart: Univ. Hohenheim.

- Hall, J. L. (2002). Cellular mechanisms for heavy metal detoxification and tolerance. *J. Experimental Botany* **53**, 1-11.
- Hamer, D. H. (1986). Metallothionein. *Annu Rev Biochem* **55**, 913-51.
- Hanahan, D. (1983). Studies on transformation of *E. coli* with plasmids. *J. Mol. Biol.* **166**, 557-580.
- Hart, J. J., Welch, R. M., Norvell, W. A., Sullivan, L. A. and Kochian, L. V. (1998). Characterization of cadmium binding, uptake, and translocation in intact seedlings of bread and durum wheat cultivars. *Plant Physiol* **116**, 1413-20.
- Harvey, B. (1995). Absorbing Possibilities: Phytoremediation. *Environmental Health Perspectives* **103**.
- Hattori, J., Labbe, H. and Miki, B. L. (1994). Construction and expression of a metallothionein-beta-glucuronidase gene fusion. *Genome* **37**, 508-12.
- Herren T. and Feller. U. (1994). Transfer of zinc from xylem to phloem in the peduncle of wheat. *J Plant Nutr* **17**, 1587-1598.
- Hirschi, K. (1999). Expression of Arabidopsis CAX1 in tobacco: altered calcium homeostasis and increased stress sensitivity. *Plant Cell Environ* **11**, 2113-2122.
- Hirschi, K., Korenkov, V.D., Wilganowski, N.L. and Wagner, G.J. (2000). Expression of Arabidopsis CAX2 in tobacco. Altered metal accumulation and increased manganese tolerance. *Plant Physiol* **124**, 125-134.
- Hoppmann, V. (2000). Targeting von rekombinanten Antikörperfragmenten in unterschiedliche Kompartimente der Pflanzenzelle. Aachen: RWTH-Aachen.
- Horsch, R., Fry, J. E., Hoffman, N., Eicholtz, D., Rogers, S. and Fraley, R. (1985). A simple and general method for transferring genes into plants. *Science* **227**, 1229-1231.
- Horton, R. M., Hunt, H. D., Ho, S. N., Pullen, J.K. and Pease, L. R. (1989). Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. *Gene* **77**, 61-68.
- Howden, R., Anderson, C. R., Goldsbrough, P. B. and Cobbett, C. S. (1995). A cadmium-sensitive, glutathione-deficient mutant of *Arabidopsis thaliana*. *Plant Physiol.* **107**, 1067-1073.
- Hsieh, H. M., Liu, W. K. and Huang, P. C. (1995). A novel stress-inducible metallothionein-like gene from rice. *Plant Mol Biol* **28**, 381-9.
- Hudspeth, R. L., Hobbs, S. L., Anderson, D. M., Rajasekaran, K. and Grula, J. W. (1996). Characterization and expression of metallothionein-like genes in cotton. *Plant Mol Biol* **31**, 701-5.
- Risser, J. A. and Baker, D. E. (1990). Testing Soils for Toxic Metals. **Chapter 11**, 275-298.

- Jesnowski, R., Naehring, J. and Wolf, K. (1995). A rapid and reliable method for PCR-based amplification of chromosomal and mitochondrial DNA from intact yeast cells. *Curr Genet* **27**, 318-9.
- Kägi, J. H. R. and Kojima Y. (1985). Metallothionein II. In *Second International Meeting on Metallothionein and Other Low Molecular Weight Metal-binding Proteins*, pp. 747. Edited by J. H. R. K. a. Y. Kojima. Zürich: Birkhäuser Verlag Basel.
- Kägi, J. H. R. and Schäfer, A. (1988). Biochemistry of metallothionein. *Biochemistry* **27**, 8509–8515.
- Kapila, J., de Rycke, R., van Montagu, M. and Agenon, G. (1996). An Agrobacterium mediated transient gene expression system for intact leaves. **122**, 101-108.
- Karenlampi, S., Schat, H., Vangronsveld, J., Verkleij, J. A. C., van der Lelie, D., Mergeay, M. and Tervahauta, A. I. (2000). Genetic engineering in the improvement of plants for phytoremediation of metal polluted soils. *Environmental Pollution* **107**, 225-231.
- Kawashima, C. G., Noji, M., Nakamura, M., Ogra, Y., Suzuki, K. T. and Saito, K. (2004). Heavy metal tolerance of transgenic tobacco plants over-expressing cysteine synthase. *Biotechnol Lett* **26**, 153-7.
- Kawashima, I., Inokuchi, Y., Chino, M., Kimura, M., and Shimizu, N. (1991). Isolation of gene for metallothionein-like protein from soybean. *Plant Cell Physiology* **32**, 913-916.
- Kawashima, I., Inokuchi, Y., Chino, M., Kimura, M., and Shimizu, N. (1992). Wheat Ec metallothionein genes; like mammalian Zn⁺ metallothionein genes, wheat Zn⁺ metallothionein genes are conspicuously expressed during embryogenesis. *Eur. J. Biochem.* **209**, 971-976.
- Kayser, A. (2000). Evaluation and enhancement of Phytoextraction of heavy metals from contaminated soils. In *Swiss Federal Institute of Technology Zürich*, pp. 153. Zürich.
- Kayser, A., Schulin, R. and Felix, H. R. (1999). Feldversuche zur Phytoremediation schwermmetallbelasteter Böden., pp. 170-182. Berlin: Pflanzenbelastung auf kontaminierten Standorten, Umweltbundesamt Berichte 1/99, Erich Schmidt Verlag,.
- Keltjens, W. G., and van Beusichem M.L. (1998). Phytochelatins as biomarkers for heavy metal stress in maize (*Zea mays* L.) and wheat (*Triticum aestivum* L.): combined effects of copper and cadmium. *Plant and Soil* **203**, 119-126.
- Klaassen, C. D. and Liu, J. (1998). Induction of metallothionein as an adaptive mechanism affecting the magnitude and progression of toxicological injury. *Environ Health Perspect* **106** Suppl 1, 297-300.
- Klaassen, C. D., Liu, J. and Choudhuri, S. (1999). Metallothionein: an intracellular protein to protect against cadmium toxicity. *Annu Rev Pharmacol Toxicol* **39**, 267-94.

