Characterization of *NcHMA4* and *NcZTP1* for metal tolerance and accumulation in *Noccaea caerulescens*

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Abstract

Metals can be essential elements for plant metabolism, but in elevated concentrations in soils can cause serious environmental damages, loss of production of important crops and affect human health. Metal hyperaccumulators are the plants that can uptake and accumulate increased amounts of metals from the contaminated soils, and in a near future the use of biotechnological approaches can become an important tool to develop plants with an enhanced metal uptake and accumulation mechanism, for phytoremediation of soils and other purposes. Meanwhile, model metal hyperaccumulator plant species have been studied in order to understand the physiological and molecular mechanisms underlying metal accumulation and tolerance. In this work I aimed to study the *NcHMA4* and *NcZTP1* genes, that are important genes in, respectively, metal transport to the shoots (Papoyan and Kochian, 2004) and storage into vacuoles (Kobae *et al.*, 2004) in *Noccaea caerulescens* (formerly known as *Thlaspi caerulescens*), a model species for hyperaccumulation. To accomplish this goal, we focused on the creation of RNAi and promoter + reporter gene constructs. Also, a parallel work with regeneration of plants in vitro and stable transformation was performed to permit further studies with stable transformation system, which is the main limiting step in understanding mechanisms related to metal homeostasis in *N. caerulescens*. The promoter sequence of *NcZTP1* has been isolated by Genome Walking and from the first attempt, an intron upstream the coding sequence was identified and two slightly different sequences were found, indicating that *NcZTP1* may be duplicated in *N. caerulescens*. Sequencing will be done of the fragments generated on the second attempt in order to obtain the promoter region. A promoter + reporter gene of *NcHMA4* construct was made, but further analysis is still needed to test whether the construct is correct. RNAi constructs for *NcHMA4* and *NcZTP1* were made and found to be correct and *N. caerulescens* plants were transformed with those constructs. Gene expression analysis of wild type plants and transformants showed that *NcZTP1* was knocked down, but none of the plants transformed with the *NcHMA4* construct were silenced. Furthermore, stable transformation and regeneration of *N. caerulescens* was tested following the method described by Guan *et al* (2008), but neither transformants nor regenerated plants were obtained, and further improvements on the method are discussed. Therefore, the data presented on this thesis can be used in further studies for elucidating the role and localization of *NcHMA4* and *NcZTP1* and to develop a suitable method for stable transformation and regeneration of *N. caerulescens.*

Introduction

Metal Toxicity and Hyperaccumulator species

In normal conditions, many metals are essential to plants as micronutrients, such as Fe, Cu, Mn, Ni and Zn, but when in elevated concentrations in soil they may be toxic (Epstein and Bloom, 2005), and become an environmental problem because plants are unable to grow due to toxicity (Assunção *et al*, 2001). Zn is an essential component of several enzymes, like RNA polymerase, Cu/Zn superoxide dismutase and carbonic anhydrase. Also, a large number of proteins contain Zn-binding structural domains such as the zinc finger domain. Although Zn is essential for plants, supraoptimal concentrations of Zn can affect the metabolism. For instance, uncontrolled binding can render enzymes non-functional and the redox activity can elicit the generation of reactive oxygen species (Kobae *et al.*, 2004). Copper (Cu) is important in many structural and functional roles in plants. Many oxidative enzymes are dependent to Cu, and it also plays an important role in cell wall lignification (Bell and Dell, 2008). Cu deficiency can inhibit photosynthesis and reduce availability of leaf carbohydrates (Hassan and Aarts 2010). Iron (Fe), is also an important element for plants, and plays a key role in the synthesis of chlorophyll, ethylene and in construction of cell wall (Bell and Dell 2008). At elevated concentrations, Fe can cause oxidative stress (Guerinot and Yi, 1994). Ni is an essential micronutrient, although needed in small amounts. Plant needs Ni as a co-factor of the enzyme urease. Both deficiency and excess can cause injuries to plants, such as leaf necrosis when in debt (Gerendas *et al.*, 1999) and loss of chlorophyll content, growth inhibition of shoots and roots when in excess (Freeman *et al.*, 2004 in Hassan and Aarts, 2010). Finally, cadmium (Cd) is not an essential element, but an important environmental pollutant and a potent toxicant in plants. Cd may enter in plants following Zn or Fe uptake machinery (Pence *et al.*, 2000; Plaza *et al.*, 2007) and accumulation can cause several damages to plants, as DNA repair inhibition, reduction in photosynthesis, water and nutrient uptake, and visible symptoms of injury, chlorosis, growth inhibition, browning of root tips and death (Hassan and Aarts, 2010).

Some species, however, are able to survive in contaminated soils, by many different strategies (Kupper and Kochian, 2009). One of the strategies developed by plants is to actively take up heavy metals and accumulate them. These kind of plants

are called metal hyperaccumulators, and over 450 species are known for following this kind of strategy (Maestri *et al.*, 2010). The hyperaccumulation mechanism is characterized by two basic processes: high metal accumulation and high metal tolerance (Gustin *et al*, 2009). Hyperaccumulators have been studied with different approaches, not only to understand the physiological, structural and molecular mechanisms involved (Gustin *et al.*, 2009; Hanikenne *et al.*, 2008, Assunção *et al.*, 2001), but also to use this knowledge for the further development of useful strategies such as phytoextraction, phytoremediation and phytomining. Furthermore, more recently discussed, transformed plants may be useful to improve nutritional value of vegetables consumed as food (Grusak, 2002).

Over 34 different plant families are comprised of hyperaccumulators. Many of these species belong to the family Brassicaceae, mainly the genera *Alyssum* and *Noccaea* (Verbruggen *et al.*, 2009). Zn and Cd hyperaccumulators species are practically all confined to the genera Brassicaceae, in particular to the genus *Noccaea* and the species *Arabidopsis halleri* (Verbruggen *et al.*, 2009). The most studied species for hyperaccumulation are *N. caerulescens* (formerly known as *Thlaspi caerulescens*) and *A. halleri*.

