Search for Metal-Responsive Genes in Plants

Putative Roles in Metal Tolerance or Accumulation

Doctoral dissertation

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ABSTRACT

A number of plant species have developed mechanisms to tolerate and/or accumulate quantities of heavy metals that would be toxic to most other plants. The molecular mechanisms are not fully understood, even though these important traits contribute both to the micronutrient content and toxic metal content of our food.

The aim of this thesis was to isolate zinc-responsive genes from the cadmium/zinc hyperaccumulator *Thlaspi caerulescens*, which might contribute to the metal accumulation or tolerance trait. Using Differential Display, 16 differentially expressed genes were isolated from two *T. caerulescens* accessions, including metallothionein (MT) type 2 and type 3 genes, a MRP-like transporter gene as well as genes of unknown function.

The role of the metal-binding proteins, metallothioneins (MTs), was studied in more detail in three plant species: the hyperaccumulator *T. caerulescens*, the non-hyperaccumulator metallophyte *Silene vulgaris*, and hybrid aspen (*Populus tremula* × *tremuloides*). The expression levels of MTs associated with the origins of the *T. caerulescens* and *S. vulgaris* accessions, with higher expression in accessions originating from metal-enriched soils. The same observation was made with intraspecies crosses, in which the F3 lines that harbored an allele from the copper-tolerant *S. vulgaris* or from a superior cadmium/zinc-accumulating *T. caerulescens* accession had higher *SvMT2b* or *TcMT2a* and *TcMT3* expression levels compared to the corresponding sensitive or less accumulating plants, respectively. The expression of *TcMTs* did not co-segregate with zinc accumulation capacity in *T. caerulescens*. Neither did the *SvMT2b* expression co-segregate with copper tolerance in crosses between sensitive and tolerant plants. However, *SvMT2b* expression co-segregated with copper tolerance in families derived from crosses between moderately tolerant *S. vulgaris* plants, suggesting that higher *MT2b* expression was able to increase copper tolerance, albeit depending on the genetic background, *MT2b* thus being a candidate for hypostatic enhancer of copper tolerance.

In yeast, MTs were able to increase cadmium and copper tolerance. Moreover, in hybrid aspen grown on a metal-contaminated site, *PttMT2b* expression correlated positively with foliar cadmium and zinc concentrations. However, Arabidopsis plants transformed with *TcMT2* or *TcMT3* did not show increased metal tolerance or accumulation; instead, they showed reduced root growth at low external cadmium or zinc exposure, implying that the *TcMT2a* and *TcMT3* when overexpressed, might disturb metal homeostasis at low metal availability.

In conclusion, these results indicate that *TcMT2a* and *TcMT3* are not primary determinants of zinc accumulation in *Thlaspi*. However, the elevated expression levels in the metallicolous accessions suggest that they contribute to the metal-adapted phenotype, possibly through improving copper homeostasis at high zinc and cadmium body burdens. Alternatively, they might function as hypostatic enhancers of copper, zinc or cadmium tolerance.
ACKNOWLEDGEMENTS

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Viivi Hassinen

Kuopio, January 2009
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AAS</td>
<td>Atomic absorption spectrophotometer</td>
</tr>
<tr>
<td>aa</td>
<td>Amino acid</td>
</tr>
<tr>
<td>Bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>Cys</td>
<td>Cysteine</td>
</tr>
<tr>
<td>Cy5</td>
<td>Cyanine fluorescent dye, red</td>
</tr>
<tr>
<td>DD</td>
<td>Differential Display</td>
</tr>
<tr>
<td>DW</td>
<td>Dry weight</td>
</tr>
<tr>
<td>FAM</td>
<td>6-carboxy-fluorescein (fluorescent dye)</td>
</tr>
<tr>
<td>ICP-OES</td>
<td>Inductively coupled plasma atomic emission spectroscopy</td>
</tr>
<tr>
<td>LC</td>
<td><em>T. caerulescens</em> accession La Calamine</td>
</tr>
<tr>
<td>LE</td>
<td><em>T. caerulescens</em> accession Lellingen</td>
</tr>
<tr>
<td>LM</td>
<td><em>T. caerulescens</em> accession St Laurent le Minier (Ganges)</td>
</tr>
<tr>
<td>MP</td>
<td><em>T. caerulescens</em> accession Monte Prinzera</td>
</tr>
<tr>
<td>MT</td>
<td>Metallothionein</td>
</tr>
<tr>
<td>NA</td>
<td>Nicotianamine</td>
</tr>
<tr>
<td>PS</td>
<td>Phytosiderophore</td>
</tr>
<tr>
<td>PC</td>
<td>Phytochelatin</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>QRT-PCR</td>
<td>Quantitative real-time PCR</td>
</tr>
<tr>
<td>QTL</td>
<td>Quantitative trait locus</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcription/transcriptase</td>
</tr>
</tbody>
</table>
LIST OF ORIGINAL PUBLICATIONS

This dissertation is based on the following publications, which are referred to in the text by their Roman numerals:


II Hassinen VH, Tuomainen M, Peräniemi S, Schat H, Kärenlampi SO, Tervahauta Al. 2009. Metallothioneins 2 and 3 contribute to metal-adapted phenotype but are not directly linked to Zn accumulation in metal hyperaccumulator, Thlaspi caerulescens. Journal of Experimental Botany 60, 187-196.


IV van Hoof NA, Hassinen VH, Hakvoort HW, Ballintijn KF, Schat H, Verkleij JA, Ernst WH, Kärenlampi SO, Tervahauta Al. 2001. Enhanced copper tolerance in Silene vulgaris (Moench) Garcke populations from copper mines is associated with increased transcript levels of a 2b-type metallothionein gene. Plant Physiology 126, 1519-1526.

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1 INTRODUCTION

Heavy metals such as Cu, Zn and Ni, are essential micronutrients required for a variety of functions, including electron transfer reactions and as cofactors in many proteins and enzymes, whereas other metals, like Cd and Pb are considered non-essential. Both essential and non-essential metals are toxic above certain concentrations. They strongly inactivate metal-sensitive enzymes, resulting in growth retardation and, in the worst case, death of the organism.

Increased metal concentrations in soils can be caused by human activities, e.g. mining and smelting. Due to their persistent nature, the contaminating heavy metals are problematic in many areas in the world even though in developed countries the emissions have declined. For example, Cd may accumulate into fields over a time due to its presence as a contaminant in fertilizers (Mortvedt, 1996).

On the other hand, mineral deficiencies are common worldwide. It has been estimated that more than 25% of the world’s population is at risk of Zn deficiency (Maret and Sandstead, 2006) which is, according to WHO, as serious as Fe and vitamin A deficiencies. Thus, increasing Zn content in cereals is an important research topic (Palmgren et al., 2008).

Both beneficial micronutrients as well as harmful heavy metals may enter the food chain from the soil via plants. Plants differ in their ability to accumulate and tolerate metals. The extreme examples are the hyperaccumulator plants, which may contain hundred-fold higher metal concentrations in their shoots compared to other plants.

The molecular mechanisms of plant metal uptake and homeostasis are not completely known, even thought these traits markedly influence human health. In this Thesis, the current knowledge of plant metal uptake and tolerance is discussed, with emphasis on Cd/Zn hyperaccumulator plant Thlaspi caerulescens.
2 REVIEW OF THE LITERATURE

2.1 Plants and heavy metals

Metals such as Zn, Cu and Ni are essential mineral nutrients required for enzymatic reactions. Some metals, like Cd, have no known function in the higher plants. Both essential and nonessential metals are toxic to plants above certain concentrations. Depending on their physical and chemical properties, heavy metals may cause oxidative damage, either through directly catalyzing the production of reactive oxygen species, typically found for Fe or Cu, or through interference with non-protein antioxidants, such as glutathione. They may also bind to protein sulphydryl groups, or displace essential metal ions from biomolecules, as observed for Cd (reviewed by Schützendübel and Polle, 2002; Hall, 2002).

Plants are sessile organisms and thus have evolved tolerance mechanisms in soils that have increased metal concentrations. Plants can be divided into three groups with regard to their metal uptake: accumulators, indicators and excluders (Baker, 1981) (Figure 1).

![Figure 1. Responses of plants to increased soil metal concentrations (Baker, 1981).](image)

Metal excluder plants have low metal concentrations in the shoots even at relatively high soil metal concentrations, due to restricted metal uptake and/or translocation from the roots to the shoots (Baker, 1981). Metals are taken up by the plant only after the plant metal homeostasis mechanisms cease to function properly. Silene vulgaris is an example of a plant species with excluder strategy (Schat and Ten Bookum, 1992).

In the indicator plants, the shoot metal contents reflect those found in the soil. As an example, Populus alba is a Cd/Zn indicator plant, which could be used for the monitoring of soil metal contamination (Madejon et al., 2004).
Metal accumulator plants have increased metal contents even at low external metal concentrations. Extreme cases are the metal hyperaccumulator plants, which accumulate very high amounts of certain heavy metals in their above-ground parts. The hyperaccumulators are discussed in more detail in Section 2.1.2.

2.1.1 Metal tolerance in plants

All plants have some level of tolerance to metals. According to the classification of Ernst et al. (2008), plants with ‘basic metal tolerance’ are those living on non-metal enriched soils (also called ‘non-metallicolous’ by Meerts and Isacker, 1997), whereas metal hypertolerant (or merely called ‘tolerant’) plants can survive and reproduce on highly metal-enriched soils. Metal tolerant plant species do not necessarily accumulate metals, as exemplified by the metal excluder *S. vulgaris*.

Many cellular mechanisms may be involved in the basic metal tolerance (Hall, 2002; Clemens, 2006). In general, tolerance is achieved either by effluxing metals from the cytoplasm or by cytosolic metal binding. In Arabidopsis, sequestration of Zn to the root vacuole by MTP3 transporter is proposed to be involved in Zn tolerance in Arabidopsis (Arrivault et al., 2006). Chelation in the cytosol may be mediated by phytocelatins (Tennstedt et al., 2009), metallothioneins (MTs) or other metal-binding proteins. However, metal hypertolerance probably involves mechanisms that are specific for the species and for the metal and may be different from basic metal tolerance. For example, metal-binding phytochelatins are important for basic Cd tolerance but not for Cd hypertolerance in *S. vulgaris* and in *T. caerulescens* (Schat et al., 2002). In *S. vulgaris*, metal hypertolerance is governed by one or two major genes, with additional modifiers controlling the level of hypertolerance (Schat and Ten Bookum, 1992; Schat et al., 1993). Efficient Cu efflux at the root plasma membrane could be the major determinant of Cu hypertolerance (van Hoof et al., 2001b), whereas MT2b seems to act as a hypostatic enhancer (van Hoof et al., 2001a).

Metal hyperaccumulation and hypertolerance are separate traits and are, at least partly, under independent genetic control (Assunção et al., 2003b, Zha et al., 2004). However, as the hyperaccumulator plants are hypertolerant to the metal involved, some processes involved in tolerance and accumulation may be partially the same in Zn and Cd hyperaccumulators. For example, efficient sequestration into the shoot vacuoles by MTP1 transporter seems to play a role in hypertolerance as well as in hyperaccumulation (Gustin et al., 2009). In addition, enhanced Zn xylem loading in roots assisted by HMA4 is essential for Zn hyperaccumulation as well as for Zn and Cd hypertolerance in the Zn hyperaccumulator, *Arabidopsis halleri* (Hanikenne et al., 2008).
2.1.2 Metal hyperaccumulation in plants

Over 400 hyperaccumulating plant species are known to date. Most of them accumulate Ni (317 taxa, as listed by Boyd, 2007), but also Zn (11), Cd (1), Co (28), Mn (9), Pb (14) and Se (20) accumulating species are known. Also arsenic hyperaccumulating fern, *Pteris vittata*, has been discovered (Ma et al., 2001).

Hyperaccumulators are defined as plants which can accumulate more than 100 mg kg\(^{-1}\) (0.01%) of Cd, Co or Cr, 1000 mg kg\(^{-1}\) (0.1%) of As, Cu, Pb, Ni, or Se, or 10,000 mg kg\(^{-1}\) (1%) of Mn or Zn in their above-ground parts on dry weight basis (Baker and Brooks, 1989). These threshold levels are around 100-fold higher compared to those in the nonaccumulator species. However, much higher metal concentrations have been found in the hyperaccumulator plants. Some of the most extreme examples include a tree from New Caledonia, *Sebertia acuminata*, which has green latex that contains up to 25 % of Ni; a single tree can contain up to 37 kg of Ni (Jaffre et al., 1976; Sagner et al., 1998). A crucial feature of hyperaccumulators, in contrast to non-hyperaccumulators, is their high rate of metal translocation from the roots to the shoot, leading to shoot to root metal concentration ratios above unity (Baker et al., 1994; Lasat et al., 1996).

The hyperaccumulators most intensively studied at the molecular level are the Zn- and Cd-hyperaccumulating species *Thlaspi caerulescens*, some Ni-hyperaccumulating species of the genus *Alyssum*, and *Arabidopsis halleri*. These hyperaccumulators have become model plants, one reason being that they are close relatives to *Arabidopsis thaliana*. In Table 1, properties of a few hyperaccumulators are shown.
### Table 1. Properties of some of the most studied Cd, Zn or Ni hyperaccumulator species compared to the non-accumulator *A. thaliana* (Peer *et al*., 2003, 2006). The metal concentrations were measured either in plants growing in their natural environments or in plants exposed to metals. As a comparison, the concentrations in non-accumulators are usually in the range of (mg kg⁻¹): Cd 0.1-3, Zn 20-400, Ni 1-10 (Boyd, 2007).

<table>
<thead>
<tr>
<th>Species (Growth habit)</th>
<th>Metal accumulation (mg kg⁻¹)</th>
<th>Genome size / homology to <em>A. thaliana</em> (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hyperaccumulators:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Thlaspi caerulescens</em></td>
<td>Zn (30 000)⁵</td>
<td>2n=14/87-88, 88.5⁵</td>
</tr>
<tr>
<td>(biennial, self-fertilizing or outcrossing)</td>
<td>Cd (14 000)⁴</td>
<td></td>
</tr>
<tr>
<td><em>Thlaspi goingense</em></td>
<td>Zn (12 400)⁶</td>
<td>2n=8x=56/87-88</td>
</tr>
<tr>
<td>(biennial, outcrossing)</td>
<td>Ni (4 700)⁴</td>
<td></td>
</tr>
<tr>
<td><em>Arabidopsis halleri</em></td>
<td>Cd (2 700)⁷</td>
<td>2n = 16/94</td>
</tr>
<tr>
<td>(biennial, outcrossing)</td>
<td>Zn (15 000)⁸</td>
<td></td>
</tr>
<tr>
<td><strong>Non-accumulator:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Arabidopsis thaliana</em></td>
<td>Zn (150)¹</td>
<td>2n=10</td>
</tr>
<tr>
<td>(annual, self-fertilizing)</td>
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</tr>
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</table>


The hyperaccumulation trait must have been evolved independently in a number of unrelated plant families. One of the proposals is that hyperaccumulation might be an element-based defense strategy against herbivores or parasitism (Boyd, 2007). This hypothesis is currently the most studied one, and it has been shown that high Cd concentrations do protect *T. caerulescens* against thrips (Jiang *et al*., 2005). However, metals do not provide protection against snails in *T. caerulescens*, as the high Zn or Cd concentrations in the leaves have no effect on feeding preferences (Noret *et al*., 2005). Thus high metal concentrations do not protect from all herbivores. Any ecological benefit from elevated metal concentrations, whether defense against herbivores or something else, could provide a basis for a progressive evolution of still greater metal accumulation.

Many details of the hyperaccumulation process are yet to be established, even though numerous genes possibly contributing to the hyperaccumulation trait have been discovered recently (Chapter 4). Hyperaccumulation is an interesting phenomenon with many potential applications, ranging from phytoremediation to the biofortification of cereals (Guerinot and Salt, 2001).
2.1.3 Phytoremediation

Phytoremediation is the use of green plants (and associated micro-organisms) to clean-up soils or waters or render organic or inorganic contaminants harmless (recent reviews: Krämer, 2005; Pilon-Smits, 2005; Chaney et al., 2007; Doty, 2008; Koopmans et al., 2008). In addition to phytoextraction, phytostabilization, phytovolatilization and rhizofiltration can be used for inorganic contaminants. In phytostabilization, the contaminants are immobilized and their availability is reduced by contaminant accumulation into the plant roots or by binding to plant-borne organic matter. In phytoextraction, the contaminants are taken up by the plant roots and released as volatile chemicals from the roots or shoots. This process can occur naturally in the plants, as exemplified by inorganic Se, which is transformed enzymatically into volatile forms in plants. Volatilization of Hg has been successful in transgenic Arabidopsis expressing bacterial mercuric reductase (Rugh et al., 1996). Rhizofiltration uses plant roots to filter contaminants from waste waters, in a hydroponic setting or from a constructed wetland. In phytoextraction, plants take up contaminants, mainly inorganics, into their harvestable parts. After harvesting, the biomass may be incinerated. The classification of remediation types is somewhat artificial as, for example, the contaminants may be extracted and volatilized at the same time. The complete remediation of the site to fully functional soil is called phytorestoration (Bradshaw, 1997).