- Koide, Y., Hirano, H., Matsuoka, K. and Nakamura, K. (1997). The N-terminal propeptide of the precursor to sporamin acts as a vacuole-targeting signal even at the C terminus of the mature part in tobacco cells. *Plant Physiol* **114**, 863-70.
- Koncz, C. and Schell, J. (1986). The promoter of TL-DNA gene 5 controls the tissue-specific expression of chimaeric genes carried by a novel type of Agrobacterium binary vector. *Mol. Gen. Genet.* **204**, 382-396.
- Kotrba, P., Macek, T., and Ruml T. (1999). Heavy Metal-Binding peptides and proteins in plants. *Collect. Czech. Chem. Commun.* **64**, 1057-1086.
- Kotrba, P. and Ruml, T. (2000). Bioremediation of Heavy metal pollution exploiting constituents, metabolites and metabolic pathways of livings. *Collect. Czech. Chem. Commun.* **65**, 1205-1241.
- Krämer, U., Cotter-Howells, J. D., Charnock, J. M., Baker, A.J.M., and. Smitt, J.A.C (1996). Free histidine as a metal chelator in plants that accumulate nickel. *Nature* **379**, 635-638.
- Krämer, U., Pickering, I. J., Prince, R. C., Raskin I and Salt, D. E. (2000). Subcellular localization and speciation of Ni in hyperaccumulator and non-accumulator *Thlaspi* species. *Plant Physiol* **122**, 1343-1353.
- Krämer, U. and. Chardonens, A. N. (2001). The use of transgenic plants in the bioremediation of soils contaminated with trace elements. *Appl Microbiol Biotechnol.* **55**, 661-672.
- Kubota H., S. K., Yamada T., and Maitani T. (1995). Phytochelatins (class III Metallothioneins) and their desglycyl peptides induced by cadmium in normal root cultures of *Rubia tinctorum* L. *Plant Science* **106**, 157-166.
- Lasat, M. M., Baker, A. J. M. and Kochian, L. V. (1998). Altered Zn compartmentation in the root symplasm and stimulated Zn absorption into the leaf as mechanisms involved in Zn hyperaccumulation in *Thlaspi caerulescens*. *Plant Physiol* **118**, 875-883.
- Lasat, M., Pence, N. S., Garvin, D. F., Ebbs, S. D. and Kochian, L. V. (2000a). Molecular physiology of zinc transport in the Zn hyperaccumulator *Thlaspi caerulescens*. *J Exp Bot* **51**, 71-79.
- Lasat, M. M. (2000b). The use of plants for the removal of toxic metals from contaminated soils: (SuDoc EP 1.2:2002011154): U.S. Environmental Protection Agency.
- Lasat, M. M. (2002). Phytoextraction of toxic metals: a review of biological mechanisms. *J Environ Qual* **31**, 109-20.
- Lefebvre, D. D., Miki, B.L. and Laliberte, J. F. (1987). Mammalian metallothionein functions in plants. *Bio/Technology* **5**, 1053-1056.
- Lewandowski, J., Leitschuh, S. and Koß, V. (1997). Schadstoffe im Boden. Heidelberg: Springer-Verlag.