N. caerulescens: a model for hyperaccumulation patterns studies

The species *N. caerulescens* can take up metals in concentrations at least 100 times higher than normal species, especially Zn and Cd. These plants in nature need more Zn^{2+} (104-fold) then non-hyperaccumulator species needs to grow normally (Hong-Bo *et al.*, 2010). In *N. caerulescens*, hyperaccumulation is constitutive to Zn at the specie level, but not to Cd and Ni (Assunção *et al.*, 2003a). Different populations of *N. caerulescens* exhibit uncorrelated and metal-specific variation in uptake, root to shoot translocation and tolerance of Zn, Cd and Ni. *N. caerulescens* was indicated as a model to understand the hyperaccumulation mechanisms (Assunção *et al.*, 2003a), in part because of its proximity to the general plant model *Arabidopsis thaliana*, with an identity of about 88.5% in the coding region (Peer *et al.*, 2003; Rigola *et al.*, 2006) , but also because of its self-compatibility and easy out-crossing, and more importantly, because of the high level of variation in the degrees and metal specificities of metal

accumulation, root-to-shoot transport and tolerance traits between different accessions (Assunção *et al.*, 2003a).

Assunção *et al.* (2001), studying expression of metal transporters genes in three accessions of *N. caerulescens* – La Calamine and Monte Prinzera, from metalliferous soil, and Lellingen , from a non-metalliferous soil –, observed a high and independent inter-accession variability for Zn uptake, shoot-to-root concentration ratio and tolerance. The metallicolous accessions, LC and MP, demonstrated elevated tolerance to Ni and Zn, respectively. The authors associated the high level of Zn tolerance in LC plants with decreased uptake and transport of this metal, compared to the non-metallicolous accession LE. Otherwise, MP plants showed an increased rather than decreased uptake of Ni and Zn, compared to LE. These results indicated that the metal tolerance and metal uptake are independent traits.

However, the relation between metal tolerance, accumulation and root to shoot translocation in hyperaccumulators is not well understood yet. Assunção *et al.* (2003b) compared the same populations cited above to study the different patterns of tolerance, accumulation and root to shoot translocation of Zn, Cd and Ni, but the results showed a largely uncorrelated variation between these factors among *N. caerulescens* populations. Comparisons at the transcriptional level by microarray between the ecotypes La Calamine and Lellingen were also done by Plessl *et al.* (2009). The results showed different patterns of gene expression among the two different ecotypes. LC had a higher expression of genes related to avoidance of oxidative stress, genes encoding for water channel components and for a Zn transporter (ZTP1), while for metal chelator genes, LC showed less expression. These results contribute to explain the higher tolerance of LC compared to LE, but further studies with the putative genes involved in Zn adaption are needed.

The above mentioned and many other studies demonstrate that the different phenotypes of *N. caerulescens* and the possibility of the generation of new genotypes have been opening many possibilities for comparative transcriptome, proteome and metabolome analyses, and also for Quantitative Trait Loci mapping studies that may contribute to the further understanding of hyperaccumulation and tolerance traits.

Important genes involved in metal hyperaccumulation and tolerance physiology

Plants have evolved a complex network of homeostatic mechanisms that controls the uptake, accumulation, trafficking and detoxification of metals in order to control the concentration of metals and maintain stable condition (Clemens, 2001). The metal hyperaccumulation mechanism involve at least four important physiological events: stimulated metal uptake from the soil, reduced metal sequestration in the root vacuoles, increased xylem loading for transport to the shoots and, finally, increased sequestration and storage in the leaves (Milner and Kochian, 2008). The enhanced metal uptake of metals from soil in *N. caerulescens* seems to be driven by natural high expression of members of the ZIP family of transport proteins in roots – *ZNT1* and *ZNT2* – (Pence *et al.*, 2000; Assunção *et al*, 2001), that are homologues of *AtZIP4*. This high expression was confirmed in microarray analyses (Hammond *et al.*, 2006; van de Mortel *et al.*, 2006).

HMA4

The next important characteristics for the hyperaccumulation mechanism are a reduced metal sequestration in the root vacuoles and an efficient metal translocation to the shoot. *N. caerulescens* presents lower accumulation and a faster release of Zn from the root vacuoles than a non-hyperaccumulator species *T. arvense* (Verbruggen *et al.*, 2009). Xylem sap from *N. caerulescens* was found to contain approximately a 5-fold higher Zn concentration compared with the xylem sap from *T. arvense* for plants grown on the same Zn level (Lasat *et al.*, 1998). Several types of transporters are important for the xylem metal loading. One of the gene families that is involved in this process is the P-type ATPase-HMA. Genes of this family play an important role in transporting transition metal ions against their electrochemical gradient using the energy provided by ATP hydrolysis. HMAs can be clustered into two classes: those transporting monovalent cations (Cu/Ag group) and those transporting divalent cations (Zn/Co/Cd/Pb) (Rensing *et al.*, 1999), and the P1B subfamily is believed to be involved in the transport of the second group.

HMA4 is a member of the P1B subfamily of P-type ATPase superfamily. In *A. thaliana*, *HMA4* is expressed throughout the plant, but with stronger expression in roots (Mills *et al.*, 2003). *A. thaliana hma2 hma4* double mutants showed normal metal

uptake from the soil, but also showed less accumulation in shoot tissues, demonstrating their role in translocation of metals to the shoot (Hussain *et al.*, 2004). Furthermore, *A. thaliana HMA4* T-DNA insertion mutants are sensitive to elevated levels of Cd and Zn, and also confer Cd resistance to the yeast species *S. cerevisiae*. Thus HMA4 seemed to be functioning as an efflux transporter serving to expel the Cd taken by the yeast cell (Mills *et al.*, 2005). Overexpression of *AtHMA4* also lead to increased accumulation of Zn and Cd in the shoots of the transgenic Arabidopsis plants, further suggesting that HMA4 plays a role in loading metals into the xylem (Verret *et al.*, 2004).

In *N. caerulescens*, the gene *HMA4* is 71% identical to *AtHMA4* and contains many of the same predicted motifs found in *AtHMA4* and other heavy metal ATP-ases (Papoyan and Kochian, 2004), and its expression occurs strongly and almost exclusively in roots, showing up-regulation by exposure to high concentrations of Cd and Zn, and also by Zn deficiency (Papoyan and Kochian, 2004). Metal accumulation experiments in *NcHMA4*-transformed yeast showed decreased accumulation of Cd and Pb, and also Zn and Cu compared to wild-type cells. This is consistent with the function of *NcHMA4* operating at yeast plasma membrane to pump metals out of the cell, conferring metal tolerance. Furthermore, up-regulation under Zn deficiency conditions also indicates an important role for maintaining shoot Zn status to allow plant reproduction (Papoyan and Kochian, 2004).