Phytoextraction has gained plenty of attention as a potential low-cost method for in situ remediation of contaminated sites. The optimal plant in phytoextraction would be fast-growing with a high biomass that would accumulate high concentrations of elements in its harvestable parts. Moreover, the plant should have a high tolerance to several metals and have dense root system that would allow metal extraction from a wide area. Currently, no such plants are known. In the phytoextraction experiments, either plants with high biomass and low metal accumulation (e.g. Brassica juncea) or high metal accumulation and low biomass (hyperaccumulators) have been used. There are more studies with high-biomass and low-accumulating plants. The hyperaccumulators are generally considered poor candidates for use in phytoextraction due to their small size as the total amount of contaminant uptake is dependent not only on the elemental concentration but also on the plant biomass. On the other hand, it has been estimated that T. caerulescens could be a viable alternative in Cd phytoextraction from topsoil in a moderately polluted site regardless of the poor growth characteristics of the plant (Zhao et al., 2003). Moreover, some Ni hyperaccumulators, like Berkheya coddii, have a relatively rapid biomass production, showing potential to be used for Ni phytoremediation (Robinson et al., 1997).

The time required to remediate a contaminated site is usually estimated to be more than ten years. The factual metal removal rates may be lower than the values estimated from short-term experiments. Thus it has been speculated that phytoextraction is not feasible in practice with the currently available knowledge (Van Nevel et al., 2007). Phytoextraction method needs further optimization and probably will be suited only to...
certain sites with moderate contamination levels. Possible applications could be the use of intensively managed short rotation coppice cultures of *Populus* in the removal of Al, Cd and Zn (Laureysens *et al.*, 2005). Another potential application could be the removal of Cd from the agronomic soils by using a non-food or -feed crop in rotation with the food crops.

Phytoremediation is an interdisciplinary technology that will benefit from research developments in many different areas. Increased knowledge about the detoxification and accumulation mechanisms in the plant can lead to the development of transgenic or non-transgenic plants that are better suited for remediation.

### 2.2 *Thlaspi caerulescens* - a model plant for metal hyperaccumulation

*T. caerulescens* (Alpine pennycress) (Figure 2) is a short-lived, nonmycorrhizal biennial or perennial plant that grows throughout central and western Europe and in Scandinavia. It is often found at Zn and Pb mining and/or smelting sites, but it may also occur in non-metalliferous soils, thus being a facultative metallophyte.

![Figure 2. *Thlaspi caerulescens* flowering in springtime in the backyard of the University of Kuopio](image)

*T. caerulescens* is extremely tolerant to Zn and, when grown hydroponically, it can accumulate 30 mg Zn g⁻¹ shoot dry weight (3%) without toxicity symptoms (Brown *et al.*, 1995). Moreover, several *Thlaspi* populations show Cd or Ni hyperaccumulation capacity of up to 14 mg Cd g⁻¹ (Lombi *et al.*, 2000) and 4.7 mg Ni g⁻¹ dry weight (Schat *et al.*, 2000). Typical for hyperaccumulators, metals are efficiently transported into the above-ground tissues.

Interesting metallicolous and nonmetallicolous populations (i.e. populations of a facultative metallophyte species growing either in a heavy-metal contaminated or
nonmetalliferous soil, respectively) with differential Cd, Zn and Ni tolerance, uptake and translocation capabilities have been found for *T. caerulescens*, which explains why this plant is one of the most intensively studied hyperaccumulator plants. *T. caerulescens* is even considered to be the model plant for hyperaccumulation (Assunção *et al.*, 2003c; Peer *et al.*, 2003). This plant can be easily grown in the greenhouse as it has a compact growth habit with about 20-30 weeks to flowering. It is a close relative of the non-hyperaccumulator model plant *Arabidopsis thaliana*, with about 88% sequence identity (Rigola *et al.*, 2006). The chromosome number is *n*=7 as opposed to *n*=5 in Arabidopsis (Peer *et al.*, 2003).

Moreover, *T. caerulescens* is amenable to *Agrobacterium*-mediated transformation (Peer *et al.*, 2003) even though transformation by floral dipping is not very efficient due to small number of flowers and seeds produced. Recently, the *Agrobacterium*-mediated transformation has been successful in *T. caerulescens* suspension cell cultures (Klein *et al.*, 2008). Shoot regeneration after transformation has been also achieved through callus phase (Guan *et al.*, 2008). Efficient transformation of hyperaccumulators will be crucial in elucidating the role of genes important for this trait.

2.2.1 Diversity of *T. caerulescens* populations

The *T. caerulescens* populations studied originate throughout the Europe from soils enriched with different combinations of heavy metals or from unpolluted soils, and are known to vary in their metal tolerance and uptake characteristics. Examples of local *T. caerulescens* populations, also called accessions, include ‘La Calamine’ (LC) and ‘Monte Prinzer’ (MP) originating from metal-enriched soils (Schat *et al.*, 2000) and ‘Lellingen’ (LE) from a nonmetalliferous soil (Meerts and van Isacker, 1997) (Table 3, Section 4.1). Metal tolerance and uptake capacities of the accessions LE, LC and MP have been extensively characterized by Assunção *et al.* (2003a). More recently, accessions from Zn/Cd/Pb enriched (i.e. calamine) soils in Southern France, the region around the village of Ganges (GA), have been discovered, which exhibit a superior foliar Cd accumulation capacity (Lombi *et al.*, 2000). In this accession, Zn does not compete with Cd during uptake (Lombi *et al.*, 2000), suggesting that there are different, accession-specific mechanisms for Cd uptake. Peer *et al.* (2003) proposed one of the *T. caerulescens* accession from the Ganges region, i.e. the one from the mine at St Félix de Pallières, as the model hyperaccumulator plant.

High levels of Zn tolerance and Zn accumulation are considered to be constitutive at the species level in *T. caerulescens*, in spite of a pronounced variation between the accessions. In general, higher Zn accumulation but lower Zn tolerance has been found in *T. caerulescens* accessions originating from non-contaminated soils compared to those from metalliferous soils (Meerts and Van Isacker, 1997; Escarré *et al.*, 2000; Assunção *et al.*, 2003).
al., 2003b; Frérot et al., 2003). For example, tolerance to Zn is much higher in the calaminous LC and GA accessions than in the nonmetallicolous accession LE (Assunção et al., 2003a). On the other hand, Zn accumulation and transport to the shoots is higher in LE than in LC especially at low Zn concentrations. Higher Zn tolerance in LC compared to LE is associated with higher Zn sequestration capacity within the plant and lower uptake (Assunção et al., 2003b). The differential metal uptake properties among different T. caerulescens accessions provide a basis for molecular biological studies of metal uptake mechanisms.

In addition to differences in metal tolerance and translocation, metallicolous and non-metallicolous populations show differences in their life cycle. In a study of Dechamps et al. (2007), metallicolous populations were more often annual or biennial, whereas nonmetallicolous populations were biennial or perennial. This raises the question of a possible cost of metal tolerance. In a two-year study, no cost of higher tolerance to reproductive traits was demonstrated in metallicolous populations (Dechamps et al., 2007). However, metallicolous populations showed increased damage due to herbivory in a nonmetallicolous environment, implying a possible cost of adaptation due to reduced defence (Dechamps et al., 2008).

2.2.2 Structural adaptations in T. caerulescens

T. caerulescens has two structural adaptations that may contribute to high metal uptake. First, T. caerulescens has been shown to target the root development to soil patches that have more available Zn (Whiting et al., 2000). This so-called ‘zincophilic root foraging’ has been attributed to an increased requirement for Zn (Haines, 2002).

Second, T. caerulescens shows increased lignification of the root inner cortical cell layer. It has been suggested that the function of this layer is not to block metal movement into the xylem, but to minimize the backward movement of metals accumulated in the stele, resulting in efficient metal loading into the xylem (van de Mortel et al., 2006).

Differences between the metal hyperaccumulator T. carulescens and the non-accumulator Arabidopsis thaliana have been addressed using suspension cell cultures. Compared to Arabidopsis, Thlaspi suspension cells had higher growth requirements for Zn, higher Zn and Cd tolerance and enhanced expression of specific metal transport-related genes. Moreover, differences in metal fluxes were observed (Klein et al., 2008). Thus it seems apparent that the traits are already expressed at the single cell level.
2.2.3 Localization of metals in *T. caerulescens*

Heavy metals are generally stored in the metabolically less active organelles like vacuoles and cell walls. Non-hyperaccumulator plants store excess metals mainly in the root vacuoles, whereas hyperaccumulators transport metals to the shoot.

In *T. caerulescens* roots, Cd can be found in the cell wall as well as in epidermal cells and in the xylem parenchyma (Wójcik *et al*., 2005b). In the leaves, highest concentrations of Cd and Zn are found in the epidermal cells, especially in the vacuoles (Küpper *et al*., 1999; Frey *et al*., 2000; Cosio *et al*., 2005; Ma *et al*., 2005; Wójcik *et al*., 2005b). This may suggest that the large vacuolated epidermal cells serve as a Zn storage site, while metal concentrations are kept at lower levels in the photosynthetically active mesophyll cells. However, the majority (65 to 70%) of the total leaf Cd and Zn is accumulated in the vacuoles of the mesophyll cells due to small biomass of the epidermis (Ma *et al*., 2005). A high concentration of Zn was also found in the cell walls of epidermal and mesophyll cells in *T. caerulescens* (Frey *et al*., 2000) and in the apoplast of epidermal cells in a related hyperaccumulator plant, *T. praecox* (Vogel-Mikus *et al*., 2008). Effluxing into apoplast and association with the cell wall seems to be one of the mechanisms employed by *T. caerulescens*. However, *T. caerulescens* accessions differ in metal efflux characteristics. In the leaf mesophyll cells of the ‘Prayon’ accession, greater Cd efflux to the apoplast is observed than in ‘Ganges’. This suggests that the superior accumulating ‘Ganges’ accession sequesters Cd more effectively within the cell, decreasing the need for efflux to achieve homeostasis. This may reflect differences in metal transporter expression between these accessions, and suggests that binding to the cell wall is not the main cause of superior Cd accumulation in ‘Ganges’ (Ebbs *et al*., 2008).

Even though the epidermal cells have higher Zn concentrations compared to the mesophyll cells, Zn is virtually absent from the guard cells (Frey *et al*., 2000). Guard cells have important roles in maintaining the plant water status and may be protected from heavy metal-induced damages. This could be achieved via metal efflux transporters or specific metal import transporters; however, these mechanisms are currently not known.

2.3 Molecular mechanisms of metal hyperaccumulation

The search for genes involved in metal hyperaccumulation was first started by analyzing genes known to be involved in metal homeostasis or uptake in other species, for example in yeast, or in non-accumulator plants (Pence *et al*., 2000; Assunção *et al*., 2001). The use of transcriptomics has led to the identification of many genes putatively involved in hyperaccumulation, from the viewpoint that the genes more highly expressed in the hyperaccumulators compared to the non-accumulators may be important in hyperaccumulation.
So far, only one custom-tailored *T. caerulescens* cDNA array has been made, with the limited number of about 1900 genes spotted (Plessl et al., 2005). *T. caerulescens* genes share a high degree of homology to Arabidopsis genes, which has enabled the use of commercial Arabidopsis microarray chips for expression studies. Hammond et al. (2006) compared shoot transcriptomes between two related *Thlaspi* species, *T. caerulescens* and a non-hyperaccumulator *T. arvense*, using the Affymetrix *A. thaliana* array, and found that many metal transporter genes from ZIP, CDF and HMA families were more highly expressed in *T. caerulescens*. The root transcriptomes of *T. caerulescens* versus *A. thaliana* have been compared with and without Zn and Cd exposure using Arabidopsis oligonucleotide microarray. In addition to transporter genes, a set of transcription factors as well as genes leading to lignin biosynthesis, were more highly expressed in *T. caerulescens* (van de Mortel et al., 2006, 2008). The transcriptome comparison of another Zn/Cd hyperaccumulator, *A. halleri*, also took advantage of *A. thaliana* microarrays (Becher et al., 2004; Weber et al., 2004; Chiang et al., 2006; Talke et al., 2006). Some of the transporter genes from ZIP, CDF and HMA families were more highly expressed also in *A. halleri*, suggesting that these genes are important in hyperaccumulation trait. The large-scale studies have increased our understanding of genes putatively involved in the metal hyperaccumulation. However, the function of these genes in metal transport must be confirmed.

In the next Sections, the molecular mechanisms of Zn and Cd uptake, translocation and tolerance are discussed, with emphasis in the genes known to be relevant in metal hyperaccumulation especially in *T. caerulescens*.

### 2.3.1 Genes involved in metal uptake, transport and tolerance

Genes important in hyperaccumulation are expected to be involved in enhanced metal uptake into the root, transport to the shoot and finally to the storage organelles such as vacuoles of leaf epidermal and mesophyll cells. Plants that are capable of hyperaccumulation are also tolerant to these metals due to efficient transport and detoxification mechanisms within the plant. Thus genes important in enhanced accumulation may also be important for metal tolerance.

Transporters are crucial in the uptake of metal ions from the soil solution and in the transport of metals throughout the plant. Plants have evolved many large metal transporter families, which differ in their expression pattern, substrate specificity and localization at the levels of the cell, tissue and organ. Transporters of essential metals may also transport non-essential metals like Cd, which can result in the accumulation of toxic elements. In Table 2, the properties of the most studied transporters in *T. caerulescens* are shown.
The first metal transporter isolated from *T. caerulescens* was ZNT1, which was able to mediate high-affinity Zn and low-affinity Cd uptake when expressed in yeast (Pence et al., 2000). ZNT1 belongs to the ZIP-(ZRT/IRT) family of transporters, in which 15 members are known in Arabidopsis (Mäser et al., 2001). ZNT1 is closely related to Arabidopsis AtZIP4, and is expressed at higher levels in *T. caerulescens* compared to Arabidopsis or *T. arvense* (Pence et al., 2000; Assunção et al., 2001). Microarrays have revealed that also many other ZIP-type transporters are more highly expressed in *T. caerulescens* compared to Arabidopsis, including homologs of *AtIRT3* and *AtZIP10* (van de Mortel et al., 2006). High expression of ZNT1 was also seen in microarrays of *T. caerulescens* and in the hyperaccumulator *A. halleri* (van de Mortel et al., 2006; Hammond et al., 2006). ZNT1 is expressed under Zn-limiting as well as in Zn-sufficient conditions, in contrast to the non-accumulators, where expression is observed only under Zn-deficiency (Assunção et al., 2001). The enhanced ZNT1 activity under normal Zn levels is suggested to be one of the factors mediating enhanced Zn and Cd uptake from the soil (Pence et al., 2000).

Xylem loading is the next and very important step in metal translocation in the hyperaccumulator plants. The HMA2 and HMA4 transporters play an important role in metal loading to the xylem. In Arabidopsis, AtHMA4 is mainly expressed in root stele, and overexpression of AtHMA4 leads to increased Cd and Zn accumulation in the shoots (Verret et al., 2004). Loss of function of both AtHMA2 and AtHMA4 almost completely blocks the movement of Cd to the shoot (Wong and Cobbett, 2009). In *T. caerulescens*, TcHMA4 is mainly expressed in the roots and is induced by Zn deficiency and high Zn as well as by Cd (Papoyan and Kochian, 2004). TcHMA4 confers Cd tolerance in yeast (Bernard et al., 2004; Papoyan and Kochian, 2004). In the hyperaccumulator *A. halleri*, a major QTL for Cd tolerance co-localized with AhHMA4 (Courbot et al., 2007). When AhHMA4 was knocked down in *A. halleri* using RNAi, plants translocated less Zn and were more sensitive to Cd and Zn (Hanikenne et al., 2008). This was direct evidence of the role of HMA in hyperaccumulator plant. These studies strongly suggest that HMA4 is important in the Cd and Zn translocation to the shoot as well as in Cd and Zn tolerance.

Another protein transporter family, the Yellow Stripe 1 (YSL)-like protein family, mediates metal loading to the vessels. Three YSL genes, TcYSL3, 5 and 7, are expressed at higher levels in *T. caerulescens*, compared to Arabidopsis. Localization of TcYSL3 and TcYSL7 in the root stele and the ability of TcYSL3 to transport Ni-NA and Fe-NA complexes into yeast suggests that TcYSLs are involved in vessel loading and unloading of metal-nicotianamine (NA) complexes (Gendre et al., 2007). The role of YSL transporters and NA in metal distribution in plants has been recently reviewed (Curie et al., 2009).