- Life Extension. (2003). Heavy Metal Toxicity. <http://www.lef.org/protocols/prtcl-156.shtml>
- Liu, J. R., Suh., M. C. and Choi, D. (2000). Phytoremediation of cadmium contamination: Overexpression of metallothionein in transgenic tobacco plants. *Bundesgesundheitsbl-Gesundheitsforsch- Gesundheitsschutz* **43**, 126-130.
- Loeppert, R. H. and Suarez, D. L. (1996). Carbonate and Gypsum. In *Methods of Soil Analysis. Part 3. Chemical Methods*, pp. 437-474. Edited by J. M. Bigham. Madison: Soil Science Society of America (SSSA) and American Society of Agronomy (ASA).
- Lombi, E., Zhao F. J., Dunham S. J. and McGrath S. P. (2000). Cadmium accumulation in populations of *Thlaspi caerulescens* and *Thlaspi goesingense*. *New Phytol* **145**, 11-20.
- Macek, T. M., M., Pavlikova, D., Szakova, J., Truska, M., Cundy, S., Kotrba, A., Yancey, N., and Scouten, W.H. (2002). Accumulation of cadmium by transgenic tobacco. *Acta Biotechnol.* **22**, 101-106.
- Maitani, T., Kubota, H., Sato, K. and Yamada, T. (1996). The Composition of Metals Bound to Class III Metallothionein (Phytochelatin and Its Desglycyl Peptide) Induced by Various Metals in Root Cultures of *Rubia tinctorum*. *Plant Physiol.* **110**, 1145-1150.
- Maiti, I. B., Wagner, G. J., Yeagan, R. and Hunt, A. G. (1989). et al., Inheritance and expression of the mouse metallothionein gene in tobacco. *Plant Physiol.* **91**.
- Marschner, H. (1995). Mineral Nutrition of Higher Plants, 2nd Edition edn. San Diego CA, USA. Academic Press.
- Matsuoka, K., Matsumoto, S., Hattori, T., Machida, Y. and Nakamura, K. (1990). Vacuolar targeting and posttranslational processing of the precursor to the sweet potato tuberous root storage protein in heterologous plant cells. *J Biol Chem* **265**, 19750-7.
- Mejare, M. and Bulow, L. (2001). Metal-binding proteins and peptides in bioremediation and phytoremediation of heavy metals. *Trends in Biotechnology* **19**, 67-73.
- Messerle, B. A., Schäffer, A., Vasak, M., Kagi, J. H. and Wuethrich, K. (1990a). Three-dimensional structure of human [113Cd7] metallothionein-2 in solution determined by nuclear magnetic resonance spectroscopy. *J Mol Biol* **214**, 765-79.
- Messerle, B. A., Bos, M., Schäffer, A., Vasak, M., Kagi, J. H. and Wuethrich, K. (1990b). Amide proton exchange in human metallothionein-2 measured by nuclear magnetic resonance spectroscopy. *J Mol Biol* **214**, 781-6.
- Miller, R. R. (1996). Phytoremediation: Ground Water Remediation Technology Analysis Center.
- Misra, S. and Gedamu, L. (1989). Heavy metal tolerant transgenic *Brassica napus* L. and *Nicotiana tabacum* L. plants. *Theorit. Appl. Genet.* **78**, 161-168.
- Müller, H., Ziegler, B. and Schweizer, B. (1993). UV/VIS-Spektroskopie in der Nukleinsäureanalytik. *BioTec.* **4**, 25-29.

- Nelson, D. W. and Sommers., L. E. (1996). Total Carbon, Organic Carbon, and Organic Matter. In *Methods of Soil Analysis. Part 3. Chemical Methods*, pp. 961-1010. Edited by J. M. Bigham. Madison: Soil Science Society of America (SSSA) and American Society of Agronomy (ASA).
- Nordberg, M. (1998). Metallothioneins: historical review and state of knowledge. *Talanta* **46**, 243-254.
- Oztürk, L., Eker, S. Özkutlu F. and Cakmak, I. (2003). Effect of cadmium on growth and concentrations of cadmium, ascorbic acid and sulphhydryl groups in durum wheat cultivars. *Turk J Agric For.* **27**, 161-168.
- Pan, A., Tie, F., Duau, Z., Yang, M., Wang, Z., Li, L., Chen, Z. and Ru, B. (1994a). Alpha-domain of human metallothionein IA can bind to metals in transgenic tobacco plants. *Molecular & General Genetics* **242**, 666-674.
- Pan, A., Yang M, Tie F., Li L., Chen Z. and Ru B. (1994b). Expression of mouse metallothionein-I gene confers cadmium resistance in transgenic tobacco plants. *Plant Mol Biol* **24**, 341-352.
- Pearson, J. and Rengel, Z. (1995). Uptake and distribution of ⁶⁵Zn and ⁵⁴Mn in wheat grown at sufficient and deficient levels of Zn and Mn. II. During grain development. *J Exp Bot* **46**, 841-845.
- Pence, N., Larsen, P. B., Ebbs, S. D., Letham, D. L. D., Lasat, M. M., Garvin, D. F., Eide, D. and Kochian, L. V. (2000). The molecular physiology of heavy metal transport in the Zn/Cd hyperaccumulator *Thlaspi caerulescens*. *Proc Natl Acad Sci USA* **97**, 4956-4960.
- Peterson, P. (1983). In Metals and micronutrients: Uptake and Utilization by Plants. In *Adaptation to toxic metals.*, pp. 51-69. Edited by W. P. DA Robb. London: Academic Press.
- Pilon-Smits, E. and Pilon M (2000). Breeding mercury-breathing plants for environmental clean-up. *Trends Plant Sci.* **5**, 235-236.
- Prasad, V. N. M. and Freitas O. D. M. H. (1999). Feasible Biotechnological and Bioremediation Strategies for Serpaentine soils and Mine Spoils. *Nature Biotechnology* **2**.
- Prober, J. M., Trainor, G. L., Dam, R. J., Hobbs, F. W., Robertson, C. W., Zagursky, R. J., Cocuzza, A. J., Jensen, M. A. and Baumeister, K. (1987). A system for rapid DNA sequencing with fluorescent chain-terminating dideoxynucleotides *Science* **238**, 336-341.
- Raskin I, Kumar P. B. A. N., Dushenkov, S. and Salt, D. (1994). Bioconcentration of heavy metals by plants. *Curr. Op. Biotechnology*, **5**, 285-290.
- Raskin , (1996).I Plant genetic engineering may help with environmental clean-up. *Proc Natl Acad Sci USA*, **93**, 3164-3166.