A. thaliana transformed with *Arabidopsis halleri HMA4* demonstrated a more efficient transfer of metals from roots to leaves compared to non-transgenic *A. thaliana*, but also showed shoot sensitivity to high metal concentrations (Hanikenne *et al.*, 2008). These results demonstrate that *HMA4* plays an important role in metal translocation from roots to shoots of the hyperaccumulators, but also urge for the need of additional genes for shoot metal detoxification in non accumulators (Hanikenne *et al.*, 2008). Many questions remain unanswered concerning the P1B-ATPases, particularly in relation to their full range of roles in plants, their regulation, how do they work and also their potential for genetic manipulation of non accumulators for phytoremediation purposes (Williams and Mills, 2005).

ZTP1

The last important characteristic that distinguishes hyperaccumulators from non-accumulators plants is the storage of metals in the vacuoles of leaf cells. Several

families of transporters are involved in the storage process, like the CDF family. The AtMTP1 (*Arabidopsis thaliana* Metal Transporter Proteins), also called ZAT (Zinc Transporter of *A. thaliana*), was the first plant CDF protein identified (Assunção *et al.*, 2001; Maser *et al.*, 2001; Persans *et al.*, 2001; van der Zaal *et al.*, 1999) and showed low levels of expression in whole seedlings growing under normal or excess of Zn (Kobae *et al.*, 2004). It is localized to the vacuolar membranes in root and leaf cells in *A. thaliana* (Kobae *et al.*, 2004; Desbrosses-Fonrouge *et al.*, 2004) and seems to function as a housekeeping protein in *A. thaliana* (Kobae *et al.*, 2004).

Plants of *Arabidopsis thaliana* showed increased resistance to Zn by overexpression or heterologous expression of *AtMTP1* (van der Zaal *et al.*,1999), *PtdMTP1* (Blaudez *et al.*, 2003), *AtMTP3* (Arrivault *et al.*, 2006), *TgMTP1* (Gustin *et al.*, 2009), giving consistent evidences of the important role of this gene in Zn accumulation by enhanced sequestration into the vacuole. Furthermore, gene suppression is needed, because a loss-of function mutant is more appropriate in order to define the role of *MTP1* in plants. T-DNA inactivation mutants of *A. thaliana* not expressing *AtMTP1* are more sensitive to Zn (Kobae *et al.*, 2004), and also, silencing by RNA interference (RNAi) of *AtMTP1* showed hypersensitiveness to Zn (Desbrosses-Fonrouge *et al.*, 2005).

N. caerulescens contains a *ZTP1* gene (Assunção *et al.*, 2001; Rigola *et al.*, 2006), which is the closest homologue of the Arabidopsis *MTP1* (*ZAT*), and showed higher expression when compared to *T. arvense*, a non-accumulator species (Assunção *et al.*, 2001). The zinc transporter *ZTP1* is constitutively expressed both in root and shoots in *N. caerulescens,* and shows higher expression in La Calamine than in Lellingen (Plessl *et al.*, 2009). Furthermore, the highest expression in roots of LC seems to be when Zn was deficient and decreased with increasing Zn concentrations, while in the shoot, the level of expression was higher in all Zn concentrations (Plessl *et al,* 2009). Recent data showed that the level of expression of *ZTP1* in *N. caerulescens* (Ganges population) is not only dependent on metal nutrition/toxicity, but also depends on the plant stage of development, with higher *NcZTP1* mRNA abundance found in young leaves of young and mature plants (Kupper and Kochian, 2009).

Transformation of *N. caerulescens*

To study a gene function in plants, one of the best approaches is to verify the phenotype of knocked-down mutants by using RNAi constructs. Knocking down genes in hyperaccumulators, disturbed in the important steps mentioned above, will help to elucidate the function of the targeted gene in this process. Also, another interesting approach is to transform plants with constructs containing the promoter of the gene of interest fused to a reporter gene, like GFP and GUS. This approach allows tracking the promoter activity in plants and helps to elucidate the pattern and localization of gene expression.

Although *N. caerulescens* has been widely studied and indicated as a model for hyperaccumulation trait in plants, differently from *A. thaliana*, it has not a well established and reproducible protocol for stable transformation. *Agrobacterium rhizogenes* mediated transformation (hairy root) has been used, but despite it being very useful and applicable, the results and conclusions are limited and based on indirect inferences in some cases. Guan et al (2008) described a method of stable transformation via *Agrobacterium tumefaciens*, which appears interesting enough to try and reproduce.

Aim of this thesis

The aims of this work were: 1) to isolate the *NcZTP1* promoter; 2) to make constructs of the *NcHMA4* promoter + Reporter Gene and the *NcZTP1* promoter + Reporter Gene to track their activity in the different tissues; 3) to investigate the essentiality of *NcHMA4* and *NcZTP1* for root metal tolerance and root and shoot accumulation in *N. caerulescens* through RNAi; and 4) to develop a suitable protocol for *N. caerulescens* transformation and regeneration. The hypotheses of this MSc work is that *NcHMA4* plays a critical role in heavy metal transport to the shoot by xylem metal loading during hyperaccumulation and deletion will seriously hinder Zn and Cd root-to-shoot movement, as well as root and shoot metal tolerance. Also, we hypothesize that *NcZTP1* is an essential gene for metal tolerance and accumulation in *N. caerulescens* and without its function in roots, the plant is unable to withstand high Zn concentrations.

Material and Methods

NcZTP1 promoter isolation

The *N. caerulescens* accession La Calamine total DNA was extracted by the Nucleon Extraction Protocol (Barker, 1995). In order to isolate the *NcZTP1* promoter, APAgene TM GOLD Genome Walking Kit (BIOS&T) was used. The kit procedure is based on a 2-step primary PCR and two nested PCRs. The kit provides four random tagging primers (DRT) and two universal tagging primers (UAP). Also, 3 gene-specific primers (GSP) were designed on regions of ZTP1 gene (Table 1).

Table 1. Gene Specific Primers (GSP) used for ZTP1 walking to promoter isolation

GSPa: Z025	CAAGACATAACACCACCGCG
GSPb: Z024	GACTTTACTTGCCACAGCCC
GSPc: Z023	AATGATGTGACTTGAAGACTCC

After the second nested PCR a 1 % agarose gel was run and five bands, ranging from 250 bp to 1 kb approximately (Figure 1), were excised from gel and purified. Purified fragments were cloned into pGEM®-T Easy Vector (PROMEGA) by ligation with T4 DNA ligase. The cloning product was transformed into *E. coli* DH5α chemically competent cells by heat shock. The transformed DH5α were selected on Lysogeny Broth (LB) agar plates containing 100 mg/L Ampicilin, 0.5 mM IPTG and 80 µg/ml X-Gal. White colonies were picked up and cultured in 3 ml LB containing 100 mg/L Ampicilin at 37 °C overnight. Then miniprep was conducted with QIAprep Spin Miniprep Kit (QIAGEN).