*T. caerulescens* and Arabidopsis NRAMP3 and 4, localized at the vacuolar membrane, mediate Fe, Mn, Cd and Zn (only NRAMP4 transports Zn) influx from vacuole to cytoplasm. Overexpression of AtNRAMP3 leads to Cd hypersensitivity and Fe overaccumulation in *A. thaliana* (Thomine et al., 2000). *T. caerulescens* TcNRAMP3 and TcNRAMP4 are more expressed than are their *A. thaliana* homologues, both in the roots and in the shoots.
Inactivation of *AtNRAMP3* and *AtNRAMP4* in *A. thaliana* resulted in strong Cd and Zn hypersensitivity, which was proposed to be due to imbalances in the mobilization of essential metals from the vacuole (Oomen *et al.*., 2009). At the functional level TcNRAMP3 and 4 do not seem to differ from their Arabidopsis orthologues. It is unclear whether increased expression of NRAMPs is a cause or a consequence of metal hyperaccumulation.

In the shoots, metals are translocated into the vacuoles (Figure 3). In *T. caerulescens*, ZAT1 (TcMTP1) is proposed to mediate the vacuolar Zn transport (Assunção *et al.*, 2001). MTP proteins have been isolated from other hyperaccumulators as well, including *T. goesingense*. TgMTP1 accumulates to high levels in the vacuolar membranes of the shoots where it likely acts in transporting Zn into the vacuole, enhancing both Zn accumulation and tolerance (Gustin *et al.*, 2009). Moreover, *A. thaliana* expressing TgMTP1 showed an enhanced Zn accumulation to the shoot and enhanced expression of Zn transporters (ZIP3, ZIP4, ZIP5, and ZIP9) in both shoot and root tissues, probably due to a Zn-deficiency response mediated by MTP1 (Gustin *et al.*, 2009).

There are also several other metal transporter families which may have a role in metal hyperaccumulators. For example ABC transporters, with members capable of transporting various xenobiotics and metals to the vacuole, are more expressed in *T. caerulescens* compared to *A. thaliana* (van de Mortel *et al.*, 2006). Also the cation exchanger (CAX) proteins could be involved in the transport of Cd into the vacuole, as was observed in *Nicotiana tabacum* overexpressing AtCAX2 and AtCAX4 (Korenkov *et al.*, 2007).

Expression of metal-related genes in the hyperaccumulators is regulated by the plant metal status; however, the regulation of these genes may differ compared to the non-accumulating plants. It has been suggested that transcription factors mediate the higher expression in the hyperaccumulators. Also gene duplications may have contributed to the higher expression, as is the case for *AhHMA4* (Hanikenne *et al.*, 2008) and *AhMTP1* (Dräger *et al.*, 2004), however, in *T. caerulescens* gene duplications remain to be demonstrated. Epigenetic changes and microRNAs may also contribute to heavy-metal mediated expression. Cd responsive micro-RNAs have been isolated from rice, some of them putatively regulating transcription factors (Huang *et al.*, 2009).

Recently, QTL analyses have been performed in order to study metal accumulation in *T. caerulescens* (Assunção *et al.*, 2006; Deniau *et al.*, 2006). In a cross between non-metallicolous and calaminous accession (LE x LC), two QTLs were found with the trait-enhancing alleles from both parents, which together explained about 40% of the root Zn accumulation (Assunção *et al.*, 2006). In a cross between calaminous and superior-accumulating calaminous accession (LC x GA), eight QTLs for shoot and root Zn and Cd accumulation were found (Deniau *et al.*, 2006). Two QTLs were found for both Cd and Zn accumulation in the root, and three and one for shoot Zn and Cd accumulation, respectively. The trait-enhancing alleles originated from the superior-accumulating accession (GA), except for three QTLs for Zn accumulation. As indicated above, there was an overlap in these traits, with one common QTL for Zn and Cd accumulation in the roots.
It will be interesting to identify the genes behind these QTLs. In *A. halleri*, QTLs for Zn tolerance co-localize with *AhHMA4* and two copies of *AhMTP1*, while *AhHMA4* is also the major QTL for Cd tolerance (Willems *et al.*, 2007; Courbot *et al.*, 2007). Based on QTL data, Roosens *et al.* (2008) suggested that the most promising candidates for Zn accumulation and tolerance in *A. halleri* are *ZIP6*, *ZIP9*, *HMA3*, *HMA4*, *MTP1a*, *MTP1b* and *NRAMP3*. In *T. caerulescens*, other genes might be responsible for Cd/Zn accumulation and tolerance than in *A. halleri*. However, the evidence from cross-species transcriptome comparisons suggests a high degree of convergence between *T. caerulescens* and *A. halleri* (Verbruggen *et al.*, 2009).

Although there are indications for some of the transporter genes to be involved in plant metal tolerance or accumulation, information about transporter chemistry are scarce. For example, the transfer mechanisms and kinetics, specificity, localization, regulation and especially interaction partners of the transporters are largely undiscovered. More research on the protein properties such as metal-binding regions, recently studied for *HMA2* (Wong *et al.*, 2009), are needed.

<table>
<thead>
<tr>
<th>Name/synonym (Plant orthologues)</th>
<th>L: Localization/ expression</th>
<th>P: Phenotype</th>
<th>R: Proposed role</th>
<th>Refs</th>
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<tbody>
<tr>
<td><strong>ZIP (Zn, Fe regulated, ZRT/IRT) family transporters</strong></td>
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<tr>
<td>ZIP1/TcZIP4</td>
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<tr>
<td>(AtZIP4)</td>
<td>PM/roots, shoots (AtZIP4: root stele)</td>
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<td></td>
<td>1-5, 8, 23</td>
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<td></td>
<td>I: low and sufficient Zn (AtZIP4: low Zn, Cu)</td>
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<td></td>
<td>P: Zn, Cd uptake in Sc (AtZIP4: Cu uptake)</td>
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<td></td>
<td>R: Zn, Cd uptake from soil (AtZIP4: trans-root metal transport?)</td>
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<tr>
<td><strong>ZNT1/TcIRT3 (AtIRT3)</strong></td>
<td>L: shoots</td>
<td></td>
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<td>8,</td>
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<td></td>
<td>R: Zn uptake from soil ?</td>
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<tr>
<td><strong>TcIRT1 (AtIRT1)</strong></td>
<td>P: Fe, Zn, Mn, Co, Cd uptake in Sc; Zn, Cd accumulation in low Fe in At (AtIRT1)</td>
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<td>6, 23</td>
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<td></td>
<td>R: root Fe uptake transporter</td>
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<tr>
<td><strong>CDF (Cation Diffusion Facilitator) family transporters:</strong> cytoplasmic efflux of transition metal cations</td>
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<tr>
<td>ZTP1/TcMTP1 (At:ZAT1/MTP1)</td>
<td>L: VM (TgMTP1: PM)</td>
<td>R: Zn loading into the vacuole (AtMTP1:QTL of Zn tolerance)</td>
<td></td>
<td>7-11, 22, 24</td>
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<tr>
<td><strong>P₁₈ATPases:</strong> translocate cations out of the cytoplasm across biological membranes using energy from ATP</td>
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<tr>
<td>TcHMA4 (AhHMA4)</td>
<td>L: higher in roots than in shoots</td>
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<td></td>
<td>11-17,</td>
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<tr>
<td>(AhHMA4)</td>
<td>I: low Zn, high Cd, high Zn</td>
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<td>20, 25</td>
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<td></td>
<td>P: Cd tolerance in Sc</td>
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<td></td>
<td>R: xylem loading, root-to-shoot Cd translocation (AhHMA4: QTL for Cd and Zn tolerance)</td>
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<tr>
<td><strong>NRAMP (The Natural Resistance Associated Macrophage Protein) family:</strong> transport divalent metal cations into the cytoplasm</td>
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<td>TcNRAMP3, 4 (AtNRAMP3, 3, 4)</td>
<td>L: VM (NRAMP3)</td>
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<td></td>
<td>18, 21</td>
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<tr>
<td></td>
<td>P: Cd uptake in Sc</td>
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<tr>
<td><strong>YSL (Yellow Stripe-Like) family:</strong> cellular uptake of metal-NA complexes</td>
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<tr>
<td>TcYSL3, 5, 7 (AtYSLs)</td>
<td>L: root stele, xylem (TcYSL3, 7)</td>
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<td>19</td>
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<tr>
<td></td>
<td>P: transport Ni-NA and Fe-NA complexes in Sc (TcYSL3)</td>
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<td></td>
<td>R: long-distance Ni translocation, vessel Ni-Na loading/unloading</td>
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</table>

$^1$Pence et al., 2000; $^2$Küpper et al., 2007; $^3$Grotz et al., 1998; $^4$Milner and Kochian, 2008; $^5$Wintz et al., 2003; $^6$Lombi et al., 2002; $^7$van der Zaal et al., 1999; $^8$Assunção et al., 2001; $^9$Käser et al., 2001; $^{10}$Willems et al., 2007; $^{11}$Bernard et al., 2004; $^{12}$Papoyan and Kochian, 2004; $^{13}$Hussain et al., 2004; $^{14}$Verret et al., 2004; $^{15}$Sinclair et al., 2007; $^{16}$Courbot et al., 2007; $^{17}$Omen et al., 2008; $^{18}$Gendre et al., 2007; $^{19}$Hanikenne et al., 2008; $^{20}$Thomine et al., 2000; $^{21}$Gustin et al., 2009; $^{22}$Plaza et al., 2007; $^{23}$Kobae et al., 2004; $^{24}$Wong and Cobbett, 2009
2.3.2 Small ligands and their roles in metal hyperaccumulation

It is generally believed that metals are present as chelates inside the plants. Metal-binding ligands, such as amino acids (histidine, nicotianamine), organic acids (malate,
citrate) as well as peptides (phytochelatins) and proteins (metallothioneins) may be important in metal chelation in the hyperaccumulator plants, hence contributing to metal tolerance. However, the importance of metal chelation in the hyperaccumulator plants is currently incomplete. Chelation may vary depending on the metals, plant species and even age of tissue (Salt et al., 1999).

Nicotianamine (NA) has gained plenty of interest recently as a metal-binding ligand involved in hyperaccumulation. Nicotianamine is a non-proteinogenic amino acid and a precursor of phytosiderophores (PS) in grasses, and is capable of binding many metals, including Fe, Zn, Ni, and Cu (for a recent review, see Curie et al., 2009). In Thlaspi hyperaccumulators, correlation exists between the Ni and NA concentrations in foliar tissues, suggesting that NA is involved in the hyperaccumulation of Ni; similar correlation was not found for Zn (Callahan et al., 2007). The nicotianamine synthase (TcNAS) gene was found from T. caerulescens library because it conferred tolerance to Ni in yeast (Vacchina et al., 2003). In Arabidopsis, overexpression of TcNAS resulted in increased Ni tolerance and accumulation (Piannel et al., 2005). Ni induces the accumulation of NA in the roots by inducing transport from the shoots via the phloem (Mari et al., 2006). From the roots, NA as a Ni-NA complex is redirected to the shoots via the xylem. This Ni-NA circulation is absent from the non-hyperaccumulator T. arvense, suggesting that Ni complexation with NA for xylem transport is an important mechanism in Ni hyperaccumulation (Mari et al., 2006). In T. caerulescens, three out of the four TcNAS genes are expressed at higher levels compared to A. thaliana (van de Mortel et al., 2006). The Yellow Stripe 1 (YSL)-like family proteins are involved in vessel loading and unloading of metal-NA complexes. Three YSL proteins, i.e. TcYSL3, 5, 7, are expressed at higher levels in T. caerulescens compared to Arabidopsis (Gendre et al., 2007).

Hyperaccumulator plants contain high levels of malate and citrate, and it is presumed that chelation by organic acids is important for metal storage. In the extreme Ni-accumulating tree, Sebertia acuminata, Ni is bound mainly to citrate in the latex (Schaumlöffel et al., 2003). In T. caerulescens, Cd was found to be mainly associated with malate in the intact leaves (Ueno et al., 2005). The high malate concentration in the vacuole was not affected by Cd exposure. The authors suggested that, since malate is not a strong Cd ligand, the malate-Cd complex is exclusively formed in the vacuole, where sulfur-based ligands are barely present. According to Callahan et al. (2006), organic acids may play a role in sequestering metals to the vacuole but as they do not bind metals very strongly they are probably not involved in the long-distance transport of metals.

Histidine has high affinity for metals, and it has been implicated in Ni hyperaccumulation. Ni induces His levels in the xylem sap in the Ni hyperaccumulator Alyssum lesbiacum, which has markedly higher His levels than does the non-hyperaccumulator A. montanum. Moreover, when A. montanum plants were treated with His, both Ni tolerance and root to shoot transport of Ni increased, suggesting that the high constitutive root His concentration is responsible for both the higher Ni tolerance and the higher Ni
translocation in *A. lesbiacum* (Krämer *et al.*, 1996). *A. lesbiacum* shows constitutively elevated expression of genes in the His biosynthetic pathway, in particular the one encoding the first enzyme of the pathway, ATP-phosphoribosyl transferase, which largely controls the rate of His synthesis. Overexpression of this gene in Arabidopsis increased Ni tolerance, but did not result in increased Ni accumulation in the leaves or xylem sap (Ingle *et al.*, 2005), suggesting that high His levels are important for Ni tolerance, but not for Ni translocation in Arabidopsis. In this respect, Arabidopsis seems to differ from *A. montanum* and *Brassica juncea*, in which Ni translocation can be strongly enhanced by exogenous His supply (Krämer *et al.*, 1996; Kerkeb and Krämer, 2003). Recently, Richau *et al.* (2009) showed that a species’ amenability to His-imposed enhancement of Ni xylem loading is determined by its capacity to retain His-complexed Ni in root cell vacuoles. In contrast to *A. montanum*, the non-hyperaccumulator *T. arvense* was not capable for enhanced xylem loading of Ni in response to exogenously supplied His (Richau *et al.*, 2009).

Organic acids have been suggested as the major Zn complexing molecule in aerial tissues, but the second most abundant Zn complex in the shoots in Zn-His. In the roots, 70% of Zn is complexed with His, suggesting that His plays an important role in Zn homeostasis in the roots in *T. caerulescens* (Salt *et al.*, 1999). It is well conceivable that His promotes xylem loading of Zn, in the same way as it promotes xylem loading of Ni (see above). The speciation of Zn in *T. caerulescens* appears to be related to tissue age, Zn-His and Cd-S complexes being more important in younger tissues. This may indicate a requirement for stronger ligands in the younger tissues compared to weaker interaction with vacuolar organic acids found in older tissues (Küpper *et al.*, 2004).

Phytochelatins (PCs) are small, Cys-rich polypeptides with a general formula of (GluCys)$_n$Gly ($n=2-11$). Hypersensitivity to Cd in PC-deficient cad1 mutants shows that PCs are important in basic Cd tolerance (Howden *et al.*, 1995). Moreover, PCs appear to be important in Zn tolerance and accumulation in Arabidopsis (Tennstedt *et al.*, 2009). However, PCs may not contribute to the hyperaccumulation phenotype, as *T. caerulescens* and the non-hyperaccumulator *T. arvense* have similar phytochelatin levels under Cd exposure (Ebbs *et al.*, 2002). Moreover, when PC accumulation in *T. caerulescens* was blocked by buthionine sulfoximine (BSO) treatment, Cd sensitivity was affected neither in the accession ‘Plombières’ (Wójcik *et al.*, 2005a), nor in ‘Ganges’. However, Cd sensitivity was increased in accession ‘Prayon’ (Hernandez-Allica *et al.*, 2006), and, more strongly so, in ‘Monte Prinzera’, originating from serpentine soil (Schat *et al.*, 2002). This may imply that different populations rely on different strategies in Cd detoxification. In general, there seems to be an inverse correlation between the PC concentrations, or the plant-internal PC-thiol to Cd molar ratios, and the Cd tolerance level among the accessions, which is also found among accessions of non-hyperaccumulator metallophytes. In addition, the response to BSO treatment also seems to be inversely correlated with Cd tolerance among accessions, again exactly as found in
the non-hyperaccumulator metallophytes. This suggests that PC-based Cd tolerance is only significant in less Cd-tolerant accessions (Schat et al., 2002). Recent studies have shown that PCs, probably as PC-metal complexes, can be transported between root and shoot, in both directions, via the phloem (Chen et al., 2006). In Brassica juncea there is much more Cd in the phloem than in the xylem, suggesting that PC-mediated Cd transport through the phloem might significantly contribute to Cd accumulation in the shoot (Mendoza-Cozatl et al., 2008). In hyperaccumulators, however, most of the metal transport will be unidirectional via the xylem.