- Raskin, I., Smith R. D. and Salt D. E. (1997). Phytoremediation of metals: using plants to remove pollutants from the environment. *Curr Op Biotechnology* **8**, 221-226.
- Reichel, C., Mathur, J., Eckes, P., Langenkemper, K., Koncz, C., Schell, J., Reiss, B. and Maas, C. (1996). Enhanced green fluorescence by the expression of an *Aequorea victoria* green fluorescent protein mutant in mono- and dicotyledonous plant cells. *Proc Natl Acad Sci U S A* **93**, 5888-93.
- Rhoades, J. D. (1996). Salinity: Electrical conductivity and total dissolved solids. In *Methods of Soil Analysis. Part 3. Chemical Methods*, pp. 417-435. Edited by J. M. Bigham. Madison: Soil Science Society of America (SSSA) and American Society of Agronomy (ASA).
- Risser, J. A. and Baker, D.E. (1990). Testing Soil for Toxic Metals. In *Soil Testing and Plant Analysis*, Third Edition edn, pp. 275-298. Madison: Soil Science Society of America (SSSA) and American Society of Agronomy (ASA).
- Saier, M. (2000). A functional-phylogenetic classification system for transmembrane solute transporters. *Microbiol Mol Biol Rev* **64**, 354-411.
- Salt, D. E., Blaylock M, Kumar P. B. A. N., Dushenkov S, Ensley B. D., Chet I. and Raskin I., (1995). Phytoremediation: A novel strategy for the removal of toxic metals from the environment using plants. *Bio/Tech* **13**, 468-474.
- Salt, D. E., Smith, R. D. and Raskin, I. (1998). Phytoremediation. *Annu Rev Plant Physiol Plant Mol Biol* **49**, 643-668.
- Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). Molecular cloning-A laboratory Manual. New York.: Cold Spring Harbor Laboratory.
- Sambrook, J., Fritsch, E. F. and Maniatis, T. (1996). Molecular cloning-A laboratory Manual., 3rd edition edn: Cold Spring Harbor Laboratory.,
- Sanger, F., Nicklen, S. and Coulson, A.R. (1977). DNA sequencing chain terminating inhibitors. *Proc. Natl. Acad. Sci. USA*, **74**, 5463-5467.
- Sauerbeck, D. (1982). Welche Schwermetallgehalte in Pflanzen dürfen überschritten werden, um Wachstumsbeeinträchtigungen zu vermeiden? pp. 109-129: Landwirtsch. Forsch., Sonderh. 39.
- Schat, H., Llugany, M., Vooijs, R., Hartley-Whitaker, J. and Bleeker, P. M. (2002). The role of phytochelatins in constitutive and adaptive heavy metal tolerances in hyperaccumulator and non-hyperaccumulator metallophytes. *J Exp Bot* **53**, 2381-92.
- Scholthof, H., Scholthof, K. and Jackson, A. (1996). Plant virus gene vectors for transient expression of foreign proteins in plants. *Annu. Rev. Phytopathol.* **34**, 299-323.
- Shaw, B. P. (1995). Effects of mercury and cadmium on the activities of antioxidative enzymes in the seedlings of *Phaseolus aureus*. *Biologica Plantarum* **24**.

- Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provencano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J. and Klenk, D. C. (1985). Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **150**, 76-85.
- Sorensen, K. and Brodbeck, U. (1986). A sensitive protein assay method using micro titer plates. *Experimentia* **42**, 161-162.
- Stillmann M. J., Shaw, I. F. C. and Suzuki K. T. (1992). Metallothioneins : Synthesis, Structure, and Properties of Metallothioneins, Phytochelatins, and Metal-Thiolate Complexes, pp. 436. Edited by M. J. Stillmann, Shaw F.C., Suzuki K. T. Weinheim: VCH publishers, Inc.
- Suh, M. C., Choi, D. and Liu, J. R. (1998). Cadmium resistance in transgenic tobacco plants expressing the *Nicotiana glutinosa* L. metallothionein-like gene. *Mol Cells* **8**, 678-84.
- Suzuki, N., Koizumi, N. and Sano, H. (2001). Screening of cadmium-responsive genes in *Arabidopsis thaliana*. *Plant Cell Environ* **24**, 1177-1188.
- Tamura, K., Shimada, T., Ono, E., Tanaka, Y., Nagatani, A., Higashi, S. I., Watanabe, M., Nishimura, M. and Hara-Nishimura, I. (2003). Why green fluorescent fusion proteins have not been observed in the vacuoles of higher plants. *Plant J* **35**, 545-55.
- Tan, H. K. (1994). Environmental Soil Science. New York: Marcel Dekker.
- Tapia, L., Gonzalez-Aguero, M., Cisternas, M. F., Suazo, M., Cambiazo, V., Uauy, R. and Gonzalez, M. (2004). Metallothionein is crucial for safe intracellular copper storage and cell survival at normal and supra-physiological exposure levels. *Biochem J* **378**, 617-24.
- Tsien, R. Y. (1998). The green fluorescent protein. *Annu Rev Biochem* **67**, 509-44.
- Utsunamyia, T. (1980). *Japanese Patent Application*, No 55-72959.
- Van der Zaal, B., Neuteboom, L. W., Pina, J. E., Chardonens, A. N. and Schat, H., Verkleij, J. A. C., Hooykaas, P. J. J. (1999). Overexpression of a novel *Arabidopsis* gene related to putative zinc-transporter genes from animals can lead to enhanced zinc resistance and accumulation. *Plant Physiol* **199**, 1047-1055.
- Vaquero, C., Sack, M., Chandler, J., Drossard, J., Schuster, F., Monecke, M., Schillberg, S. and Fischer, R. (1999). Transient expression of a tumor-specific single-chain fragment and a chimeric antibody in tobacco leaves. *Proc. Natl. Acad. Sci. USA*, **96**, 11128-11133.
- Vassilev, A., Schwitzguebel, J. P., Thewys, T., Van Der Lelie, D. and Vangronsveld, J. (2004). The use of plants for remediation of metal-contaminated soils. *ScientificWorldJournal* **4**, 9-34.
- Vázquez, M., Barceló, J., Poschenrieder, C., Mádico, J., Hatton, P., and Baker, A., Cope, G. H. (1992). Localization of zinc and cadmium in *Thlaspi caerulescens* (Brassicaceae), a metallophyte that can hyperaccumulate both metals. *J. Plant Physiol.* **140**, 350-355.

- Vögeli, L. R. and Wagner, J.G. (1990). Subcellular Localization of Cadmium and Cadmium-Binding Peptides in Tobacco Leaves. *Plant Physiology* **92**, 1086-1093.
- Walkerpeach, C. and Velten., J. (1994). *Agrobacterium*-mediated gene transfer to plant cells: cointegrate and binary vector systems. *Plant Molecular Biology Manual B1.*, 1-19.
- Wiechelman, K. J., Braun, R. D. and Fitzpatrick, J. D. (1988). Investigation of the bicinchoninic acid protein assay: identification of the groups responsible for color formation. *Anal Biochem* **175**, 231-7.
- Wu, D. Y., Ugozzoli, L., Pal, B. K., Qian, J. and Wallace, R. B. (1991). The effect of temperature and oligonucleotide primer length on the specificity and efficiency of amplification by the polymerase chain reaction. *DNA Cell Biol* **10**, 233-8.
- Xiang, C., Werner, B. L., Christensen, E. M. and Oliver, D. J. (2001). The biological functions of glutathione revisited in Arabidopsis transgenic plants with altered glutathione levels. *Plant Physiol* **126**, 564-574.
- Yeargan, R., Maiti, I. B., Nielsen, M. T., Hunt, A. G. and Wagner, G. J. (1992). Tissue partitioning of cadmium in transgenic tobacco seedlings and field grown plants expressing the mouse metallothionein I gene. *Transgenic Research* **1**, 261-267.
- Yu, L. H., Liu, J. Y., Umeda, M., and Uchimiya, H. (1998). Molecular Characterization of Metallothionein Genes in Plants. *Plant Biotechnology* **15**, 167-172.
- Zenk, M. H. (1996). Heavy metal detoxification in higher plants-a review. *Gene* **179**, 21-30.
- Zhang, X. Y., Zuo, X. F., Shan, L. and Ru, B. G. (2001). [Genetic analysis of T1 progeny of transgenic tobacco with metallothionein gene and metallothionein domain mutant alpha alpha gene]. *Yi Chuan Xue Bao* **28**, 877-83.
- Zhou, J. a. G., PB. (1995). Structure, organization and expression of the metallothionein gene family in Arabidopsis. *Mol Gen Genet* **248**, 318-328.
- Zhu, Y., Pilon-Smits, E. A. H., Jouanin, L. and Terry, N. (1999). Overexpression of glutathione synthetase in Indian mustard enhances cadmium accumulation and tolerance. *Plant Physiol* **119**, 73-79.
- Zimmer, M. (2002). Green fluorescent protein (GFP): Applications, structure, and related photophysical behavior. *Chem. Rev.* **102**.