For sequencing, 10-µl PCR reactions were set with 0.5 µl DETT dye, 300 – 500 ng plasmid DNA and 0.5 µl M13 forward or M13 reverse primer (20 pmol/µl). Temperature cycling was set as: 25 cycles of 30 sec at 94 °C, 15 sec at 50 °C, and 1 min at 60 °C. Samples were sent to Greenomics (Wageningen, The Netherlands) to be sequenced.

Construct of HMA4 Promoter + GFP/GUS Marker Gene

A Gateway D-Topo entry vector containing *NcHMA4* promoter was already available in the lab and was used in a LR reaction with the pKGWGG-RR vector (Limpens *et al.*, 2004) through Gateway® LR Clonase™ Enzyme Mix (Invitrogen™) to create an expression clone. Approximately 250 ng of entry vector was combined with about 300 ng of the destination vector in a reaction with 4 µl of 5 X LR Clonase™ Reaction Buffer completed with TE buffer pH 8.0 to 16 µl. Afterwards, 4 µl of LR Clonase™ Enzyme Mix was added and the reaction tubes were incubated at 25 °C overnight. 2 µl of Proteinase K solution was added to the samples to terminate the reaction and another incubation was conducted, at 37 °C for 10 minutes.

All of the reaction was transformed into *E. coli DH5a* chemically competent cells by heat shock. The transformed *DH5a* were selected on LB agar plates containing 100 mg/L Spectinomycin. Single colonies were picked up and cultured in 3 ml LB containing 100 mg/L Spectinomycin at 37 °C overnight, then miniprep was conducted with QIAprep Spin Miniprep Kit (QIAGEN). Digestion with the EcoRI restriction enzyme was performed to the miniprep DNAs and to the pKGWGG-RR empty vector to check insertion of the *NcHMA4* promoter (Figure 4). Additional digestions with AccI, NotI, BamHI and EcoRI were performed to the entry vector, pKGWGG-RR and the construct. For each digestion a 20-µl reaction was prepared with approximately 900 ng of plasmid DNA, 1 µl of enzyme, 2 µl of the specific buffer for the enzyme and water. After incubation for one hour at 37 °C, a 0.7% agarose gel was run.

PCR analysis for amplification of *NcHMA4* was performed with both entry and final construct, and genomic DNA as positive and water as negative control following the reaction: 94 °C 10min, 30 cycles of 94 °C 30 sec, 52 °C 30 sec and 72 °C 40 sec and 72 ° 10 min. Sequencing reaction of entry and recombinant vectors was prepared as well, following the same settings described in section 1. This construct will be used for further hairy root transformation.

NcHMA4 and NcZTP1 RNAi constructs

Making Constructs

PCR fragments of *NcHMA4* and *NcZTP1* containing CACC site at 5′ end of the forward primer were cloned into entry vectors (TOPO Gatway – Invitrogen™). The vectors were digested with ScaI and EcoRV (Figure 7). Afterwards both fragments were transferred separately to a destination vector by LR recombination reaction using the Gateway® LR Clonase™ Enzyme Mix (Invitrogen™) to create an expression clone. The LR reaction product was transformed into electro-competent DH5α *E. coli* by electroporation. Positive colonies were selected on LB agar plates containing 100 mg/L spectinomycin and confirmed by digestion of isolated plasmid DNA with restriction enzyme EcoRI (Figure 9). *Agrobacterium rhizogenes* was electroporated with the construct, checked for harbouring the desired plasmid (Figure 9) and then cultured and used for further hairy root transformation.

Hairy root transformation

Roots of *N. caerulescens* were transformed with RNAi constructs via *Agrobacterium rhizogenes* mediated transformation. Single colonies of transformed *Agrobacterium rhizogenes* were cultured in 3 mL LB media with 100 mg/L Spectinomycin at 28° C overnight. 200 µL from the liquid culture were plated in LB agar plates containing the same antibiotic and grew for 2 days at 28° C. Seeds of *N. caerulescens* accession La Calamine were sown in 4 plates of ½ MS (pH 5.8) without sugar, 25 seeds per plate. Seven-day-old plants had their roots removed just below the hypocotyl and were transformed with *A. rhizogenes*. One spot of aggregated bacteria was put at the tip of each hypocotyl for co-culturing and plates were put in the clime chambers supplying 250 µmol m^{-2} s^{-1} light at plant level during 16 hour/day period, 24 °C temperature, and 70% relative humidity. After five days, seedlings were transferred to new ½ MS (pH 5.8) without sugar supplemented with 100 mg/L tricarcillin to remove the bacteria. Transformed roots were checked every 3 days in a stereo-macroscope (Leica) through the expression of DsRED protein, and non-transformed roots were removed.

The transformed plants were transferred to pots filled with modified half-strength Hoagland's solution 42 days after hairy root transformation. The concentration of $ZnSO_4$ used was 10 µM, considered a normal concentration for *N. caerulescens.* After transfer of plants, roots were periodically check for the presence of non-transformed roots, and the non-transformed roots were cut.

Gene Expression Analysis

Roots of transformed plants and wild type were collected and stored in -80º C until RNA isolation. Samples of four HMA4 , one ZTP1 and three wild-type plants had their RNA isolated with the RNeasy kit (QIAGEN). After checking the integrity of RNA on agarose gel and the quantity and quality in NanoDrop®, cDNA was made using the iScript™cDNA Synthesis Kit (Bio-Rad) from 1000 ng of RNA. The concentration of the cDNA was measured in NanoDrop® and the 20 µl was diluted 5 times with 80 µl of water.

Specific primers for Real-Time PCR were designed for *NcHMA4* and *NcZTP1* (Table 2), and a pair of primers for the housekeeping gene NcTubulin already available in the lab was also used. The efficiency of the designed primers was tested by performing a 12 µl Real-Time PCR of 6 µl iQTM SYBR® Green Supermix (Bio-Rad), 0.25 µl of primers (100 nM), 2.5 µl of DNA and 3 µl water. Samples were prepared in duplicate, in a 2-fold dilution series of a cDNA sample from a wild type La Calamine plant and run in C1000™ Thermal Cycler (Bio-Rad) following the reaction: 3 min 95 ºC, 40 cycles of 15 sec 95 ºC and 1 min at 55ºC, followed by melt curve test starting at 62 ºC increasing 0.5 ºC each 0.05 sec until 95ºC. RNA diluted in the same 2-fold series was also included as control. The Threshold cycles (Ct) when the sensor can detect fluorescent signal from SYBR® Green dye binding to double-stranded PCR products of each gene were plotted with respective Log10 of dilution factors to obtain a regression curve using Bio-Rad CFX Manager software (Bio-Rad).