Metallothioneins (MTs) are Cys-rich proteins suggested to be involved in plant metal tolerance or homeostasis. In Arabidopsis, the variation in Cu tolerance was shown to correlate with MT2 gene expression (Murphy and Taiz, 1995). In mammals, MTs are involved in several processes including Zn homeostasis and Cd detoxification (Palmiter, 1998; Coyle et al., 2002). In plants, the precise roles of MTs in non-hyperaccumulators or hyperaccumulators are not known. In T. caerulescens, several MT types and isoforms have been isolated (Roosens et al., 2004, 2005; Hassinen et al., 2007, 2009). Expression levels of some of the MT isoforms, at least those of MT2a, MT2b and MT3, have been found to be higher in hyperaccumulators compared to non-accumulators in several microarrays. For example, T. caerulescens MT2a and MT2b expression was higher in the roots compared to A. thaliana (van de Mortel et al., 2006), whereas higher MT2a and MT3 expression was observed in the shoots of T. caerulescens compared to T. arvense (Hammond et al., 2006). Higher MT2a, MT2b and MT3 expression was also seen in A. halleri seedlings compared to A. thaliana (Chiang et al., 2006). Metallothioneins are discussed in more detail in the next Section.

2.4 Metallothioneins

Metallothioneins are small Cys-rich proteins able to bind metals through the thiol groups of their Cys residues. The first MT was isolated over 50 years ago from horse kidney, as a low-molecular-weight, Cd-containing protein (Margoshes and Vallee, 1957). MTs have been found in many animals and fungi, cyanobacteria and plants. Next to Cd-dependent carbonic anhydrase in diatoms (Lane and Morel, 2000), MTs are the only proteins known to naturally contain Cd. In addition to Cd, MTs are able to bind many other elements, most commonly Zn and Cu.

2.4.1 Classification of MTs

Initially MTs were grouped into three classes based on their similarity to the originally isolated horse MT: protein with similar sequences formed class I, atypical MTs (including plant MTs) formed class II and phytochelatins, which are no longer considered MTs, formed class III. Since then the proteins from classes I and II were grouped into 15 families.
and further to subdivisions based on phylogeny and arrangement of Cys-residues. For example, family 1 contains vertebrate, family 2 mollusc and family 3 crustacean MTs. Fungal MTs are divided into the families 7-13. Plant metallothioneins belong to family 15, and are further subdivided into four subgroups based on their amino-acid arrangement (Cobbett and Goldsborough, 2002).

Arabidopsis has four MT types (Figure 4) with seven members expressed, namely MT1a, MT1c, MT2a, MT2b, MT3, MT4a and MT4b (Zhou and Goldsborough, 1994; Cobbett and Goldsborough, 2002; Guo, 2003). Plant metallothioneins differ from other metallothioneins because they have a long and variable spacer region. Generally, plant MTs are characterized by two (or three in case of type 4 MTs) Cys-rich domains that are separated by variable spacer regions, which are about 40 amino acids long in types 1-3. According to the classification of Cobbett and Goldsborough (2002), type 1 MTs have six Cys-X-Cys motifs (where X is another amino acid) equally distributed over the two domains. However, the spacer region can be much smaller in Brassicaceae species, as is the case in Arabidopsis and T. caerulescens, where the spacer is 11 amino acids.

Type 2 MTs have a highly conserved N-terminal domain (MSCCGGCNGCCS), which is characterized by a Cys-Cys motif and a Cys-Gly-Gly-Cys motif, whereas in the C-terminal domain three Cys-X-Cys motifs are present. Type 3 MTs have four Cys residues at the N terminus, the first three of which are typically arranged in a conserved motif Cys-Gly-Asn-Cys-Asp-Cys and the fourth as Gln-Cys-X-Lys-Lys-Gly. At the C terminus, six Cys residues are arranged in three Cys-X-Cys pairs.

The type 4 MTs have three Cys-rich regions separated by smaller spacer regions. Each Cys-rich region has 5 to 6 Cys residues arranged mostly as Cys-X-Cys. Arabidopsis has two MT4-type sequences, one of which has 31 additional amino acids in the N terminus before the first Cys, which can be seen in other dicot MT4s as well. So far, no type 4 MTs have been isolated from T. caerulescens. When searched against Arabidopsis MT4 sequences, no homologous sequence was found from the GenBank EST collection either.

Mammalian MTs are clustered in a single locus, for example human MTs in a single locus in chromosome 16 (Karin, 1984). The promoter region contains regulatory elements, including one or more copies of the metal responsive element (MRE) (Stuart, 1985), which acts as a binding target for the transcription activating protein factor (MTF-1) (Bruugnera, 1994).

In Arabidopsis, only MT1a and MT1c are located near each other, within a 2-kb region in chromosome I, whereas other forms are scattered over the genome: MT2b and non-transcribed MT1b locate in chromosome V, MT2a and MT3 in chromosome III and both MT4s in chromosome II.
Figure 4. Cys-domains of Arabidopsis and T. caerulescens metallothionein subtypes (Cobbett and Goldsborough, 2002). *Silene vulgaris* and *Populus* MT2 are included for comparison. Arabidopsis AtMT1a (AT1G07600), AtMT1c (AT1G07610), AtMT2a (AT3G09390), AtMT2b (AT5G02380), AtMT3 (AT3G15353), Ec types (AT2G23240 and AT2G42000); T. caerulescens MT1 (derived from EST sequence DN925102), TcMT2a-LC (FJ439656), TcMT2b-LC (FJ439647), TcMT3-LM (FJ439650), TcMT3-LC (FJ439655); *Silene vulgaris* SvMT2b (AF101825), *P. tremula x deltoides* PtdMT2 (AY594298), *Triticum aestivum* Ec (X68289 and X68291).
2.4.2 Structure of MTs

Many aspects of MT functioning in plants are not very well understood, partly because the purification of intact MT proteins is difficult and currently no plant MT protein structure is available in the databases. However, wheat Ec-1 protein structure may be soon available in the protein database (PDB) (PDB ID: 2KAK).

The structure of mammalian metallothioneins, on the other hand, has been determined using NMR or X-Ray crystallography for human, mouse, rat and rabbit MTs. Mammalian MTs bind seven divalent ions with tetrahedral Me(II)-Cys units, and form a dumbbell-like shape with two separate protein domains, where the β-subunit binds three and α-subunit four ions (Figure 5). Plant MTs, on the other hand, are thought to adopt a hairpin-like structure, where the two domains interact to form a metal-containing cluster with a total of four divalent ions (Domènech et al., 2006).

Figure 5. Metal binding properties and Cys arrangement of a) plant QsMT2, which is proposed to bind four divalent cations with hairpin structure (right) (Domènech et al., 2006) and b) two mammalian metallothioneins, mouse MT1a (top) and human MT2a (bottom), with proposed metal-binding coordination according to Zangger et al. (1999), c) NMR structure of mouse MT1 B (left) and α- (right) subunits (Zangger et al., 1999; PDB: 1DFS, 1DFT), spheres represent Cd ions.
Compared to the mammalian MTs, plant MTs have a long and variable spacer region. The function of the spacer region in plant MTs is currently not known. It has been suggested that it may be involved in the stability or may assist in protein folding and thus in metal coordination (Domènech et al., 2007). Moreover, the Cys-arrangement of MTs is more diverse in plants than in mammals, which has been proposed to result in differential metal-binding properties (Roosens et al., 2005).

2.4.3 Expression of plant MTs

The most widely studied aspects of plant MTs are their inducibility by different metals and their localization in different plant parts. The different MT classes have distinct tissue-specific expression pattern in Arabidopsis. In general, MT1s are more highly expressed in the roots, MT2s and MT3 more highly in the leaves and MT4 in the seeds (Zhou and Goldsbrough, 1994, 1995; Guo et al., 2003). More precisely, it was shown with GUS reporter constructs that the Cu-inducible MT1a and MT2b were expressed in the phloem, MT2a and MT3 in the mesophyll cells and MT4 only in the seeds of Arabidopsis. The authors suggested that MT1a and MT2b are involved in metal redistribution via the phloem, whereas MT2a and MT3 act as chaperones (Guo et al., 2003).

In Arabidopsis, the expression of MT genes is primarily inducible by Cu (Zhou and Goldsbrough, 1994; Murphy and Taiz, 1995; Guo et al., 2003), but also to some extent by Cd and Zn (Murphy and Taiz, 1995; Zhou and Goldsbrough, 1994). When expressed in yeast, plant MTs are able to rescue Cu, Cd and Zn-sensitive mutants under metal exposure (Zhou and Goldsbrough, 1994; van Hoof et al., 2001a; Roosens et al., 2004, 2005; Hassinen et al., 2007; Guo et al., 2008). In Arabidopsis, MT1 family knock-down lines, with reduced MT1a and MT1c levels, were hypersensitive to Cd and accumulated less As, Cd, and Zn to the leaves than did the wild-type, suggesting that type 1 MTs confers Cd tolerance and may be involved in Zn homeostasis (Zimeri et al., 2005). The MT1a loss-of-function plants showed reduced Cu concentrations in the roots, implying that MT1a is important in the accumulation of Cu in the root as well (Guo et al., 2008). In the study, even the mt1a mt2b double mutant had normal Cd and Cu tolerance (Guo et al., 2008). Overexpression of Arabidopsis MT2a and MT3 in bean guard cells has been shown to increase Cd tolerance (Lee et al., 2004). Cu and Cd tolerance in A. thaliana was increased by overexpression of Brassica juncea BjMT2 (Zhigang et al., 2006); however, in the absence of heavy metals the root growth of the transformants was reduced, possibly implicating problems in maintaining metal homeostasis.

In addition to metals, other conditions are known to induce MT expression. Guo et al. (2003) reported induction by senescence, and it was suggested that MTs may be involved in the mobilization of metal ions from senescing leaves. Metallothioneins were also abundant in a cDNA library derived from senescing Populus leaves (Bhalerao et al., 2003). Induced expression by oxidative stress has been reported for MT2 in cork tissue (Mir et
al., 2004). In cotton seedlings, GhMT3 mRNA was up-regulated by high salinity, drought, low temperature, abscisic acid (ABA), ethylene, reactive oxygen species (ROS), Zn and Cu (Xue et al., 2008). Moreover, Nicotiana tabacum plants overexpressing GhMT3 had an increased tolerance to ROS stress, and GhMT3a protein appeared to be capable of scavenging ROS in vitro. The authors speculated that MT might release metals during oxidative stress and may trigger a Zn-mediated antioxidant response, as has been reported in fungi by Tucker et al. (2004).

MTs may also have a function in plant development. It was recently discovered that rice metallothionein (OsMT2b) was down-regulated by cytokinins. The OsMT2b-overexpressing rice plants had decreased cytokinin levels, whereas the opposite was true for RNAi knock-down mutant plants (Yuan et al., 2008). The authors concluded that OsMT2b has a role in root development and in seed germination by regulating cytokinin levels. In a detailed study, rice MT2b expression was found to be down-regulated in the epidermal cells that undergo cell death during normal root growth (Steffens and Sauter, 2009). In the knock-down mutant plants, an inverse relationship between cell death and MT2b transcript abundance was seen. These authors suggested that MT2b is a ROS scavenger which must be down-regulated to allow the progress of epidermal cell death, mediated by ethylene and H$_2$O$_2$. Another rice MT, OsMT1a, was specifically Zn-inducible and enhanced Zn accumulation was found when OsMT1a was overexpressed in rice. Moreover, the overexpressing plants showed increased drought tolerance and higher antioxidant enzyme activities, implying that OsMT1a is involved in ROS scavenging (Yang et al., 2009).

There are thus indications that MTs function in many processes in plants, ranging from development to detoxification. Different MT isoforms have different functions, which may be cell-type specific. However, no specific function can be assigned for a specific MT isoform based on currently available data. Moreover, MT homologs from different species may have different functions.

2.5 Conclusion

In conclusion, some of the key genes identified from the hyperaccumulators so far are the intensively studied transporters involved in metal uptake, transport to the shoot and intracellular sequestration. The ZIP transporters are probably mediating metal uptake, the P-type ATPases (HMA2, HMA4) are involved in xylem loading and MTP1 is important in vacuolar sequestration. The HMA and MTP transporters seem to be crucial in metal tolerance as well.

In the leaves, metals are transported to the vacuoles, where they are bound to organic acids. Apoplast is another storage site, where cell wall may be involved in metal binding. In the cytosol, nicotinanamine is probably involved in metal buffering; however, other
chelators such as metallothioneins, may be involved. The mechanisms of cytosolic metal buffering or binding to cell wall are not completely understood. While these might not be the main determinants of hyperaccumulation, they may influence the accumulation or tolerance level of hyperaccumulator plants.
3 AIMS OF THE STUDY

Knowledge of plant metal uptake, tolerance and accumulation is increasing but understanding of the mechanisms still remains incomplete.

In this study, Zn-responsive genes were studied in the Zn/Cd hyperaccumulator, *Thlaspi caerulescens*. The members of one family, the metal-binding metallothioneins, were studied in more detail in *T. caerulescens*, in Cu-tolerant *Silene vulgaris* and, under field conditions, in hybrid aspen (*Populus tremula x tremuloides*).

The specific aims of the thesis were:

A  To isolate Zn responsive genes from *Thlaspi caerulescens* (I)

B  To study the role of metallothioneins in:

   B1) Zn hyperaccumulation by analyzing the expression of *MT2a*, *MT2b* and *MT3* in *T. caerulescens* accessions and progenies of inter-accession crosses with differential Zn accumulation capacities (II)

   B2) metal buffering under field conditions by searching for possible correlation between *MT2b* expression and foliar metal concentration in hybrid aspens growing at a metal-contaminated site (III)

   B3) Cu tolerance by analyzing *MT2b* expression in Cu-tolerant and -sensitive *Silene vulgaris* ecotypes and its co-segregation with Cu tolerance in progenies of inter-ecotypic crosses (IV).
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4 MATERIALS AND METHODS

4.1 Plant cultures

4.1.1 Plant material

In the literature, local *Thlaspi caerulescens* populations, often incorrectly termed ‘ecotypes’ are often called ‘accessions’. In this study, the term accession has been adopted.

The *T. caerulescens* accessions used (I, II) originate from metalliferous or non-metalliferous soils and have differences in metal tolerance, uptake and translocation (Table 3) (Assunção *et al.*, 2001, 2003a). The accession Lellingen (LE) is from a non-metalliferous soil at Lellingen, Luxembourg (Meerts and Van Isacker, 1997). The accession La Calamine (LC) originates from a soil contaminated with calamine ore waste (Zn, Cd, Pb) near La Calamine, Belgium, and the accession Ganges (GA) from a similar site near Ganges, France (Zhao *et al.*, 2002). In II, GA was renamed St Laurent le Minier (LM) according to the better defined origin, to distinguish it from other accessions from the Ganges region. Zn-responsive genes were isolated from the accessions LC and LE (I). *MT* expression was studied in LC, LE and LM (II). The accession LC is tolerant to metals and has low metal uptake. The non-metallicolous LE is less tolerant to metals and has a high uptake capacity. The accession GA both tolerates and accumulates metals (Assunção *et al.*, 2003a).
Table 3. Properties of *T. caerulescens* and *S. vulgaris* (IV) accessions used in this Thesis. *T. caerulescens* accessions were characterized by Assunção *et al.* (2003a), except for FP which is described by Roosens *et al.* (2003). The *S. vulgaris* accessions were characterized by Schat and Ten Bookum (1992).

<table>
<thead>
<tr>
<th>Origin</th>
<th>Site characteristics</th>
<th>Accession characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Thlaspi caerulescens</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LE</td>
<td>Lellingen, Luxembourg</td>
<td>Normal soil</td>
</tr>
<tr>
<td>LC</td>
<td>La Calamine, Belgium</td>
<td>Zn/Pb mine spoil (calamine)</td>
</tr>
<tr>
<td>LM</td>
<td>St Laurent le Minier, Ganges, France</td>
<td>Zn/Pb mine spoil (calamine)</td>
</tr>
<tr>
<td>FP</td>
<td>St Felix de Pallieres, Ganges, France</td>
<td>Zn/Pb mine spoil (calamine)</td>
</tr>
<tr>
<td>MP</td>
<td>Monte Prinzera, Italy</td>
<td>Ultramafic soil (serpentine)</td>
</tr>
<tr>
<td><strong>Silene vulgaris</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Am</td>
<td>Amsterdam, The Netherlands</td>
<td>Normal soil</td>
</tr>
<tr>
<td>Im</td>
<td>Imsbach, Germany</td>
<td>Cu mine spoil</td>
</tr>
<tr>
<td>Ma</td>
<td>Marsberg, Germany</td>
<td>Cu mine spoil</td>
</tr>
</tbody>
</table>

In II, the *F*₃ progeny of two *T. caerulescens* inter-accession crosses, selected for Zn accumulation capacity, were used in *MT* expression studies. The LC x LE crosses were described and characterized by Assunção *et al.* (2003b), and the LC x GA (=LM) crosses by Deniau *et al.* (2006). In the case of LC x LE crosses, the *F*₂ families, derived from a single self-pollinated *F*₁ plant, were characterized for Zn accumulation. In the case of LC x LM crosses, the *F*₂ plants were allowed to self-pollinate, and the *F*₃ plants were characterized. The high-accumulating lines contained over ten times more Zn than did the low-accumulating lines when grown at 10 µM ZnSO₄. Three low- and two high-, and five low- and five high-Zn accumulating *F*₃ families from the LC x LE and LC x LM crosses, respectively, were used for expression and allele analysis (II).