VII APPENDICES

VII.I Abbreviations

A	Adenin
aa	Amino acid (s)
AAS	Atomic Absorbsion Spectrophotometer
Ab	Antibody
Amp	Ampicillin
AP	Alkaline phosphatase
<i>A. tumefaciens</i>	<i>Agrobacterium tumefaciens</i>
BCIP	5-bromo-4-chloro-3-indolyl phosphate
bp	Baise pair
BSA	Bovine serum albumin
C	Cytosine
CaCl ₂	Calcium chloride
CaMV	Cauliflower mosaic virus
Carb	Carbenicillin
Cd	Cadmium
cDNA	Complementary DNA
ChMTII	Chinese hamster Metallothionein II
CHS	Chalcone synthase
CI	Chloroform/isoamyl alcohol (24:1)
Cys	Cysteine
cv	Cultivar
cV	<i>Catharantus roseus</i> vacuolar targeting signal
°C	degree Celsius
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleoside triphosphate
DTPA	Diethyl triamin penta acetic acid
dw	Dry weight
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmatic reticulum
EtOH	Ethanol
fw	Fresh weight
G	Glycine
GAM	Goat-anti-mouse (antibodies)
g	Relative Centrifugal Force (RCF)
GFP	Green Fluorescent Protein

GSH	Glutathione
GST	Glutathione S-transferase
GSM	<i>Agrobacteria</i> glycerol stock media
h	Hour(s)
HCl	Hydrochloride
his	Histidine
HPLC	High pressure liquid chromatography
kDa	Kilodalton
kg	Kilogram
Km	Kanamycin
l	Liter
LBA	Luria broth with ampicillin
M	Molarity
mAb	monoclonal antibody
MES	2-(N-morpholino)-ethanesulphonic acid
min	Minute(s)
MPBS	Non-fat skim milk powder in PBS
MgSO ₄	Magnesium sulfate
mRNA	Messenger RNA
MSMO	Murashige and Skoog medium with minimal organics
MW	Molecular weight
NaCl	Sodium chloride
nm	Nanometer
OD	Optical density
o/n	Overnight
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PBST	0.1 % (v/v) Tween-20 in PBS
PCs	Phytochelatin
PCI	Phenol/chloroform/isoamyl alcohol (25:24:1)
PCR	Polymerase chain reaction
pH	A logarithmic measure of hydrogen ion concentration
pTRA-kc	Plant expression vector kanamycin resistance
rAb	Recombinant antibody
RNA	Ribonucleic acid
Rif	Rifampicilin
rpm	Rounds per minute
RT	Room temperature
S	Sporamin A vacuolar targeting signal
ScMTII	<i>Saccharomyces cerevisiae</i> Metallothionein II
-SH	Sulphydryl groups
SOE	Splicing by Overlap Extension

scFv	Single-chain variable fragment
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
<i>Taq</i>	<i>Thermus aquaticus</i>
TEA	Triethanol amine
TBE	Tris- buffered saline electrophoresis buffer
TEMED	N, N, N', N'-tetramethylene-ethylenediamine
TMV	Tobacco mosaic virus
Tris	Trishydroxymethylaminomethane
U	Unit
UTR	Untranslated region
UV	Ultraviolet
V	Volt; variable region
v/v	Volume per volume
w/v	Weight per volume
w/w	Weight per weight

VII.2 Nucleic acid and amino acid sequence of Chinese Hamster Metallothionein II

Gen Bank Accession number: M11265

atggacccca actgctcctg tgctacagat ggatcctgct cctgcgctgg gtcttgcaaa tgcaaagagt gcaaatgcac
cacctgcaag aaaagctgct gctcctgctg ccgggtgggc tgtgcgaagt gctcccaggg ctgcgtctgc aaagaggctt
cggacaagtg cagctgctgc gcctga