Expression analysis was performed with all cDNA samples. No-template control (sterilized MilliQ water, NTC) and RNA control (RNA samples diluted to same level as cDNA samples) were included to check for primer dimerization of each primer pair and DNA contamination on RNA samples, respectively. The same settings of reaction used for primers efficiency test were repeated. Data from Real-Time PCR, the Ct values, were analyzed to get relative quantification of gene expression using the 2-

ΔΔCt method described by Livak and Schmittgen (2001), using the expression: ΔΔCt = ΔCtsample – ΔCtcalibrator = (Cttarget – Ctreference)sample – (Cttarget – Ctreference) calibrator. Fold change in gene expression, or the normalized target amount, was then equal to $2^{-\Delta\Delta Ct}$ and applied to statistic analysis to compare samples.

Table 2. Primers designed for HMA4 and ZTP1 qPCR analysis of RNAi transformed plants.

HMA4 Forward	Z034 GTGGCAGAAGAGTTACTTCGACG
HMA4 Reverse	Z035 TTTGGAACGGGGAGATGAGG
ZTP1 Forward	Z036 AGAGACCGAGAGAGCAAAGG
ZTP1 Reverse	Z037 TTGCGTTCTTTGGTATCCCC

Establish a standard protocol for stable *N. caerulescens* transformation

The method developed by Guan *et al* (2008) was repeated in order to establish a suitable protocol for stable transformation and regeneration of *N. caerulescens.* Two different accessions of *N. caerulescens* were tested – La Calamine and Ganges, transformed with the vector pCambia2301, harbouring the GUS gene with Kanamycin resistance as selection marker. Seeds were sterilized by vapor-phase sterilization for four hours with 50 mL bleach added with 15 mL hydrochloric acid (37%) and germinated on 12-cm square plates containing ½ MS Media (Duchefa) without any source of carbohydrates for 15-20 days in clime chambers supplying 250 µmol m^{-2} s^{-1} light at plant level during 16 hour/day period, 24ºC temperature, and 70% relative humidity. Different tissues were cut as explants and transferred to Basic Culture Medium (BCM – Table 3) for 3 weeks – leaves, entire hypocotyls and hypocotyls cut in two. After this period of initial regeneration, the explants tissues were separated into two groups, and one group was transferred to Regeneration Culture Media (RCM – Table 3) and other group was transferred to Pre-Culture Media (PCM – Table 3). In the first group only regeneration was tested and in the second, transformation and regeneration were carried out. All plates were tapped with Micropore® tape, to permit a better exchange of gases, differently of the Parafilm® used by Guan *et al* (2008).

Transformation

Agrobacterium tumefaciens containing vector pCambia2301 was cultured in liquid LB with kanamycin at 28 °C overnight. The optical density (OD_{600}) was measured by spectrophotometer until it reached about 0.5. 5 mL of the culture was centrifuged at 4000 rpm for 5 minutes and then diluted 1:3 with liquid MS medium containing 100 µmol of acetosyringone. Explants were immersed in the bacterial suspension for 10 minutes, blotted onto sterile filter paper to remove excess of bacteria and then transferred to Co-Culture Media (CCM – Table 3) for 4 days at 28 °C on darkness.

Selection and Regeneration

The co-cultured explants were washed three times with distilled water and once with MS liquid medium containing 100 mg/L tricarcillin. Thereafter, explants were transferred to Selection Media with 100 mg/L tricarcillin (SCM-Tc – Table 3) for 7 days, but no selection and regeneration was possible, due to plant death.

Table 3. Composition of all culture medium used for *N. caerulescens* tissue culture. BCM: Basic Culture Media; PCM: Pré-Culture Media; CCM: Co-culture media;, SCM-Tc: Selection Media and RCM: Regeneration Culture Media

BCM	MS + 30 g/L sucrose + 6 g/L agar + 300 mg/L LH + 200 mg/L inositol + 1 mg/L BA + 0.2 mg/L NAA, pH 5.8
PCM	MS + 30 g/L sucrose + 7 g/L agar + 200 mg/L inositol + 2 mg/L KT + 2 mg/L BA + 1 mg/L NAA, pH 5.2
CCM	MS + 30 g/L sucrose + 7 g/L agar + 200 mg/L inositol + 2 mg/L KT + 2 mg/L BA + 1 mg/L NAA + 100 µmol/L AS, pH 5.2
SCM-Tc	MS + 30 g/L sucrose + 6 g/L agar + 200 mg/L inositol + 2 mg/L KT + 2 mg/L BA + 1 mg/L NAA + 100 mg/L tricarcillin, pH 5.8
RCM	MS + 30 g/L sucrose + 7 g/L agar + 200 mg/L inositol + 2 mg/L KT + 2 mg/L BA + 1 mg/L NAA, pH 5.8

Results

ZTP1 Promoter Isolation

A Genome Walking approach was taken in order to isolate the *NcZTP1* promoter sequence for further studies with this gene. The fragments obtained were purified from agarose gel (Figure 1), and after cloning and sequencing, were cleaned from vector contamination using the *VecScreen* tool from NCBI (National Center of Biotechnology Information) website. Afterwards, contigs were created on Vector NTI (Invitrogen) software Contig Express tool. Two contigs were created. On comparing the two contigs to each other through BLAST2, part of the sequences were completely identical and partially divergent (Figure 2). Both contigs were also compared to a nucleotide database in BLASTn (nucleotide BLAST - NCBI), and homology with *AtMTP1* genomic sequence was detected, in a region of an intron upstream the start codon. Also there was similarity with the cDNA region of *NcZTP1* gene where the primers were designed.
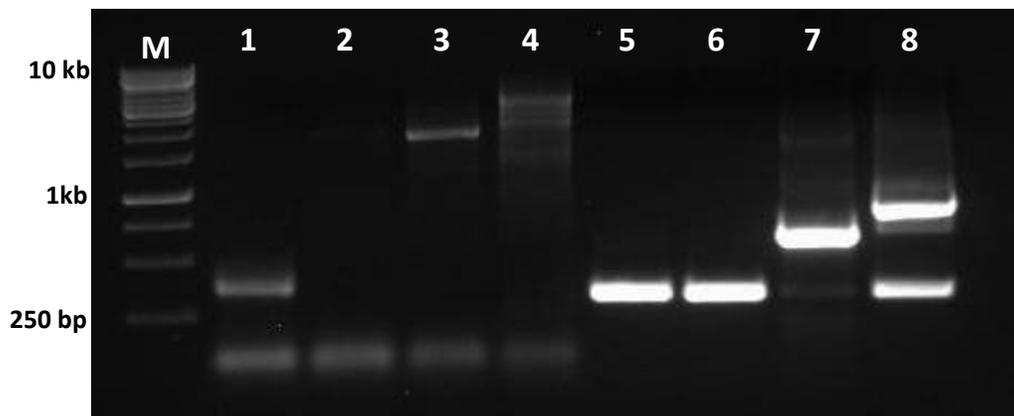