In III, commercially available (Metsämannut Oy, Finland) 2-year old clonal root cuttings of hybrid aspen (*Populus tremula x tremuloides*) were used to study metal accumulation and foliar *MT2b* expression in field conditions.

In (IV), *MT2b* expression was studied in Cu-tolerant and -sensitive *S. vulgaris*. Metallicolous populations of Cu-tolerant *Silene vulgaris* Imsbach and Marsberg originated from copper mines in Germany. The non-metallicolous Cu-sensitive population Amsterdam originated from a non-polluted site (The Netherlands) (IV) (Table 3). Expression analyses were made in *F*₃ and *F*₄ plants selected from crosses between Imsbach and Amsterdam populations. Thirty *F*₁ plants were pair-crossed, *F*₂ seeds were pooled and *F*₂ seedlings
Materials and Methods

were tested for Cu tolerance. Sensitive and tolerant seedlings were pair-crossed (sensitive x sensitive and tolerant x tolerant). Plants from nine sensitive and nine tolerant F3 lines were screened for tolerance and used for expression and allele analysis. F3 plants with different MT2 genotypes but equal tolerance levels were further pair-crossed. Plants of five F4 lines were screened for tolerance and five least tolerant and most tolerant plants were genotyped for MT2 alleles.

4.1.2 Plant growth conditions and sampling (I, II, IV)

To obtain root and shoot samples for molecular biological studies, T. caerulescens, A. thaliana and S. vulgaris seedlings were exposed to metals in hydroponics (I, II, IV). In I, six-week-old T. caerulescens seedlings from accessions LC and LE grown in soil were transferred to nutrient solution according to Shen et al. (1997; experiment 2, 0.2 µM ZnSO4). After four weeks, the plants were exposed to Zn (0.2, 25, 100 and 500 µM ZnSO4) for one week. For microarray (I) and in II, T. caerulescens cultivation was done as described by Assunção et al. (2003a), using modified Hoagland solution (Schat et al., 1996) which contained 2 µM ZnSO4. The 3-week-old LC and LE seedlings were exposed to 0, 2 (only in I), 10, 100 or 1000 (only LC in I) µM ZnSO4 for one (II) or two weeks (I) after one week of preculture in hydroponics. The experiments were performed in a climate chamber (20/15 °C day/night, 65 % relative humidity, 150 µmol m-2 s-2 light 12 h/day) and the solutions were changed twice a week. The hydroponics on Silene (IV) were performed as described by Schat et al. (1996). Copper tolerance was measured using a sequential test where the plants were exposed to increasing Cu concentrations at 2 days intervals. The lowest concentration which completely inhibited root growth (EC100) was taken as measure of tolerance.

After hydroponic exposures (I, II, IV), the roots and shoots were harvested separately, frozen in liquid nitrogen and stored at -80 °C until use.

For the analysis of metal uptake in the wild-type and transgenic Arabidopsis plants (II), similar growth conditions to those for T. caerulescens in II were used. Three-week old wild-type and T3 transgenic plants, including three independent MT2- and MT3-transformed lines, were transferred to modified half-strength Hoagland solution (Schat et al., 1996). After two weeks, the plants were transferred to similar nutrient solution (control, 2 µM ZnSO4) or to solution supplemented with 10, 25 or 100 µM ZnSO4 or with 1 or 10 µM CdSO4 for one week. The roots were desorbed in ice-cold 5 mM PbNO3 for one hour and rinsed with water. The roots and shoots were collected separately and dried at 65 °C for 40 h. For metal analysis, the shoots or roots from three plants were pooled, and two pools were analyzed.

For metal tolerance tests (II), the seeds from transgenic (T3) and wild-type Arabidopsis plants were surface-sterilized and sown on 0.5 x MS containing agar plates (control), or
plates supplemented with 300, 400 or 500 µM ZnSO\(_4\); 20, 30, 35 or 40 µM CuSO\(_4\); or 5, 10 or 15 µM CdSO\(_4\). Ten seeds were sown on each plate in three replicates, and three independent MT2-and MT3-transformed lines were analyzed. After two days at 4 °C, the plates were incubated at 22 °C with 16 h light/8 h dark at horizontal position for three days, and then turned into vertical position. The plates were photographed after 7 and 11 days of growth, and the root lengths were measured with the ImageJ software.

### 4.1.3 Field study: Hybrid aspens in contaminated area (III)

To study foliar metal accumulation and MT2 expression under field conditions, two-year old hybrid aspen trees (75 to 100 cm high) were directly planted on a metal-contaminated site at 1.5 m distances (III, Fig. 1). The experimental area (ca. 800 m\(^2\)), located in Helsinki, Finland, about 300 m from the Baltic Sea, has been contaminated with heavy metals and hydrocarbons during several decades. The sub-site where the hybrid aspen trees were planted was known to be contaminated mainly with heavy metals and have elevated Cd, Cu, Zn, Ni and V levels. The contamination has mainly arisen from the industrial use (from 1930’s until 1980’s), from landfills and from industrial and construction wastes dumped at the site. Moreover, wastewaters coming from an old dumping site and a railway yard have contaminated the sediment of the river running through the area. No fertilisation or irrigation was applied during the experiment.

Ten trees (250 to 335 cm of height) with distances between 6 and 30 m were randomly selected for the analyses. Samples were taken from the trees in mid-June 2004 and 2005. Leaf, branch and root samples were collected for metal analyses. The leaf samples (30 mature leaves per tree) were taken at the height of 100 to 150 cm. About half-way from the top a branch was cut and the basal 50 cm was taken for metal analyses. The root samples were taken from 5 to 20 mm thick lateral roots near the stem and washed with water. Three soil samples were taken from a depth of 20 to 40 cm at a distance of 20 cm from each tree, pooled and mixed, resulting in one soil sample per tree.

For MT2 expression analyses, five young leaves from each tree were collected, frozen in dry ice and stored at –80 °C until use.

As a reference, similar samples were taken from six aspen trees naturally growing in a non-polluted area about 200 to 400 m (two trees in 2004 and 2005) or 1500 m (two trees in 2005) from the experimental site.

### 4.1.4 Metal analyses

The dried plant samples (II, IV) were decomposed with nitric acid and microwave digestion. Plant Cd, Cu and Zn and yeast intracellular Cd concentrations (4.5.1) were
analyzed using a flame atomic absorption spectrophotometer (Perkin Elmer AAS 5100) (I, IV).

In the aspen field study (III), the plant samples were dried overnight at 55-60 °C, soil samples were dried at 40 °C and passed through a 2 mm mesh. The plant samples were decomposed with nitric acid. The sieved soil samples were decomposed with a 2:5 mixture of H₂O₂ (30%):HNO₃ in a microwave oven. Inductively coupled plasma atomic emission spectroscopy (ICP-OES, accredited SFS-EN ISO 11885: 1998, University of Jyväskylä, Finland) was used to determine aspen (root, stem and leaf) and soil Al, As, Cd, Cr, Cu, Pb, Fe, Ni, V and Zn concentrations, as well as soil Mn and Mo and plant P and Na content. Dry weights of the soil samples were analyzed separately by heating to 105 °C overnight, and the data were calculated on a dry weight basis.

4.2 RNA and DNA isolation

Total RNA was isolated from *T. caerulescens* or *S. vulgaris* (IV) shoots and roots or from *P. tremula x tremuloides* leaves using the RNeasy extraction kit (Qiagen) (I, II, III, IV). On-column DNase I digestion was done prior to cDNA synthesis (II, III). For DDRT-PCR (I), total RNA was isolated using the CTAB (cetyltrimethylammoniumbromide) method (Chang *et al.*, 1993).

For amplification of genomic sequences (II), DNA was isolated from *T. caerulescens* using the DNeasy plant mini kit (Qiagen).

4.3 Fluorescent DDRT-PCR

Differential display was used to find genes differentially expressed between Zn-exposed vs. non-exposed and/or between metallicolous and nonmetallicolous *T. caerulescens* accessions (I). In addition to arbitrary primers designed to amplify non pre-defined genes, two family-targeted primers, designed to amplify genes that have domains similar to those found in metallothioneins or in ZIP-type transporters, were used.

DNase-treated total RNA, isolated from the shoots of LC and LE grown at 0.2 µM (control) or 500 µM (excess) Zn, was reverse-transcribed using 12 oligo-dT primers (dT₁₈NM, where N = A, C or G; and M = A, C, G or T) into cDNA pools and amplified with the same oligo-dT primer in combination with four arbitrary and two family-targeted primers labelled with fluorochrome Cy5 (Amersham Biosciences). The metallothionein-targeted primer was designed based on the Cys-rich C-terminal domain of *MT2* genes, and the *ZIP*-targeted primer based on the trans-membrane domain VII of *ZIP*-family transporter genes (Grotz *et al.*, 1998). The DDRT-PCR program was: 4 min 94 °C; 4 cycles of (60 s 94 °C, 4 min 40 °C, 1 min 72 °C) followed by 25 cycles of (45 s 94 °C, 2 min 60 °C, 1 min 68 °C) and 6 min 72 °C.
An aliquot of the PCR sample was applied to automated sequencer (ALFexpress) and the data were analysed using fragment analyser (ALFwin, Amersham Biosciences). Whenever differences were found in the cDNA patterns, the PCR samples were subjected to manual run in 6 % polyacrylamide gel. The cDNA fragments of interest were excised from the gels, precipitated and amplified with PCR. After cloning (TOPO-TA, Invitrogen), the fragments were sequenced.

All PCR reactions in DDRT-PCR were carried out in duplicate. The absence of DNA was confirmed by including RNA without reverse transcriptase in the fluorescence differential display.

4.4 Isolation of full-length cDNAs and genes

Full-length cDNAs were isolated from *T. caerulescens* lambda cDNA library using as probes the cDNA fragments obtained from DD (I). The library was made from LC shoots at 10 μM ZnSO₄ by Assunção *et al.* (2001). *Silene MT* was isolated from a lambda library using Arabidopsis *MT2b* as a probe (IV).

The *MT2a* and *MT3* alleles from LM (Ganges) and LE were amplified (II) using primers designed on the basis of the LC sequence, which was obtained from the cDNA library. *TcMT2b* cDNA was isolated from the LC, LE and LM accessions using primers designed from *T. caerulescens* EST (DN923775) homologous to Arabidopsis *MT2b*.

The *T. caerulescens* sequences were deposited to GenBank: *Alpha-tubulin* (FJ439644), *MT2b-LM* genomic and cDNA (FJ439645 and FJ439646, respectively), *MT2b-LC* (FJ439648, FJ439647), *MT3-LM* (FJ439649, FJ439650), *MT3-LC* (FJ439651, FJ439655), *MT2a-LM* (FJ439652, FJ439653), *MT2a-LC* (FJ439654, FJ439656).

4.5 Transformation of yeast and plant

4.5.1 Yeast transformation and metal exposures

The full-length cDNAs of *T. caerulescens* and Arabidopsis *MT2a* and *MT3*, *T. caerulescens Ole e1* and *MRP10* (I) and *Silene MT2b* (IV) were cloned into the yeast shuttle vector pAJ401 (I, IV); for double transformation *Ole e1* was also cloned into pRS415 (I). The constructs were transferred into several *Saccharomyces cerevisiae* strains: the common strain DBY746; the Cd-sensitive *ycf1* mutant strain defective in vacuolar Cd transport; and the Cu-sensitive *cup1* mutant strain defective in yeast metallothionein. Transformation was done according to Gietz *et al.* (1992).
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Metal tolerance studies were performed either by streaking the yeasts directly on metal-containing plates (IV) or by applying metal-infiltrated discs on top of densely inoculated yeast cultures (I). In I, 100 µl of overnight yeast culture was mixed with top agar and poured on YPAD plates. Metal solutions (0.1 M CuSO₄ or 2 mM CdSO₄) were applied on filter discs placed on the surface of the top agar. After 1-day incubation at 30 ºC, the inhibition zones around the filter discs were measured.

The yeast intracellular Cd content (I) was analyzed after washing the cells with distilled water and EDTA (Bernard et al., 2004) after 24-h exposure to 5 µM CdSO₄.

4.5.2 Arabidopsis transformation

The TcMT2a-LC and TcMT3-LC cDNAs were cloned into the pCAMBIA2301 vector under the CaMV 35S promoter (II). Recombinant Agrobacterium tumefaciens colonies were selected on a medium containing kanamycin, rifampicin, and streptomycin. Arabidopsis thaliana (Col-0) was transformed via floral dip (Clough and Bent, 1998) and T₁ plants were selected with kanamycin in 0.5 MS medium. The presence of transgene was confirmed by PCR with vector-specific primers. Homozygous T₂ lines were further selected by segregation of kanamycin resistance.

4.6 MT alleles

The allelic distribution of TcMT genes was analyzed from five low- and five high-accumulating F₃ sibling lines from an LC x LM cross and from the parental accessions using PCR coupled to fluorescent fragment analysis (II). The PCR primers had a full identity to both alleles, but the sizes of the PCR products differed in the LC and LM alleles. DNA or cDNA, isolated from three pooled shoots was used as a template, and four replicate samples were analyzed.

TcMT2a allele fragments were amplified from cDNA using fluorescently (5' FAM) labelled reverse primer. The amplicon was designed from the 3' UTR region where the LM allele has a 3-bp insertion compared to LC, resulting in amplicon lengths of 118 and 115 bp for TcMT2a-LM and TcMT2a-LC, respectively. TcMT3 alleles were amplified from genomic DNA with a (5' FAM) labelled forward primer. The amplicon spans the first intron, which is 386 bp in the LM allele, being 16 bp longer than that in the LC allele.

After purification of the PCR products, the samples were applied to an automated sequencer (MegaBACE 750 DNA Analysis System, Amersham) along with the fluorescent size markers. The data were analyzed using fragment analysis software (Fragment Profiler, Amersham).
The *Silene* *MT* alleles (IV) were amplified using primers that amplify only the targeted allele, with amplicon lengths of 243 bp and 159 bp for Imsbach and Amsterdam alleles, respectively. The PCR was made using high annealing temperature (69 °C).

4.7 Gene expression analysis

4.7.1 Reverse northern and northern analysis

Reverse northern (macroarray) analysis was performed as described by Zhang *et al.* (1996) to study the expression of ca. 40 gene fragments isolated by differential display (I). The amplified cDNA fragments from five to ten individual clones originating from the same excised band were denatured and applied to duplicate nylon membranes by using Bio-Dot microfiltration apparatus (Bio-Rad Laboratories). Radioactively labelled cDNA probes were prepared from 10 µg of total RNA isolated from the shoots of LC and LE exposed to 0.2 µM and 500 µM Zn. Equal 32P counts of purified probes were used in the hybridizations. Two fragments that were expressed at equal intensities in all samples in the DD were used as controls to monitor equal labelling.

In the northern analysis, 20 µg of total RNA were used (I). The RNA samples were size-separated and hybridized according to standard procedures using 32P-dCTP labelled cDNA fragments as probes.

4.7.2 Quantitative real-time PCR

Quantitative real-time PCR was used to study *MT2a*, *MT2b* and *MT3* expression in *T. caerulescens* accessions (II) and *MT2b* expression in hybrid aspen (III) as it is more reliable than the hybridization-based methods, such as northern analysis, especially for gene families with many members having homologous DNA segments. The QRT-PCR primers for *T. caerulescens* *MTs* were designed to have a full sequence identity to all the accessions studied.

Alpha-tubulin was chosen as a reference gene in *T. caerulescens* and in hybrid aspen; according to Brunner *et al.* (2004) it is one of the most stably expressed housekeeping genes in *Populus* and thus suitable as a single internal control gene in QRT-PCR. Alpha-tubulin levels were found to be rather constant in *T. caerulescens* samples with equal amounts of starting material, and the gene was thus proven to be a good choice for a reference gene. Alpha-tubulin gene was also used as a control when expression of selected genes was studied in *T. caerulescens* after microarray analysis (van de Mortel *et al*., 2006).