MDPNCSCATDGSCSCAGSCKCKECKCTTCKKSCCSCCPVGCAKCSQGCVCKEASDK
CSCCA

VII.3 Nucleic acid and amino acid sequence of *Saccharomyces cerevisiae* Metallothionein II

Gen Bank Accession number: AY693077

atgttcagcg aattaattaa ctccaaaat gaaggtcatg agtgccaatg ccaatgtggt agctgcaaaa ataatgaaca
atgccaaaaa tcatgtagct gcccaacggg gtgtaacagc gacgacaaat gccctgcgg taacaagtct gaagaacca
agaagtcag ctgctctggg aatga

MFSELINFQNEGHECQCQCGSCKNNEQCQKSCSCPTGCNSDDKCPCGNKSEETKKSC
CSGK

VII.4 Nucleic acid and amino acid sequence of Sweet potato sporamin A tuberous root storage protein

Gen Bank Accession number: X15091

caatgcattc caggttcaat cccatccgcc tccccaccac acacgaacccgca

HSRFNPIRLPTTHEPA

VII.5 List of figures

	Page
Figure I-1 Processes of phytoremediation	2
Figure I-2 General limitations of phytorextraction	4
Figure I-3 Metal uptake and accumulation in plants	12
Figure I-4 Various heavy metal tolerance mechanisms in higher plants	13
Figure I-5 Metallothionein metal-clusters	16
Figure I-6 Schematic overview of the project	27
Figure III-1 PCR amplification and cloning of the ChMTII gene	55
Figure III-2 Strategy for cloning of <i>Chinese hamster</i> Metallothionein II into pTRA-kc plant expression vector for vacuolar targeting	56
Figure III-3 PCR amplification of the ScMTII gene from yeast	56
Figure III-4 Strategy for cloning of ChMTII and ScMTII genes into pUC18 vector containing the S vacuolar targeting signal by SOE PCR	57
Figure III-5 Strategy for cloning of ChMTII (A) and ScMTII (B) genes into pTRA-kc plant expression vector	58
Figure III-6 Cloning of S-ChMTII and S-ScMTII cassettes into pTRA-kc plant expression vector	59
Figure III-7 Transient expression of MTIIs in the plant cell vacuole	60
Figure III-8 Florescence analysis of transiently expressed vacuolar ChMTII-GFP by TSC-SP spectral confocal microscope	61
Figure III-9 Screening of transgenic tobacco plants expressing cV vacuolar ChMTII	62

Figure III-10	Screening of stable transgenic tobacco plants expressing S vacuolar ChMTII and ScMTII.	63
Figure III-11	Electron microscopy studies of ChMTII, ScMTII and ChMTII-GFP localisation	64
Figure III-12	Growth of wild type tobacco plants (II.2.5.2) as affected by increasing Cd supply (II.2.5.1) in nutrient solution for 15 days.	67
Figure III-13	Screening of transgenic tobacco plants expressing cV and S vacuolar MTIIs after Cd treatment.	68
Figure III-14	Effect of increasing Cd supply (II.2.5.2) on shoots (A) and roots (B) (II.2.5.2) dry weight of transgenic and wild type (SR-1) tobacco	69
Figure III-15	Effect of increasing Cd supply (II.2.5.2) in nutrient solution on concentration of Cd in shoot (A) and root (B) of transgenic and wild type SR-1 tobacco plants	70
Figure III-16	Effect of increasing Cd supply (II.2.5.2) on concentration of TSP in shoots and roots of wild type (SR-1) and transgenic tobacco plants	71
Figure III-17	Effect of increasing Cd supply (II.2.5.2) on concentration of non-protein SH-groups in shoots (A) and roots (B) of wild type (SR-1) and transgenic tobacco	72
Figure III-18	Effect of increasing Cd supply (II.2.5.2) on PCs (II.2.5.6) concentrations in shoots of wild type (A-B) and T ₂ transgenic plants expressing vacuolar ChMTII-GFP (C-D)	73
Figure III-19	Growth of tobacco plants as affected by increasing Cd supply (II.2.6.6) in soil for 45 days	75
Figure III-20	Screening of transgenic tobacco plants expressing ChMTII-GFP, ChMTII and ScMTII after Cd treatment	75
Figure III-21	Effect of Cd supply (II.2.6.6) on the dry weight of whole plant (A), leaf (B), stem (C) and flowers (D)	77
Figure III-22	Effect of increasing Cd supply (II.2.6.6) on the Cd concentration of the whole plant (A), leaf (B), stem (C) and flowers (D)	78
Figure III-23	Effect of increasing Cd supply (II.2.6.6) on concentration of non-protein SH-groups in leaves of wild type (SR-1) and transgenic tobacco	79

