Heavy metal pollution is a world-wide problem. Metal-enriched sites can occur naturally, or due to mining, smelting, industrialization and other anthropogenic activities. High soil metal concentrations are toxic to plants, which has lead to the evolution of metal hypertolerance in a number of plant species. These plants, called metallophytes, have developed special mechanisms to tolerate high metal concentrations in the soil and in (part of) their body. A minority of these plants, called hyperaccumulators, accumulate heavy metals to extraordinary concentrations in their leaves, whereas the majority of them are ‘shoot excluders’, maintaining more or less constant and relatively low foliar metal concentrations over a broad range of soil metal concentrations. A detailed understanding of the molecular and physiological basis of metal hyperaccumulation and hypertolerance is lacking to date. The work reported in this thesis aims to further elucidate several aspects of the molecular mechanisms of hyperaccumulation in the Zn/Cd hyperaccumulator model species, \textit{Noccaea caerulescens}, and Cd, Zn, or Cu hypertolerance in the shoot excluder metallophytes, \textit{Silene vulgaris} and \textit{S. paradoxa}.

In the Zn/Cd hyperaccumulator, \textit{Arabidopsis halleri}, a high level of \textit{HMA4} expression has been shown to be essential for Zn and Cd hyperaccumulation and hypertolerance. \textit{HMA4} appeared to be strongly over-expressed also in \textit{N. caerulescens}, in comparison with \textit{A. thaliana}, particularly in the shoot. In chapter 2 we characterized different promoters and gene copies of \textit{NcHMA4} through expression in the \textit{At hma2/hma4} double mutant, which is severely deficient in Zn root-to-shoot translocation. When expressed under the \textit{AtHMA4} promoter, both \textit{AtHMA4} and \textit{NcHMA4} fully complemented the mutant. Expression under either of the \textit{NcHMA4} promoters only partly restored the Zn translocation to the shoot, and did generally not ameliorate the severe symptoms of foliar Zn deficiency and the complete reproductive sterility characteristic of the mutant. On average, the \textit{NcHMA4} promoters were two times and 50 times more active in in the roots and the leaves of the transgenic plants, in comparison with the \textit{AtHMA4} promoter. The tissue specificity patterns, established by GUS staining, were similar in the root, i.e. mainly in the stele, albeit that the \textit{NcHMA4} promoters were much more active in the meristematic part of the root tip and the root cap. There was no detectable staining in the leaves when \textit{GUS} was expressed under the \textit{AtHMA4} promoter. \textit{GUS} expression under either of the \textit{NcHMA4} promoters, however, yielded intense staining of the
veins and, usually, the whole leaf blade. *HMA4* expression under *NcHMA4* promoters increased the transcript level of the Zn deficiency-responsive gene, *ZIP4*, in the leaves, i.e. about 7- and 35-fold, in comparison with the *hma2hma4* double mutant and wild-type, respectively. As expected, expressing *HMA4* under the *AtHMA4* promoter decreased *ZIP4* expression to wild-type level. These results demonstrate that *HMA4* expression under a strong *NcHMA4* promoter, even though it significantly improves the foliar Zn concentration, tends to enhance, rather than ameliorate the foliar Zn deficiency phenotype of the *hma2hma4* double mutant. This is probably due to expression of *HMA4* all over the leaf blade, leading to efflux of Zn from the mesophyll cells and, in the absence of hyperaccumulator-like rates of Zn root to shoot translocation, to more severe Zn deficiency. Based on these results, it seems possible that *NcHMA4* plays also a role in the transport of Zn to the large epidermal cells in *N. caerulescens*. The relatively small effect on Zn translocation of *HMA4* expression under *NcHMA4* promoters, in comparison with the *AtHMA4* promoter, may be owing to incorrect tissue-specificity in the *A. thaliana* background, i.e. a particularly strong expression all over the root tip, instead of only in the root cap, such as in *N. caerulescens* itself.