In II and III, *T. caerulescens* or *P. tremula x tremuloides* cDNA was synthesized using oligo(dT) primer from 0.5 to 1 µg of total RNA with DyNAmo 2 step SYBR Green qPCR kit
Materials and Methods

Quantitative real-time PCR reactions were made using the Dynamo HS SYBR Green kit (Finnzymes) in a 20 μl reaction volume with 0.5 μM of gene-specific primers and 2 μl of diluted cDNA, corresponding to 1.25 to 2.5 ng of total RNA, as a template.

*T. caerulescens* (II) or *P. tremula x tremuloides* (III) alpha-tubulin was used as a reference gene. The amplicon lengths for *T. caerulescens* were (II): 297 bp for alpha-tubulin, 115 bp for MT2a, 194 bp for MT2b and 121 bp for MT3, and for *P. tremula x tremuloides* (III) 216 bp and 192 bp for alpha-tubulin and MT2b, respectively. The *T. caerulescens* (II) TcMT2a amplicon was from the 3’ UTR region, while the TcMT2b and TcMT3 primers spanned an intron. All primers had full identity to both LM and LC alleles. In III, the *P. tremula x tremuloides* alpha-tubulin primers spanned an intron of 93 bp. The MT2 primers were from the translated region.

The reactions were made in an iCycler iQ Real-time PCR (Bio-Rad) in triplicate. The PCR reactions were (II, III): 95 °C 15 min, 35 cycles (95 °C 15 s, 58 °C 20 s, 72 °C 20 s), 72 °C 5 min. After redenaturation (95 °C 1 min), a melt curve analysis was done by cooling from 95 to 60 °C at 0.5 °C intervals. The fold-change in gene expression was calculated using the comparative Ct method (2^(-ΔΔCt)) (Livak and Schmittgen, 2001).

4.7.3 Microarray

The cDNA fragments isolated here using DD were included in a microarray experiment together with other cDNAs isolated from *T. caerulescens*. For microarray analysis (I), a different set of Zn-exposed LC and LE plants was used. The growth conditions were as described by Assunção et al. (2003a). RNA extraction, microarray design and analysis are described by Plessl et al. (2005). The means of four separate experiments derived from a homogenized pool of 9 to 11 plants per treatment and accession were calculated.

4.8 Development of antibodies and immunofluorescence staining

Whole-mount immunofluorescence staining was used to localize MT2 protein in *T. caerulescens* and in Arabidopsis roots (II). Antibodies against MT2 were made using two synthetic peptides (GGCKRNPDGLGYSGE and VLGVAPAMKNQYEASGE), designed for the spacer region between *T. caerulescens* TcMT2a-LC Cys-rich domains. The rabbit was immunized with 100 μg of both peptides three times in 4-week intervals. The antibodies were collected from the serum 6 weeks after the last injection. The antibodies were purified by affinity chromatography and tested with ELISA.

Four day-old Arabidopsis and seven day-old *T. caerulescens* seedlings grown in sterile conditions in Petri dishes were fixed in 4 % paraformaldehyde followed by digestion with cellulase and macerozyme. Before incubation with the primary antibody, the samples...
were blocked with Image-iT Signal Enhancer (Invitrogen) and with blocking solution (2 % milk powder, 0.5 % bovine serum albumin). The plants were incubated with the 1:50 diluted, affinity-purified MT2 antibody followed by a secondary antibody (goat anti-rabbit IgG Alexa Fluor 488, Invitrogen). After counterstaining with propidium iodine, the seedlings were analyzed with a confocal laser scanning microscope Eclipse-TE300 (Nikon Corporation, Japan)/Ultra VIEW (Perkin-Elmer, UK). As a negative control, purified MT2 antibody pre-adsorbed on the peptides used for immunization was included in each experiment. The images were processed using ImageJ software (Abramoff et al., 2004).
5 RESULTS AND DISCUSSION

5.1 Differentially expressed genes and hyperaccumulation

The mechanisms of plant metal hyperaccumulation are still partly unresolved despite an increasing number of publications during the past few years. Cross-species microarrays have been performed using Arabidopsis chips for \textit{T. caerulescens} (van de Mortel \textit{et al.}, 2006, 2008) and for \textit{A. halleri} (Becher \textit{et al.}, 2004; Weber \textit{et al.}, 2004), with hundreds of putative candidate genes more highly expressed in the metal hyperaccumulators. The rationale behind these studies is that the genes differentially expressed in the hyperaccumulators compared to non-accumulators and/or in metal exposure might play a role in hyperaccumulation. A difficulty is that the genes differentially expressed between different species might reflect differences other than those linked to metal hyperaccumulation. Moreover, there is a possibility that important genes with low sequence identity will not be detected.

In order to isolate genes that are specific for \textit{T. caerulescens} and are regulated by the hyperaccumulating metal, differential display was used in this Thesis to isolate Zn-responsive genes from the shoots of two \textit{T. caerulescens} accessions with differential Zn tolerance and uptake characteristics.

5.2 Differential display in the isolation of differentially expressed genes

Differential display (DD) is a non-targeted PCR-based method for the comparison of differential gene expression. This method was chosen as several samples can be compared simultaneously and it has the potential to reveal novel genes without prior sequence information of the organisms under study.

The DD method was first published in 1992 and used for the fast and accurate detection of altered gene expression (Liang and Pardee, 1992). In this Thesis, two major modifications were made: first, fluorescent primers were used together with fragment analysis, which improved the throughput and efficacy; second, family-targeted primers were used in addition to arbitrary primers. In DD, total cDNA is amplified with arbitrary and poly-T primers to produce cDNA pools that are separated on polyacrylamide gel, and the fragment patterns are analyzed visually. However, this is very labour-intensive if several samples are compared simultaneously. In this Thesis, the fluorescently-labelled PCR products were applied to capillary electrophoresis used in the sequencing facility. The data were analyzed using fragment analyzer programme, and only the samples that showed difference in expression pattern were run on manual acrylamide gels, from which the PCR fragments were isolated, purified and eventually sequenced (Fig. 6).
Differential display has some limitations. Being a PCR-based method, it can generate errors. To minimize false positive results, the PCR reactions were made in duplicate; the difference in fragment patterns was considered significant only when present in both samples. A reaction without reverse transcriptase was included in the analysis to confirm that the samples were free from contaminating DNA. The differential expression of the isolated fragments was verified using reverse northern blotting (macroarray).

Another limitation in DD is the high number of primer pairs required for near-complete sequence coverage, which varies from tens to hundreds; the complete sequence coverage may not even be possible with this method (Lievens et al., 2001). Thus fragments isolated in the present study represent only a limited fraction of the differentially expressed genes. However, the use of family-targeted primers may have enhanced the probability of finding relevant genes. The MT primer (named MtU) was designed from the Cys-rich domain of Arabidopsis MT2 sequence and the ZIP family primer (named ZnU) from the conservative trans-membrane domain VII of ZIP-type transporters. These families were chosen since metallothioneins are metal-regulated genes which may have a role in hyperaccumulation as metal-binding proteins. The ZIP-family transporters such as the TcZNT1 are important in root metal uptake, but may have a role in shoot metal transport.
as their expression is constitutively higher also in the shoots of *T. caerulescens* compared with the non-hyperaccumulator (Pence *et al.*, 2000; Assunção *et al.*, 2001). Moreover, as the annealing temperatures in DD are low, the family-targeted primers might amplify sequences with some similarity to the metal-binding domain of MTs or the transmembrane domain of the ZIP transporters. For example, *MT3* was isolated using *MT*-targeted primer and an *MRP* transporter using *ZIP*-transporter-targeted primer (I) (Table 4).

Presently, DD is not widely used due to the increased availability of sequence data and microarray facilities. However, DD has been successfully used in many experiments, for example in the identification of Cd-responsive genes from Arabidopsis (Suzuki *et al.*, 2001) and, more recently, of differentially expressed genes in cotton anthers (Zhang *et al.*, 2008) and of oxygen-responsive transcripts in orange fruit tissues (Pasetsis *et al.*, 2007). Moreover, as demonstrated by Mandaokar *et al.* (2003), additional differentially expressed genes were found with the PCR-based DD method compared to a hybridization-based microarray. Thus, even though DD or related methods such as cDNA-AFLP (Vuylsteke *et al.*, 2006) cannot compete with genome-wide transcription analysis like microarray, they are still valuable methods, and especially suitable for targeted analysis of a species for which sequence data are scarce.

### 5.3 Zn-responsive genes isolated from *T. caerulescens*

Forty differentially expressed cDNA fragments were isolated from *T. caerulescens* when shoot mRNAs of metallicolous LC and non-metallicolous LE were compared with and without Zn exposure (I). Of these, 16 cDNA clones were successively confirmed to be differentially expressed by reverse northern blot or northern blot (Table 4). Nine of the clones had the highest expression in, and were isolated from the accession LE, (seven from 0.2 μM Zn and two from the 500 μM Zn treatment) and seven from the accession LC (two from 0.2 μM Zn and five from 500 μM Zn treatment). The isolated genes showed differences in expression between the Zn treatments within the accessions, but some fragments also showed differential expression between the accessions in either of the Zn treatments. For some fragments, expression patterns with exactly the same size were not found in the other accession (marked as ND in Table 4), which could be due to sequence differences between the accessions in the 3’ UTR region.
Table 4. Genes isolated with differential display using arbitrary (A) or family-targeted primers (ZIP-family targeted ZnU or metallothionein targeted MtU). Relative expression levels in DD in Zn-exposed (Zn) and non-exposed (control, C) shoots of *T. caerulescens* accessions LE and LC are indicated by signs (- to ++, reflecting increasing expression from very low to highest observed level) and shading. ND: expression not detected.

<table>
<thead>
<tr>
<th>Best homology</th>
<th>Primers in DD</th>
<th>Expression in DD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LE C</td>
<td>LE Zn</td>
</tr>
<tr>
<td><strong>Isolated from Zn-exposed LC</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No homology found (fragment no. 2)</td>
<td>A</td>
<td>+</td>
</tr>
<tr>
<td><em>AtMT2a</em></td>
<td>A</td>
<td>ND</td>
</tr>
<tr>
<td>Pectinesterase</td>
<td>ZnU</td>
<td>-</td>
</tr>
<tr>
<td><em>AtMT3</em></td>
<td>MtU</td>
<td>ND</td>
</tr>
<tr>
<td><em>AtMRP10</em></td>
<td>ZnU</td>
<td>ND</td>
</tr>
<tr>
<td><strong>Isolated from Zn-exposed LE</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>A. thaliana</em> chloroplast</td>
<td>MtU</td>
<td>+</td>
</tr>
<tr>
<td><em>A. thaliana</em> expressed protein</td>
<td>ZnU</td>
<td>-</td>
</tr>
<tr>
<td><strong>Isolated from non-exposed LC</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Regulator of chromosome condensation (RCC1) family protein</td>
<td>A</td>
<td>-</td>
</tr>
<tr>
<td><em>A. thaliana</em> expressed protein</td>
<td>A</td>
<td>++</td>
</tr>
<tr>
<td><strong>Isolated from non-exposed LE</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ole e1 allergen and extensin family</td>
<td>A</td>
<td>+</td>
</tr>
<tr>
<td>Respiratory burst oxidase protein D</td>
<td>A</td>
<td>+</td>
</tr>
<tr>
<td>Nuclear RNA binding protein (RGGA)</td>
<td>ZnU</td>
<td>++</td>
</tr>
<tr>
<td><em>A. thaliana</em> hypothetical gene</td>
<td>ZnU</td>
<td>++</td>
</tr>
<tr>
<td>ATP-dependent Clp protease subunit</td>
<td>A</td>
<td>+</td>
</tr>
<tr>
<td>No homology found (fragment no. 34)</td>
<td>ZnU</td>
<td>+</td>
</tr>
<tr>
<td>No homology found (fragment no. 37)</td>
<td>ZnU</td>
<td>++</td>
</tr>
</tbody>
</table>

The Zn concentrations in this study were 0.2 µM (control) or 500 µM Zn (Zn-exposed) (Shen *et al.*, 1997). Basal level of 2 µM Zn has been used more recently for *T. caerulescens* (Assunção *et al.*, 2001, Rigola *et al.*, 2006), and was used also in I and II. As the accession LE is more sensitive to Zn than is the accession LC, the high Zn exposure may have posed a bigger stress to the non-metallicolous accession LE (Assunção *et al.*, 2003). However, no Zn toxicity or deficiency symptoms, such as chlorosis or curling of the leaves (Talukdar and Aarts, 2007) were observed.

Even though several genes were isolated using the ZIP family-targeted primer, including *MRP* transporter, there were several mismatches with the primer and the sequence. No ZIP-type domain was evident among the isolated genes. In contrast, the MT family-targeted primer amplified the *MT3* gene with full sequence identity. *MT2* was isolated with MT family-targeted primers with one bp mismatch but it was also isolated with
arbitrary primers. Thus the MT3 was the only gene isolated solely with the family-targeted primers having the desired domain.

Among the isolated fragments were genes without sequence similarity with known plant sequences and genes without known function. Also genes possibly related to oxidative stress response (Respiratory burst oxidase protein D, Clp protease) and gene regulation (Regulator of chromosome condensation, Nuclear RNA binding protein) were found.

A subset of the isolated genes were studied further, including Ole e1, MRP, PME, MT2 and MT3 genes, and are discussed in more detail below.

5.3.1 Ole e1-like gene

One of the fragments isolated was homologous to Arabidopsis Ole e1-like gene. The protein is known to consist of two domains, i.e. the Ole e1 allergen (extensin-family of proteins) and short N-terminal TonB signature. The bacterial TonB-dependent receptor proteins are involved in the energized transport of siderophore-metal complexes as well as other large complexes through the membranes (Postle and Kadner, 2003). The TonB system has not been characterized in plants.

The T. caerulescens Ole e1-like gene had the highest expression under Zn-deficiency in LE. This was observed both in DD (shoots) and in microarray (roots) (I). Based on northern analysis, the Ole e1-like gene was induced by excess Zn in LC shoots, but no induction with Zn was evident in the accessions LC, LE and LM using the more specific QRT-PCR method (Hassinen et al., unpublished). This shows that T. caerulescens Ole e1 is not primarily Zn-regulated. However, differential expression between the accessions was evident with ca. two-fold higher expression in LM shoots compared to LE shoots. Interestingly, this gene was expressed over eight times more in the shoots of compost- or agar-grown T. caerulescens compared to the non-accumulator T. arvense (Hammond et al., 2006).

Interestingly, when expressed in yeast, TcOle e1 enhanced the toxic effects of Cu and, even more pronounced, of those of Cd (I). However, the yeast Cd content was not altered significantly compared to the control yeasts, suggesting that T. caerulescens Ole e1 is not directly involved in Cd uptake or intracellular binding, and that the enhanced toxicity was not due to higher Cd concentrations inside the yeast cell.

When TcOle e1 was introduced into Arabidopsis, the overexpressor plants showed increased Zn tolerance, but not Cd or Cu tolerance, compared to the wild-type plants (Hassinen et al., unpublished). The different effect of the gene when introduced into yeast or Arabidopsis may be due to different localization of the protein, different folding or lack of interaction partners in the yeast. Currently, Arabidopsis RNAi knock-down
plants as well as plants with Arabidopsis Ole e 1 promoter-GFP constructs are underway. The latter one should reveal the localization of expression of the gene, which could provide clues of its function.

5.3.2 MRP transporter

An MRP transporter was isolated from LC that had a high homology to Arabidopsis MRP10 and MRP4, and was named TcMRP10. TcMRP10 was able to complement Cd sensitivity in the ycf1 mutant yeast strain, at least to some extent (I). The TcMRP10 was up-regulated by high Zn concentrations in the roots and shoots of both LC and LE, with higher expression in the more Zn-tolerant LC.

The MRP transporters belong to a large family of ABC transporters, which have been implicated in Cd tolerance as possible transporters of Cd-phytochelatin complexes into the vacuole (Kim et al., 2006, 2007). Some MRP transporters, including AtMRP4, are able to complement Cd sensitivity in a mutant yeast strain defective in Cd detoxification (Tommasini et al., 1998), and are also Cd-inducible in Arabidopsis (Bovet et al., 2003). We have also isolated MRP4 from Cu-tolerant birch (Keinänen et al., 2007), with stronger up-regulation by Cu in the roots and shoots of the Cu-tolerant birch clone compared to a more sensitive one. In T. caerulescens, MRP4 was found to be expressed at higher levels than in Arabidopsis (van de Mortel et al., 2008). Thus the MRP transporter isolated was chosen for further studies.