VII.6 List of tables

Table I-1	Currently found concentration ranges and regulatory guidelines for important metal contaminants in US soils in the order of relative importance	3
Table I-2	Cadmium accumulations in plants on a Cd contaminated soil	6
Table I-3	Expression of metallothionein in transgenic plants	22
Table II-1	Names, suppliers and genotypes of <i>Escherichia coli</i> strains used throughout the work	29
Table II-2	Measurement conditions	50
Table II-3	Properties of soil used in the experiment	52
Table III-1	Cadmium toxicity symptom scale	66
Table III-2	Cadmium toxicity symptoms scale on soil experiments	74

Acknowledgements

Prof. Dr. Andreas Schaeffer for the opportunity to complete this thesis at the Institut für Biologie V in Aachen, for his helpful suggestions and corrections of this thesis. I thank him very much for the help he offered me when I started living in Aachen.

Prof. Dr. Rainer Fisher for giving me a chance to work at the Institut für Biologie VII in Aachen, for his guidance, for reviewing the thesis and agreeing to be co-examiner.

Prof. Dr. Ingolf Schupan for agreeing to act as a co-examiner and Dr. Ulrich Commandeur for offering the opportunity to perform the molecular biology experiments in his lab.

My special thanks go to Dr. Stefano di Fiore for his suggestions, comments, help and encouragement during writing of this thesis. He was the person I could always turn to. Dr. Greta Nölke and Dr. Rong Ji for reading through my thesis and for all the valuable suggestions.

Many thanks go to Oliver, Dr. Estella Terrada, Dr. Mehmet Kemal Tur, Nursen Sözer, Dr. Simone Dorfmueller for their helps and the valuable suggestions. Special thanks go to Dr. Flora Schuster for her help in stable transformation of tobacco plants. Dr. Elsa Arcalis for help microscopic works.

Dr. Martin Zimmermann for offering the opportunity to perform the Phytochelatins assay in the Institute für Biologie IV.

I would like to thank Michael Evangelou for our publication from his master thesis. We worked in peace with good Turkish-Greek relationship.

Special thanks go to Siham, Meltem, Greta, Sonal, Richa, Rajan, Adel, Markus, Stefano, for all those lighter moments we had in the lab. Flora, Carmen, Karo, Kerstin, Estella, for their friendship and the funny times in the box 254.

I would like to thank all my friends Dr. Derya Önder, Fahriye Özberisoy, Kemal Dogan, Dr. Sermet Önder, Dr. Nurcan Köleli, Atalay Albayrak, Canan Tokatli, Ümit Agirman, Perihan and Nursen Sözer and Sevgi Kabak, for being good friends during all these years in Aachen. “Dostlugumuzun kollari birbirimizi dunyanin bir ucundan bir ucuna kucaklayabilecek kadar uzundur.”

Meltem for her patience and understanding during all these years that we were together. Canim dostum seni cok seviyorum iyi ki varsin.

My sincere thanks are due to all members of the Institut für Biologie V and Biologie VII for their cooperation, kindness and for providing a conducive working atmosphere.

Most of all, I thank my parents, especially my elder brother and all members of my family for their untiring support, love and the seemingly unlimited belief in me. “Canim ailem, hepinizi cok seviyorum, desteginiz ve guveniniz icin tesekkurler.”

Lebenslauf

Persönliche Daten

Familienname: Daghan
Vorname: Hatice
Geburtsdatum: 28.10.1971
Geburtsort: Adana, Türkei
Staatsangehörigkeit: Türkisch

Ausbildung:

1978-1982	Grundschule in Adana, Türkei.
1982-1989	Gymnasium in Adana, Türkei.
1989-1993	Studium der Bodenkunde an der Cukurova Universität in Adana, Türkei. Abschluß: Diplom Ingenieur-Agronom Thema der Arbeit: Classification of soil in Ceylanpinar Region.
1993-1997	Studium der Bodenkunde an der Cukurova Universität in Adana, Türkei. Abschluß: Master of Science. Thema der Arbeit: "Effect of Zinc Deficiency of Root and Root Rot Disease's Pathogens."
1997-1999	Studium der Bodenkunde an der Cukurova Universität in Adana, Türkei. Thema der Arbeit: "Plant Nutritional Statues relationship cotton diseases in the Amik Plain, Hatay, Turkey."
2000-2004	Promotion am Institut für Biologie V der RWTH Aachen unter Anleitung von Prof. Dr. Andreas Schäffer.

Berufserfahrung

1995-1999	Mustafa Kemal Universität, Hatay, Türkei.
2000-2001	Wissenschaftliche Hilfskraft am Institut für Biologie VII der RWTH Aachen.
2001-2003	Wissenschaftliche Angestellte am Institute für Biologie V der RWTH Aachen
01/03-31/07/04	Wissenschaftliche Hilfskraft am Institut für Biologie V und Biologie VII der RWTH Aachen.