Figure 1. Agarose gel (1.0%) of the amplified fragments on the first (1-4) and second (5-8) Nested PCR from ZTP1 walking for promoter isolation. Fragments ranging from 300 bp to 1 kb of the second nested PCR were excised and isolated from gel. M: 1kb marker
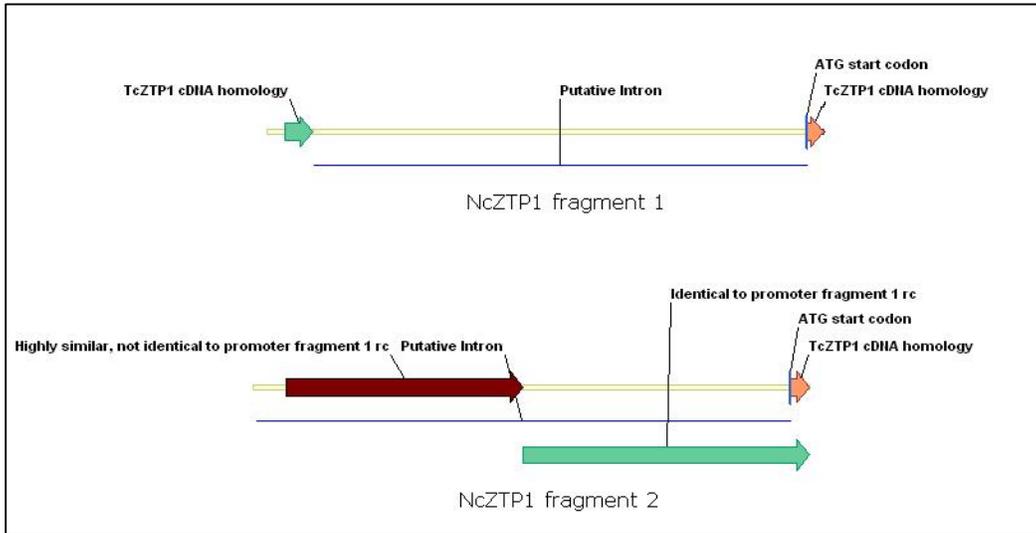
Figure 2. Two contigs formed by the assembly of the 5 fragments isolated from gene walking of *NcZTP1*. The isolated region showed similarity with an intron upstream the coding sequence of the *Arabidopsis thaliana* homologous gene *AtMTP1*. BLAST2 showed that part of the sequences were identical between the two contigs (green arrow – fragment 2), and part of sequence was highly similar but contains differences (red arrow – fragment 2). Also, BLAST with the *NcZTP1* cDNA sequence showed similarity with the region where the primers were designed (orange arrow).

A second round of genome walking was performed, in order to isolate the sequence upstream the intron. Specific primers were designed (Table 4) for each fragment identified in the previous gene walk. After the second nested PCR, an agarose (1%) gel was run (Figure 3) and the same steps described for the first round were followed to obtain the sequence of the fragments, however at miniprep step a very low yield was obtained and therefore no sequencing was possible. The DNA purified from gel will be cloned in a different vector, D-TOPO® (INVITROGEN), and further transformation of *E. coli* competent cells, miniprep and sequencing will be performed to isolate the promoter sequence of *NcZTP1*.

Table 4. Specific primers designed for each of the isolated fragments from the first gene walk attempt

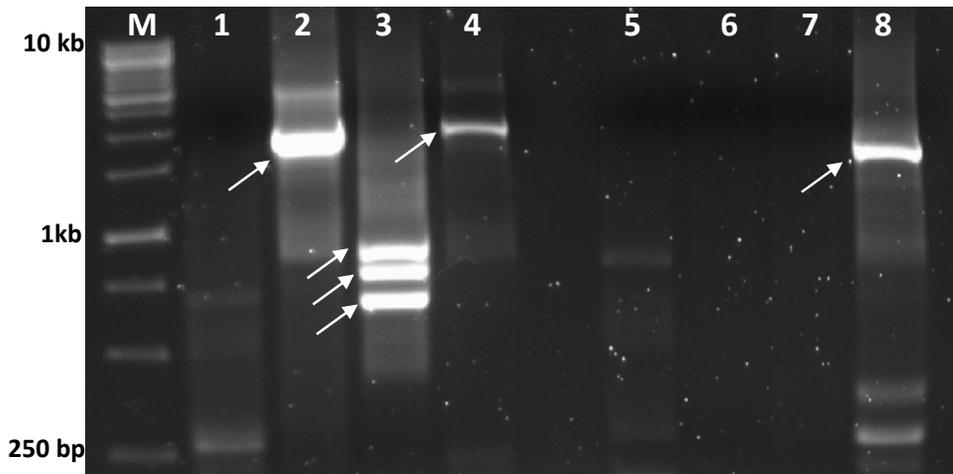|	**ZTP1 Fragment 1**	**ZTP1 fragment 2**
GSPa	Z028 GGAGCACCTAAAGGAGTAGAGC	Z030 TTGTGAAGAGACCTCAAAAACCC
GSPb	Z026 TAAGACCTGCCTTTTCTGGG	Z031 CCTAAAAAAACGTGGCCGCC
GSPc	Z027 TCCCTTCCTTTGCTCTCTCG	Z029 AATCGAAAGAAAGGAGGTAAA

Figure 3. Agarose gel (1,0%) of the amplified fragments of the first (1-4) and second (5-8) fragments in the second *NcZTP1* walking for promoter isolation. Bands ranging from 700 bp to 2 kb that are indicated with white arrow were isolated from gel for further sequencing. M: 1kb marker

NcHMA4 **Promoter + Reporter gene Construct**

An entry vector containing a 2.1 kb fragment of the *NcHMA4* promoter region was recombined by LR reaction with a pKGWGG-RR destination vector, which contains a translational fusion of the GFP and GUS gene to track promoter activity, and also a DsRED marker gene, which confers a red fluorescence to the transformed roots and is useful to identify and select transformants. The construct will be used for further experiments to study the activity of the *NcHMA4* promoter.