In subsequent studies, alterations in Cd or Zn tolerance or uptake in three Arabidopsis AtMRP4 (At2g47800) or AtMRP10 (At3g62700) T-DNA knock-out lines were not observed (H Schat, pers. com.). Recent studies have suggested that AtMRP4 is localized prefentially in the plasma membrane and appears to be associated with the regulation of guard cell function (Klein et al., 2004). As the MRPs transport a wide variety of substrates, the role of TcMRP10 in T. caerulescens remains elusive.

5.3.3 Pectin methylesterase

Pectin methylesterase-encoding gene (PME) was isolated that showed highest expression in the Zn-exposed calaminous accession LC.

PMEs demethylate cell wall pectins, revealing free carboxyl groups which can bind cations. The enzymes have many distinct functions throughout the plant life cycle (for a recent review see Pelloux et al., 2007). In Arabidopsis, overexpression of PME resulted in a higher Al content (Schmohl et al., 2000). In a cross-species microarray, PME was ca. 30 times more highly expressed in T. caerulescens roots compared to Arabidopsis.
irrespective of the Zn supply level (van de Mortel et al., 2006), implying that increased PME expression is important for *T. caerulescens*.

A fraction of the accumulated metals is known to be stored in the apoplast in *T. caerulescens* (Frey et al., 2000), but the processes involved in apoplastic metal binding are currently not known. The cell wall components, including hydroxyl and carboxyl groups of cellulose, hemicellulose and pectin may provide ligands for metal detoxification (Küpper et al., 2004). Cd has been shown to alter the distribution of acidic and esterified pectins, which was suggested to be due to PME activity (Douchiche et al., 2007).

To further study the possible role of PME in metal accumulation, we characterized four Arabidopsis loss-of-function mutant lines where the Arabidopsis homologue of the *PME* (At3g59010) was inactivated by an exon-located T-DNA element. However, the role of PME in metal accumulation could not be confirmed in Arabidopsis as the tolerance to Zn, Cd or Al was not altered based on root length measurements (Korhonen, 2007; Hassinen et al., unpublished). Neither did the shoot metal concentrations differ from those in the wild-type plants. However, as *PME* gene belongs to a multi-gene family with ca. 65 members in Arabidopsis genome (Micheli, 2001), the inactivation of a single gene may not be sufficient to give rise to the expected phenotype. Alternatively, the effect may be highly localized and thus not observed with the rather insensitive methods used.

5.3.4 Metallothioneins

In this study, two metallothioneins, *i.e.* *MT2a* and *MT3*, were isolated from Zn-exposed LC. Finding of some *MT* sequences was not unexpected, as *MT* family-targeted primers were used in DD. Moreover, *MT* sequences are quite abundant in plants and the genes have been isolated in various expression screening studies (Bhalerao et al., 2003; Wu et al., 2005; Xue et al., 2008). In a *T. caerulescens* EST collection, *MT1c* was the most abundant form with 18 ESTs, followed by 3 ESTs for *MT2a* and *MT3*, and one for *MT2b* EST (Rigola et al., 2006). No *MT1* type sequences were isolated in this study.

*MTs* have been implicated in metal detoxification as their expression is metal-inducible (Zhou and Goldsborough, 1994; Murphy and Taiz, 1995; Guo et al., 2003). Moreover, plant *MTs* of type 2 and 3 have been shown to increase Cd and Cu tolerance in yeast (Zhou and Goldsborough, 1994; van Hoof et al., 2001a; Roosens et al., 2004, 2005; Hassinen et al., 2007) and in plants when overexpressed (Lee et al., 2004; Zhigang et al., 2006).

In this Thesis, the roles for metallothioneins were studied in more detail in the hyperaccumulator *T. caerulescens* (II); in field-grown hybrid aspen (III); and in *Silene vulgaris* (IV), and are further discussed in the next Section.
5.4 Metallothioneins

5.4.1 MT sequences are different among T. caerulescens accessions

The full-length MT2a-LC and MT3-LC cDNAs were isolated from a T. caerulescens cDNA library (accession LC), using the DD fragments as probes (I). The MT2b sequence was searched from an EST database. A T. caerulescens EST with a high similarity to Arabidopsis MT2b was found and used to design primers for T. caerulescens MT2b. The TcMT2a, TcMT2b and TcMT3 cDNAs and genes were sequenced from the LE, LC, and LM accessions (II).

The TcMT2a, TcMT2b and TcMT3 genes from the LE, LC, and LM accessions were sequenced (II). Differences were found to the genes previously isolated from a different accession (Roosens et al., 2004, 2005). The isolation of MT2a and MT2b cDNAs from the same accessions indicates that there are two translated MT2 isoforms in T. caerulescens in agreement with the findings in A. thaliana. As TcMT2b-LC has about 88% and 83% identity with Arabidopsis AtMT2b and AtMT2a, respectively, it is considered to be an MT2b. T. caerulescens MT2a and MT2b show higher sequence identity with each other than do the Arabidopsis type 2a and 2b genes.

The MT sequences from the accessions from the Ganges region, i.e. FP (Roosens et al., 2004, 2005) and LM (II), show some differences from those isolated from other T. caerulescens accessions (II, Fig. 1). The TcMT2a-LM has two amino acid difference compared to accessions LC and LE (II). The TcMT2-FP (Roosens et al., 2005) is a type 2b sequence and is identical to TcMT2b-LC, differing by one amino acid from TcMT2b-LM. To our knowledge, no type 2a sequence has been isolated from FP. The amino acid substitutions in the MT2 proteins do not affect the Cys arrangement which is suggested to be critical in metal binding. The MT3 proteins of the two accessions from the Ganges region, TcMT3-LM (II) and TcMT3-FP (Roosens et al., 2004) differ by four amino acids, two of them being Cys-residues, from the sequences of the accessions LC and LE analyzed in II.

The MT3 Cys arrangement found in the two accessions from Ganges regions (FP and LM) may change the metal-binding properties of this protein compared to the one with Cys arrangement found in LC and in LE. When expressed in yeast, TcMT3-FP increased tolerance to Cu, but not to Cd or Zn, to a higher degree than did Arabidopsis MT3 (Roosens et al., 2004). On the other hand, TcMT3-LC and AtMT3 increased Cu and Cd tolerance to similar levels (I). This suggests that TcMT3-FP protein (and hence TcMT3-LM due to identical amino acid sequence) has an increased capacity to bind Cu compared to AtMT3 or TcMT3-LC. This may result in differences in MT3 function and, as suggested by Roosens et al. (2004), may reflect an increased pressure on Cu homeostasis owing to the extreme rates of Cd accumulation in these accessions.
5.4.2 MT expression is highest in metallicolous accessions

The MT2a expression levels varied between the accessions, with higher expression in the shoots and in the roots of the calaminous accession LC compared to the non-metallicolous LE at all Zn levels, according to northern and microarray analyses (I). This higher expression was also evident in QRT-PCR analysis. The highest expression was seen in the shoots of the calaminous, superior-accumulating accession LM, with about 8 and 4 times higher expression at 10 µM Zn compared to LE and LC, respectively (II). Interestingly, about two-fold higher MT2a expression has been found in the leaves of T. caerulescens compared to the related non-accumulator T. arvense (Hammond et al., 2006). Higher MT2a, MT2b and MT3 expression was also seen in the hyperaccumulator A. halleri seedlings compared to A. thaliana (Chiang et al., 2006). In the roots, MT2a expression was about 30-fold and about 80-fold higher in T. caerulescens than in Arabidopsis, under conditions of Zn deficiency/sufficiency and under excess Zn, respectively (van de Mortel et al., 2006). Higher expression in the hyperaccumulators compared to non-accumulators and in the superior-accumulating T. caerulescens accession suggests that MT2a may contribute to the accumulation trait.

In northern and microarray analyses, MT2a expression turned out to be Zn-inducible in LC shoots and in LE roots (I). A two-fold TcMT2a induction in the shoots by 10 or 50 µM Zn was also reported by Roosens et al. (2005) in northern analysis for the superior-accumulating FP accession originating from the Ganges region. However, the more specific QRT-PCR analysis showed that MT2a expression was not Zn-responsive in the shoots or in the roots of the three parental accessions LC, LE or LM, nor in the F3 sibling lines at 0, 10, 100 or 1000 µM Zn (II). These results indicate that MT2a expression is not only slightly influenced by Zn levels in T. caerulescens.

Even though the MT3 transcript levels were higher in the LC and LE shoots at elevated Zn exposures when studied by northern analysis (I), no response to Zn could be detected in the QRT-PCR (II), indicating that MT3 expression is not primarily affected by Zn either. The expression levels were higher in the shoots than in the roots (II). Remarkably, QRT-PCR revealed that MT3 expression was ca. ten times higher in the shoots of superior-accumulating LM than those in LC or LE at all Zn concentrations used (II). In the roots, the MT3 expression levels were similar in all accessions studied (II), suggesting that higher MT3 expression might be involved in the superior foliar accumulation capacity observed in the LM accession. Approximately six times higher MT3 expression has been previously observed in the shoots of the calamine FP accession from the Ganges region, as compared to a calamine accession from another region (‘Prayon’), and a serpentine accession (‘Puente Basadre’, PB) both under control conditions and under Cd exposure (Roosens et al., 2004). Interestingly, Cu exposure strongly induced MT3 expression in Prayon and PB, up to the constitutive level observed in the FP accession (Roosens et al., 2004), indicating that MT3 expression in these superior-accumulating accessions is at higher levels in the
shoots irrespective of Zn or Cu levels. About 2-fold MT3 expression in the shoots was also shown for *T. caerulescens* compared to the non-accumulator *T. arvense* (Hammond et al., 2006).

The expression of MT2b was also studied using QRT-PCR (II). Like MT2a and MT3, the highest expression was seen in the shoots of the accession LM; however, differences in the expression levels between the accessions were small. The expression levels of MT2b were rather constant at various Zn exposure levels, especially in LC and LE.

In the non-hyperaccumulator metallophyte, *Silene vulgaris*, the highest SvMT2b expression was seen in the Cu-tolerant metallicolous populations. Much higher expression was seen in both roots and shoots of the tolerant populations Lmsbach and Marsberg than in those from copper-sensitive Amsterdam, with and without Cu exposure, as evidenced by northern analysis and quantitative PCR (IV). Similarly, the *T. caerulescens* accessions showed variation in the TcMT2a, TcMT2b and TcMT3 expression levels, with the lowest expression in the accession LE from an uncontaminated site and the highest expression in the shoots of the superior-accumulating calamine accession LM from the Ganges region (II). This was especially clear for TcMT3, which had about a ten-fold higher expression in LM shoots compared to the shoots of the other accessions.

In *Silene*, the higher MT2b expression in Cu-tolerant plants may be due to amplification of the MT2b gene (IV). In *T. caerulescens*, no evidence for difference in TcMT2a copy number between the accessions LM and LC was obtained with Southern analysis (data not shown). This should be tested with TcMT3 as well. Higher MT expression might be due to differential regulation of the MT genes; however, the promoter regions of *T. caerulescens* MTs have not been studied so far.

### 5.4.3 MT expression in intraspecific crosses of *Thlaspi* and *Silene*

Besides studying plants that ectopically express the gene of interest, segregation of the gene in intraspecific crosses with contrasting characteristics is a valuable tool to study the importance of a given gene in a given trait.

The expression of TcMT2a and TcMT3 was analyzed in intraspecific crosses of *T. caerulescens* segregating for Zn accumulation (II). The expression was studied in five low and five high Zn accumulating F3 families originating from an LC x LM cross. Only MT2a expression was studied in families originating from an LC x LE cross, as MT3 expression is rather similar in these two accessions. On the other hand, expression analysis of MT3 in the LC x LM crosses was particularly meaningful, since the expression of MT3 varied markedly between these accessions, being more than ten-fold higher in the shoots of the superior-accumulating accession LM from Ganges region compared to the low-accumulating accession LC. The difference in MT2a expression in the parents was less
Results and Discussion

pronounced, being still more than two-fold higher in LM compared to the other accessions.

Despite the large differences in MT3 expression between the parental accessions, the differences in the expression levels in the F3 lines were less pronounced. MT3 or MT2a expression did not co-segregate with Zn accumulation capacity in the LC x LM cross, i.e. the high-Zn-accumulating lines did not have higher expression compared to the low-accumulating ones.

As the MT alleles in LM and LC differ, it was possible to establish the allele origins of the plants of the F3 lines of the LC x LM cross. The LM allele of MT2a has a 3 bp insertion in the 3’ UTR, whereas MT3-LM has 16 bp insertion in the first intron. These differences were used to differentiate between the lengths of the PCR products using capillary sequencing instrument and fragment analysis.

When the alleles of 12 plants per accession or F3 line were studied, the parental accessions contained exclusively one allele (i.e., were homozygous for the gene), whereas the F3 plants had either both alleles or only one of the alleles. Five F3 lines contained exclusively the MT2a-LM allele (three high- and two low-accumulating lines), two lines contained exclusively the MT2a-LC allele (both low-accumulating lines), and three lines contained both alleles (one low- and two high-accumulating lines). For MT3, four lines contained exclusively the MT3-LM allele (two high- and two low-accumulating lines), and the remaining lines contained both alleles (three high- and three low-accumulating lines).

The high MT2a and MT3 expression in the shoots of F3 lines was clearly associated with the LM allele, with lines containing LM MT sequence having higher expression levels, on average. This may imply differences in cis-regulation of the genes.

Like in T. caerulescens, in S. vulgaris higher SvMT2b expression was observed in the metalloicous accession from Imsbach than in the non-metalloicous accession from Amsterdam (IV). In a cross between Cu-tolerant and Cu-sensitive plants, the F3 sibling lines did not show cosegregation of MT2b expression and Cu tolerance, as high MT2b expression was distributed evenly among the Cu-sensitive and Cu-tolerant F3 lines. However, when F3 plants of equal tolerance, approximately intermediate between the parent levels, but with different MT2 genotypes, were further pair-crossed, the SvMT2b expression correlated with the Cu tolerance in the F4 progenies, with higher expression in the more Cu-tolerant lines. The results imply that SvMT2b is not the primary gene conferring Cu tolerance but is rather a modifier gene influencing the Cu tolerance level of already tolerant plants. Like in T. caerulescens crosses, the expression of the gene depended on allele origin, with high expression strictly associated with the Imsbach allele.
5.4.4 MT2b expression in hybrid aspen correlates with foliar Cd and Zn levels

MT2b expression was analyzed using QRT-PCR from the leaves of hybrid aspen (P. tremula x tremuloides) grown in a contaminated area to establish any correlation between MT2b expression and the metal content in the soil and/or in the leaves of the plants. MT2 was chosen for this study, because type 2 MTs are known to respond to metal excess. For example, MT2 inducibility by Zn and, to a lesser extent, by Cd and Cu has been shown in hybrid poplar P. trichocarpa x deltoides (Kohler et al., 2004). To our knowledge, correlation of MT2 expression levels with metal concentrations in field-grown trees has not been previously reported.

The highest MT2b expression was seen in the leaves of aspen trees grown in the most contaminated site, with about 3-fold higher expression compared to that in the tree grown in the least contaminated spot (III). The trees growing in the most contaminated spot had also the highest foliar Cd and Zn content among all the trees under study. Overall, the foliar MT2b expression levels of all the field-grown hybrid aspens correlated with the foliar Cd and Zn concentrations, and also roughly correlated with the soil Cd, Zn and Cu contamination levels.

It is not possible to conclude with certainty which metals were responsible for the increased expression of MT2b at this site with multiple metal contaminants. Thus it cannot be concluded that the foliar Cd or Zn concentrations alone, even though correlated with the MT2b expression, were responsible for the increased MT expression at the most contaminated site. It is possible that the other metals present in the soil, their combined effect or yet other unknown factors contributed to the expression levels. However, the results suggest that the increased expression of MT2b is one of the means for the acclimation of hybrid aspen to chronic metal exposure.

5.4.5 Effect of MT overexpression on metal tolerance in yeast and Arabidopsis

The TcMT2a, TcMT3 and SvMT2b were transferred into yeasts to study their effects on metal tolerance (I, IV) or, in case of TcMT2a and TcMT3 genes, on intracellular Cd content (I).

All genes were able to increase Cd and Cu tolerance of the yeast, as anticipated. The capacity of TcMT2a-LC and TcMT3-LC to increase tolerance did not differ from that of Arabidopsis MTs. The capacity of plant MTs to increase yeast tolerance to metals, especially Cd and Cu, has been reported for example for AtMT2a (Zhou and Goldsborough, 1994), and Cd tolerance by AtMT2a and AtMT3 (Lee et al., 2004). More recently, all expressed MTs of Arabidopsis, i.e. AtMT1a, AtMT2a, AtMT2b, AtMT3, AtMT4a and AtMT4b, were able to increase Cu tolerance and to some extent also Zn tolerance, and all
Results and Discussion

MTs except for AtMT4a increased Cd tolerance (Guo et al., 2008). This has been shown also for T. caerulescens MTs; TcMT1a, TcMT2 and TcMT3 were able to complement Cd and Cu sensitivity (Roosens et al., 2004, 2005). Additionally, TcMT1a and TcMT2 are able to complement Zn sensitivity (Roosens et al., 2005).