In chapter 3 we described the isolation of an *AtHMA2*-like gene from *S. vulgaris* and *S. paradoxa*, and investigated its potential role in Cd and Zn hypertolerance through co-segregation analysis of progenies of crosses between metallicolous and non-metallicolous plants. Both *SvHMA2* and *SpHMA2* were about 6-fold more strongly expressed in plants from the metallicolous populations than in non-metallicolous plants. In segregating crosses of both *S. paradoxa* and *S. vulgaris* the high *HMA2* expression level co-segregated with Cd hypertolerance, but not with Zn hypertolerance. The segregation patterns obtained for Cd and Zn tolerance in the *S. paradoxa* cross were identical with those previously obtained for *S. vulgaris*, pointing to monogenic control of Cd hypertolerance and digenic control of Zn tolerance, respectively. These results suggest that *HMA2* over-expression is the mechanism of Cd hypertolerance in both species, which is a remarkable example of independent parallel evolution. *HMA2* had five- and two-fold more genomic copies in the metallicolous populations, in comparison with the non-metallicolous populations, in *S. vulgaris* and *S. paradoxa*, respectively. Copy number expansion is sufficient to explain the enhanced expression level in the metallicolous population of *S. vulgaris*, but not in that of *S. paradoxa*. If *HMA2* itself is the Cd hypertolerance gene indeed, then the individual gene copies should be in a tandem array, inheriting as a single gene, in both species.
In chapter 4 we described the isolation of two orthologs of the Cu transporting ATPase-encoding gene AtHMA5, SvHMA5-1 and SvHMA5-2, from S. vulgaris and SpHMA5-1 from S. paradoxa, and analyzed their expression and genomic copy numbers in cupricolous and non-cupricolous populations of both species. We also analysed the expression and genomic copy numbers of MT2b in cupricolous and non-cupricolous populations of both species. Both HMA5-1 and HMA5-2 were much higher expressed in the cupricolous S. vulgaris population than they were in a non-metallicolous or a calamine one. As revealed by qPCR on gDNA, there seem to be four genomic copies of HMA5-1 and about 20 copies of HMA5-2 in cupricolous plants and only one of each in non-metallicolous plants of S. vulgaris, whereas there is only one in all the populations of S. paradoxa under study. MT2b expression was significantly higher in cupricolous than in non-metallicolous populations of both S. vulgaris and S. paradoxa. Genomic MT2b copy number expansion, in comparison with the non-metallicolous reference populations was apparent in all the cupriculous populations of both species. We also analysed the co-segregation of HMA5-1 and HMA5-2 expression with Cu hypertolerance in segregating F_2 and F_3 progenies of a cross between cupricolous and non-metallicolous S. vulgaris. Both HMA5-1 and HMA5-2 expression co-segregated significantly with Cu tolerance. Expression of one copy of SvHMA5-1, under a native SvHMA5-1 promoter from a cupricolous plant, in the Cu-hypersensitive At hma5 mutant yielded an expression level in the root that was comparable to that of wild-type AtHMA5, suggesting that the high expression level in the cupricolous plants is solely due to copy number expansion. Expression of SvHMA5-1 under the 35S CMV promoter yielded a low degree of Cu hypertolerance, as estimated from the root growth response. It is argued that high-level Cu hypertolerance in S. vulgaris is a genetically complex trait, involving over-expression of at least two HMA5 paralogues and MT2b. Also in S. paradoxa Cu hypertolerance in cupricolous populations was strictly associated with enhanced expression of SpHMA5 and SpMT2b, which is, again, a remarkable example of independent parallel evolution in different species.

In conclusion, expression of HMA4 under a hyperaccumulator HMA4 promoter does not yield a hyperaccumulator-like phenotype for Zn translocation in the A. thaliana genetic background, possibly due to incorrect tissue-specificity. Furthermore, the molecular mechanisms underlying Cd and Cu hypertolerance in S. vulgaris and S. paradoxa seem to be very similar, if not identical. Natural selection under the pressure of heavy metal toxicity
apparently targets the same genes in different congeneric species. Finally, research in metal hypertolerance in excluder metallophytes would strongly benefit from a genetically accessible model species.