The recombinant vectors were digested with EcoRI restriction enzyme (Figure 4), which should produce three fragments, two from EcoRI sites in the sequence of the vector and one in the promoter sequence. However, digestion of many different constructs showed a similar pattern missing one expected fragment, for instance shown at figure 4. Digestion with AccI was also performed with the construct, using the pKGWGG-RR vector as a control. This enzyme should produce four fragments on the pKGWGG-RR and three on the construct, but, despite the pattern for the construct that seemed to be as expected, the control showed five instead of the four expected fragments (Figure 5A). The pKGWGG-RR destination vector was also digested with BamHI and EcoRI, and four fragments instead of the five expected for BamHI (Figure

5B) were observed, but for EcoRI the fragments observed were consistent with the expected. Independent and double digestion with EcoRI and NotI was performed to the entry vector (Figure 5C) containing the *NcHMA4* promoter, with one fragment expected for each single digestion and two for the double digestion. However, double cut was observed in NotI individual digestion and single fragments observed in EcoRI single digestion and NotI+EcoRI double digestion.

Since the results from digestion were not consistent with the expected, PCR analysis was conduced to confirm the insert of *NcHMA4* in the construct and entry vector. Primers flanking the EcoRI site in the promoter sequence were designed, but amplification of the 192 bp expected fragment was not observed for both entry vector and construct, neither for the genomic DNA (Figure 6), due to primer dimmer.

In addition, sequencing was done for both entry vector and the destination construct in order to check insertion and the presence of EcoRI site in the insert.
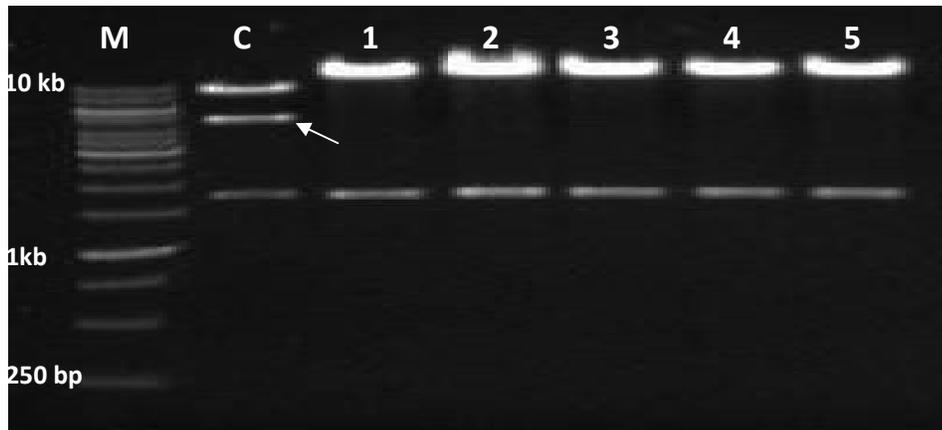

Figure 4. Gel picture of 5 samples of *NcHMA4* promoter construct with GFP/GUS and DsRED and empty pKGWGG-RR vector (C) digested with EcoRI. White arrow shows the 4 kb fragment that is produced when ccdB site is cut. *NcHMA4* promoter sequence also contains a EcoRI site, but the similar expected band was not visualized. M: 1 kb DNA Marker.
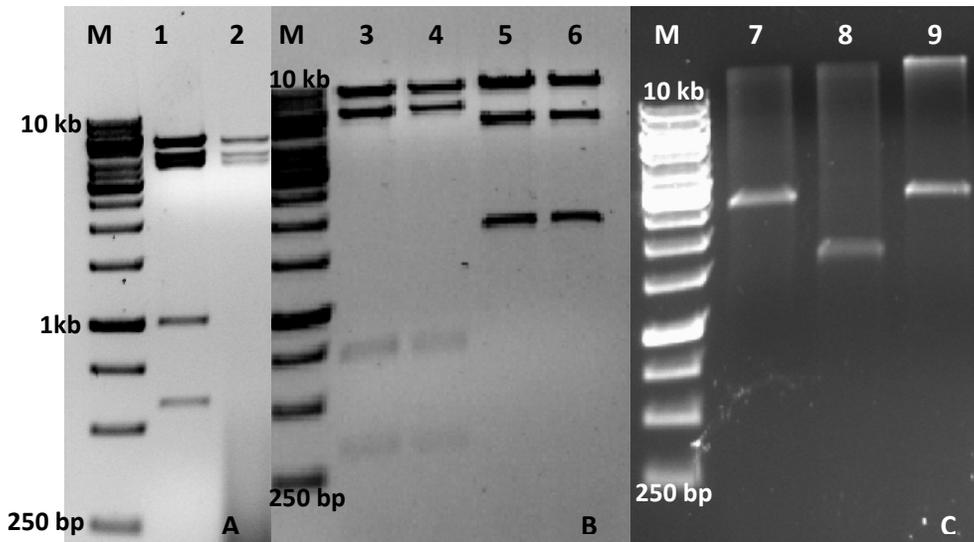
Figure 5. 0.7% agarose gel pictures of vectors digestions. **A**: digestion of pKGWGG-RR (1) and *NcHMA4* promoter construct (2) with AccI, with an unexpected 5 fragments of pKGWGG-RR while for the construct the pattern was as expected. **B**: pKGWGG-RR vector digestion with BamHI (3 and 4) showing four instead of the five expected fragments, and EcoRI (5 and 6) showing the expected pattern. **C**: entry vector double digestion with EcoRI and NotI (7), digestion with EcoRI (8) and digestion with NotI (9). M: 1 kb marker.
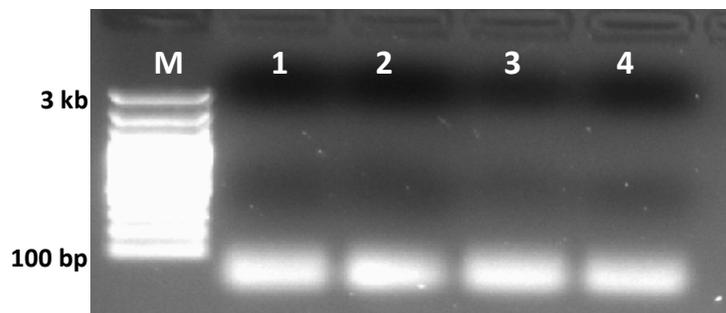
HMA4 promoter construct (2). Water (3) and genomic DNA (4) were used as control. None of the samples amplified the 192-bp expected fragment. Primer dimers were formed. M: 100 bp plus.