The intracellular Cd content of TcMT2a- and TcMT3-transformed yeast was higher than that in the control yeast, implying that MTs were able to bind metals, resulting in increased tolerance and Cd content. Again, T. caerulescens and Arabidopsis MTs did not differ in their capacity to increase Cd concentration.

These results indicate that SvMT2b, TcMT2a and TcMT3 are functional proteins capable of conferring Cd and Zn tolerance in yeast and, moreover, may bind Cd, as the intracellular Cd content of TcMT-expressing yeast was higher than in the control yeast. However, as their ability to increase tolerance did not differ from that of Arabidopsis MTs, T. caerulescens MT2a-LC and MT3-LC do not seem to have an increased capacity to bind metals compared to AtMTs. This seems not to be the case for TcMT3-FP, which increases Cu tolerance, but not Cd or Zn tolerance, more than AtMT3 does, possibly due to differences in the Cys arrangement of the C-terminal metal-binding domain (Roosens et al., 2004).

T. caerulescens MT2a-LC and MT3-LC were not able to increase tolerance to Cd, Cu or Zn when overexpressed in Arabidopsis under 35S promoter, as judged from root length measurements. The Cd and Zn contents of the Arabidopsis expressing TcMT2a or TcMT3 were not altered either (II).

Increased tolerance to metals in plants has been previously reported for Arabidopsis MT2a and MT3, which enhanced Cd tolerance when expressed in Vicia faba guard cells (Lee et al., 2004), and for Brassica juncea MT2 which increased tolerance to Cd and Cu in Arabidopsis, as inferred from the increased leaf chlorophyll content and survival of the overexpressor plants (Zhigang et al., 2006). However, similarly to our experiment, the root length was not altered under metal exposure compared to wild-type (Zhigang et al., 2006). Even though the chlorophyll content was not measured, the TcMT2a or TcMT3 overexpressor plants did not show markedly better growth or survival under metal exposure in our study.

Interestingly, the root length of TcMT2a and TcMT3 Arabidopsis overexpressors was reduced compared to the wild-type plants in the control medium with low metal concentrations. This was also reported for Arabidopsis overexpressing Brassica juncea MT2 (Zhigang et al., 2006). These results suggest that, at low metal levels, the overexpressed MT proteins may interfere with metal homeostasis by binding essential metals and making them unavailable for normal metabolism.
Our results imply that overexpression of *Thlaspi MT2a-LC* or *MT3-LC* in Arabidopsis does not enhance the basic level of metal tolerance in wild-type plants, nor is it able to increase metal accumulation in a non-hyperaccumulator background. However, TcMT2a and TcMT3 are functional proteins as they were able to increase metal tolerance and Cd accumulation in yeast. The growth reduction of TcMT-transformed Arabidopsis at low metal availability may imply that overexpressed TcMT2a and TcMT3 interfere with metal homeostasis.

**5.4.6 MT2 protein is localized in roots epidermis**

The TcMT2 protein was localized in intact *T. caerulescens* or Arabidopsis roots with whole-mount immunohistochemistry using a TcMT2a-targeted anti-peptide antibody coupled to fluorescent-labelled secondary antibody. The TcMT2a antibody was made against two peptides designed for the spacer region between the Cys-rich domains, with two and three amino-acid mismatches to TcMT2b. The antibody is also able to detect Arabidopsis MT2, as the first peptide has three amino acid mismatch compared to AtMT2a while the second peptide is identical to AtMT2a.

In the roots of transgenic Arabidopsis expressing 35S-TcMT2a, a strong fluorescence signal was observed especially at the root tip compared to the wild-type Arabidopsis plants, indicating that the transformants produce the TcMT2 protein. In the roots of *T. caerulescens* and in wild-type *A. thaliana*, MT2 protein is localized mainly in the epidermal cells and in root hairs with a high abundance near the root tip. Lower MT2 protein abundance was observed at the older parts of the roots near the stem. The localization is in line with the previous findings where Arabidopsis MT2a mRNA co-localized with the MT2 protein in the root tip, and it was concluded that MT2a acts as a chaperone protecting the root apex, the first tissue to absorb excess metals, whereas MT2b was found to be localized in the phloem (Guo et al., 2003). Thus the localization supports the role of TcMT2 in the buffering rather than in the accumulation of metals.

**5.4.7 Putative role of MTs**

In the Thesis, MTs were studied in plant species which have different strategies with respect to metal uptake. *T. caerulescens* is a Zn/Cd (Ni) hyperaccumulator plant, *S. vulgaris* has accessions with extreme metal tolerance and hybrid aspen (*P. tremula x tremuloides*) could be a metal (mainly Cd and Zn) indicator plant like *P. alba* (Madejon et al., 2004). Even though these species have different strategies, intracellular metal detoxification is important in all of them.

The expression of *T. caerulescens* MT genes did not co-segregate with Zn accumulation (II). When overexpressed in Arabidopsis, TcMT2a and TcMT3 did not increase tolerance to
Zn, Cd or Cu, nor did it increase Zn or Cd accumulation (II). These results indicate that TcMT2a and TcMT3 are not directly involved in Zn accumulation.

However, TcMT2a and TcMT3 are functional proteins capable of binding Cu and Cd, at least when expressed in yeast (I). Moreover, their increased expression in metallicolous and especially in the superior accumulating accession (II) suggests that they are important in hyperaccumulation in *T. caerulescens*.

A possibility is that *T. caerulescens* MTs would be involved in metal tolerance, which was observed for Silene *MT2b*. High SvMT2b expression in the offspring from crosses of moderately tolerant plants correlated with tolerance. Thus SvMT2b seems to act as a hypostatic factor in metal tolerance, enhancing the tolerance level of already tolerant plants (IV). The TcMT2a and TcMT3 could also act as modifier genes, which might explain why TcMTs could not influence metal tolerance when expressed ectopically in Arabidopsis. The expression of TcMT2a and TcMT3 follows roughly the tolerance to Cd and Zn in the analyzed accessions, the non-metallicolous accession having the lowest expression. However, the tolerance hypothesis does not explain the ten-fold higher TcMT3 expression seen in the shoots of superior Cd accumulating accession (LM) compared to metallicolous accession (LC), as these accessions have similar tolerance capacities.

Another explanation might be that TcMTs are involved in the homeostasis of metals, possibly that of Cu. Arabidopsis overexpressing TcMTs had reduced root growth at low metal availability but not in metal exposure, suggesting that overexpressed MTs influenced essential metal availability. The localization of MT2 in root epidermis and root hairs (II) also suggests a role in metal buffering. No immunofluorescence staining was seen around the vessels, indicating that MT2 is not involved in xylem loading.

It is also possible that MTs contribute to processes not directly involved in metal binding. They may be involved in free radical scavenging, like in the case of MT3 in cotton (Xue et al., 2008). High MT3 expression, if involved in free radical scavenging, could be an evolutionary response to high Cd accumulation capacity in the accession Ganges. However, this remains to be tested.

Overexpression or loss-of-function studies in Arabidopsis may not unravel the function of MTs in *T. caerulescens*. Thus, to understand the role of MTs in metal hyperaccumulation, knock-down experiments should be done directly in *T. caerulescens*.

The expression of *T. caerulescens* MTs, i.e. TcMT2a, TcMT2b and TcMT3, was largely unaffected by Zn treatment (II). In the Cu-tolerant *Silene* populations (IV), SvMT2b was not Cu-responsive either. However, in hybrid aspen, MT2b expression reflected the soil metal (largely Zn, Cd) contamination and also correlated with foliar Cd and Zn concentrations. This is interesting as hybrid aspen is potentially an indicator plant of soil metal contamination.
5.5 Metal accumulation capacity of hybrid aspen grown in a contaminated site

Hybrid aspen (Populus tremula x tremuloides) trees were planted in a metal-contaminated area in order to study the metal uptake capacity of this fast-growing tree species (III). As the metals were anticipated to be heterogeneously deposited at the site, typically for a contaminated area, the concentration of nine metals and metalloids (Al, As, Cd, Cr, Cu, Pb, Ni, Zn, V) were measured around each tree as well as in the roots, stems and leaves of ten trees in two consecutive years.

The soil contained several heavy metals at moderate concentrations, 5.1 mg kg\(^{-1}\) Cd, 80 mg kg\(^{-1}\) Cr, 180 mg kg\(^{-1}\) Cu, 81 mg kg\(^{-1}\) Ni, 240 mg kg\(^{-1}\) V and 520 mg kg\(^{-1}\) Zn, on average. Four of the ten sites studied were metal hotspots where Cd, Cr, Cu, Ni, V or Zn concentrations exceeded the national upper guideline values used for industrial sites, and the site can thus be classified as contaminated even for industrial purposes. However, no growth reduction was seen in the trees grown in the hotspots compared to those growing in the moderately contaminated soils.

In these conditions, hybrid aspen was able to accumulate quite high levels of Zn and Cd into its leaves, with mean concentrations of 970 and 11 mg/kg (Table 5). These concentrations are comparable to or higher than those reported in a previous study on Populus spp. grown in a contaminated site, in which ca. 400-700 mg/kg Zn and 3-8 mg/kg Cd were found in mature leaves (Laureysens et al., 2004). The maximal Cd and Zn concentrations measured in the present study were 35 and 2400 mg/kg, which are rather high considering that the limit for hyperaccumulation is 100 and 10 000 mg/kg, respectively.

Table 5. Mean (years 2004 and 2005) and maximal observed soil metal concentrations (mg/kg, dry weight) in ten sampling points and in the leaves and roots of ten hybrid aspen plants (mg/kg, dry weight).

<table>
<thead>
<tr>
<th></th>
<th>Cd</th>
<th>Cu</th>
<th>Ni</th>
<th>Zn</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soil</td>
<td>5,1 (29)</td>
<td>180 (920)</td>
<td>82 (290)</td>
<td>520 (2200)</td>
</tr>
<tr>
<td>Aspen leaves</td>
<td>11 (35)</td>
<td>7,6 (11)</td>
<td>4,4 (16)</td>
<td>970 (2400)</td>
</tr>
<tr>
<td>Aspen roots</td>
<td>2,4 (9,6)</td>
<td>11 (32)</td>
<td>2,9 (8,2)</td>
<td>82 (180)</td>
</tr>
<tr>
<td>Roots/soil</td>
<td>0,69</td>
<td>0,13</td>
<td>0,05</td>
<td>0,29</td>
</tr>
<tr>
<td>Leaves/soil</td>
<td>3,20</td>
<td>0,08</td>
<td>0,06</td>
<td>2,60</td>
</tr>
</tbody>
</table>
Results and Discussion

The foliar Zn and Cd concentrations were about fivefold and tenfold higher in the leaves than in the roots, respectively. Also Ni translocated to the leaves, whereas Al, Cr, V and, to a lesser extent, Cu were largely retained in the roots.

Overall, hybrid aspen accumulated Cd and Zn very efficiently in the leaves, with a mean bioaccumulation factor (metal concentration in the leaves vs. metal concentration in soil) of 3.3 and 2.6, respectively. The ca. 3-fold bioconcentration factor for the leaves is comparable to the 2.6-fold enrichment reported by Hermle et al. (2006) for Cd and Zn. The bioaccumulation factors were very low for other metals, indicating that aspen does not concentrate other metals in the above-ground tissues. Interestingly, the bioaccumulation factors for Cd and Zn correlated strongly in both years among the sampling sites, which suggests that the transport of these metals share common mechanisms for uptake and/or translocation.

It is important to analyze the elemental concentrations in situ. In a contaminated site the plants are affected by several environmental challenges. Compared to laboratory experiments, the contaminants are not uniformly distributed, and the growth environment is not optimal (nutrient availability, herbivores, water imbalance). Compared to metal hyperaccumulators, trees do not accumulate high amounts of heavy metals, but they may provide economic benefits through the production of biomass. Poplars (Populus spp.) are especially interesting as they have a high biomass production and are able to concentrate Cd and Zn (French et al., 2006), as was confirmed in this Thesis. Trees may also reduce erosion and leaching, which makes them interesting candidates for phytoremediation.

In conclusion, P. tremula x tremuloides has potential to withstand relatively high, mixed metal concentrations, which may be partially contributed by inducible MT. Moreover, hybrid aspen is able to concentrate high levels of Zn and Cd in the above-ground tissues, making it an interesting candidate for phytoextraction studies, along with other Populus ssp. Hybrid aspen leaves could be used as bioanalytical tools to estimate Cd and Zn contamination, as was suggested for P. alba (Madejon et al., 2004).
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6 CONCLUSION

Plant metal tolerance and accumulation are important topics worldwide, as they influence the content of nutritional or toxic metals in the crops, including food crops. Understanding the mechanisms of heavy metal homeostasis may enable the breeding of more tolerant varieties or varieties with increased concentrations of essential minerals or decreased concentrations of heavy metals in the edible parts of the crops. Moreover, such knowledge could be used for developing plants for phytoremediation.

One of the aims of the work reported in this Thesis was to isolate genes possibly related to the metal hyperaccumulation trait in *Thlaspi caerulescens*. This was done by comparing mRNA expression of two Zn-exposed *T. caerulescens* accessions using Differential Display, with the assumption that Zn-responsive genes that show differential expression between accessions with differential metal uptake and tolerance capacities might be important in the hyperaccumulation trait. Altogether 16 genes were isolated in this Thesis. Several interesting genes, possibly related to metal uptake, tolerance or homeostasis, were isolated. When expressed in yeast, these genes (*TcMT2a*, *TcMT3*, *TcMRP*, *TcOle e1-like*) were able to influence metal tolerance or uptake.

The *T. caerulescens* *MT2a* and *MT3* were studied in more detail with mRNA expression analysis in several accessions and crosses segregating for Zn accumulation, by studying yeast and Arabidopsis *MT* overexpressors under metal exposure and by localizing MT2 protein in intact roots. These studies suggest that even though *T. caerulescens* MTs are able to increase metal tolerance and accumulation in yeast, overexpression in Arabidopsis does not affect these traits. The observation that the growth of *MT*-transformed plants was negatively influenced at low metal levels but not at excess amount of metals suggests that overexpression of *MT2a* or *MT3* may disturb essential metal homeostasis.

However, overexpression or loss-of-function studies in Arabidopsis may not give sufficient information about the role of MTs in *T. caerulescens*, as the non-hyperaccumulators and hyperaccumulators appear to have different primary processes of metal detoxification. For a complete understanding of the role of MTs or other genes putatively involved in hyperaccumulation, overexpression and loss-of-function studies should be conducted in *T. caerulescens*.

Taken together, metallothioneins appear not to be the major determinants of metal hyperaccumulation. However, as they are expressed at higher levels in metallicolous *T. caerulescens* and *S. vulgaris* accessions, as compared to non-metallicolous ones, they might be involved in metal tolerance as modifiers rather than as primary determinant. This may be the case of *MT2b* in *S. vulgaris*. Alternatively they may be involved in the homeostasis of copper in a high-Zn or high-Cd cellular environment.
Large-scale transcriptomic analyses have been conducted recently, with hundreds of genes expressed at higher levels in the hyperaccumulators compared to non-accumulators (Becher et al., 2004; Weber et al., 2004; van de Mortel et al., 2006, 2008). These studies have increased and will increase our knowledge on plant heavy metal hyperaccumulation. However, the genes differently expressed between a non-accumulator/accumulator or in response to metal excess may not necessarily be the ones responsible for metal accumulation or tolerance as these traits seem to be constitutive in *T. caerulescens*. Thus the role of candidate genes has to be studied one by one for example with heterologous expression in yeast and *in planta*, as was done for metallothioneins (MTs) in this Thesis.

Moreover, transcriptomic studies have usually been conducted at the organ level, i.e. on the above-ground parts or on the roots. This gives only a partial view of the biochemical processes, as changes occurring within one cell type can be masked by changes taking place in another cell type. This is especially the case for MTs, as they belong to a multi-gene family with members having probably different functions in different cell types. Thus, for a thorough understanding of the transcriptomic changes, one should aim at the tissue level, and finally, at the level of a single cell.

In addition to transcriptomics, other profiling techniques such as proteomics (Tuomainen et al., 2006) and metabolomics should be used to have a more complete picture of the processes. Moreover, the most challenging task is to combine the data of all of these omic techniques in a systems biology approach to unravel the pathways involved and their regulatory mechanisms.
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