NcZTP1 and *NcHMA4* RNAi constructs

RNAi constructs of *NcHMA4* and *NcZTP1* were developed for further transformation into *N. caerulescens* plants and to study knocked down plants. Entry vectors containing *NcZTP1* and *NcHMA4* fragments separately were digested with ScaI and EcoRV (Figure 7). After confirmation of the entry vector, both fragments were transferred separately to a destination vector, pk7GWIWG2 (II) RR, that contains two attR1/attR2 recombination sites, in opposite directions and separated by an intron, under the control of the Cauliflower Mosaic Virus (CaMV) 35S promoter (p35S) and the 35S terminator (T35S), and the DsRED selectable marker gene, encoding a red fluorescent protein, under control of the promoter derived from the polyubiquitin, UBQ10 of *A. thaliana* (pUB) and the terminator derived from the noptaline synthase gene (nos) (Figure 8). This construct, when expressed in the plant, produces an mRNA stem-loop structure, triggering the RNAi mechanism for silencing the target gene.

After confirmation by digestion of the plasmids isolated from the transformed *E. coli* with restriction enzyme EcoRI (Figure 9), *Agrobacterium rhizogenes* was electroporated with both constructs separately and was also checked for harbouring the desired plasmid by digestion with EcoRI (Figure 9). The two new constructs (*NcZTP1* and *NcHMA4*) were named pBG0084 and pBG0085, respectively (Figure 10). These constructs were used for hairy root transformation experiment and will be used in future experiments.
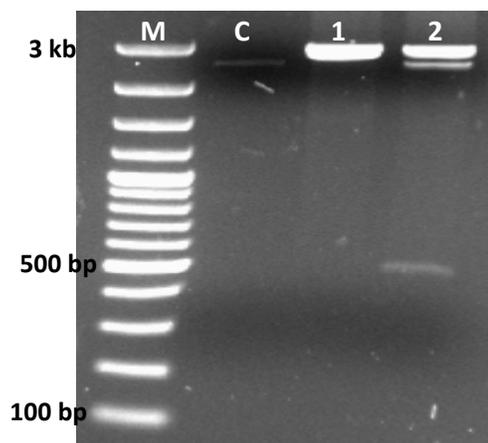

Figure 7. Digestion of D-TOPO entry vectors containing NcZTP1 (1) and NcHMA4. (2) cDNA with EcoRV and ScaI. C: empty vector control. M: 100 bp plus Marker.
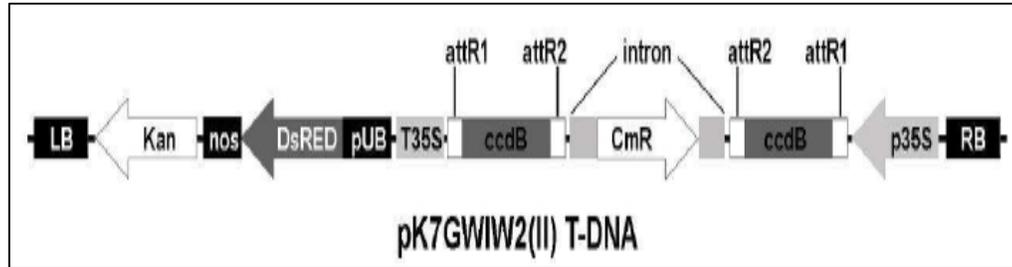
Figure 8. Picture of pK7GWIW2 (II) RNAi destination vector, that contains two attR1/attR2 recombination sites, in opposite directions and separated by an intron, under the control of the Cauliflower Mosaic Virus (CaMV) 35S promoter (p35S) and the 35S terminator (T35S), and the DsRED selectable marker gene, encoding a red fluorescent protein, under control of the promoter derived from the polyubiquitin, UBQ10, of *A. thaliana* (pUB) and the terminator derived from the noptaline synthase gene (nos).
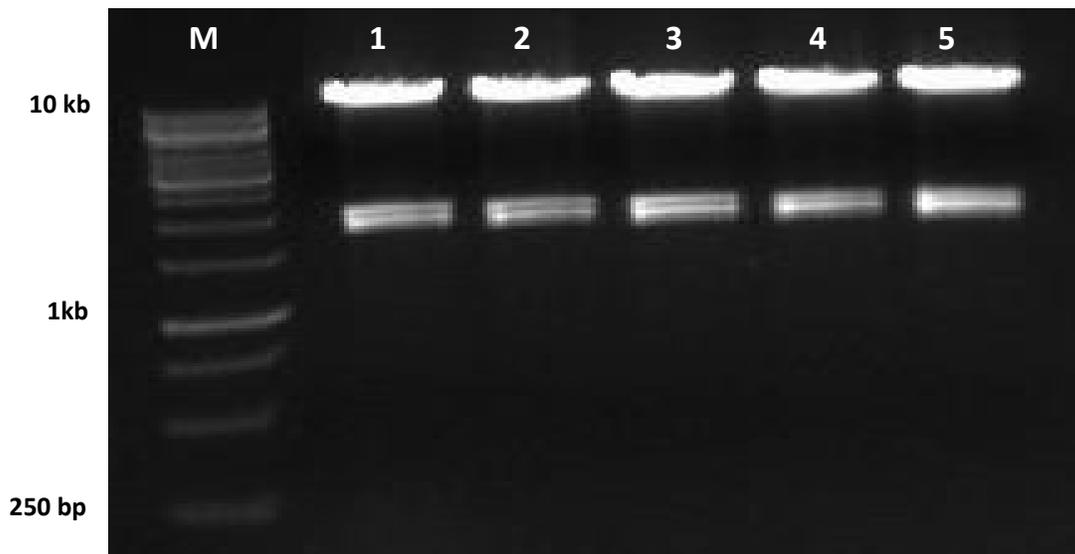

Figure 9. Digestion of *NcHMA4* (1-3) and *NcZTP1* (4-5) RNAi constructs with EcoRI for confirmation of *Agrobacterium rhizogenes* transformation. Digestion produced the expected 3 fragments with approximately 10 kb, 1.4 kb and 1.8 kb. M: 1kb marker.
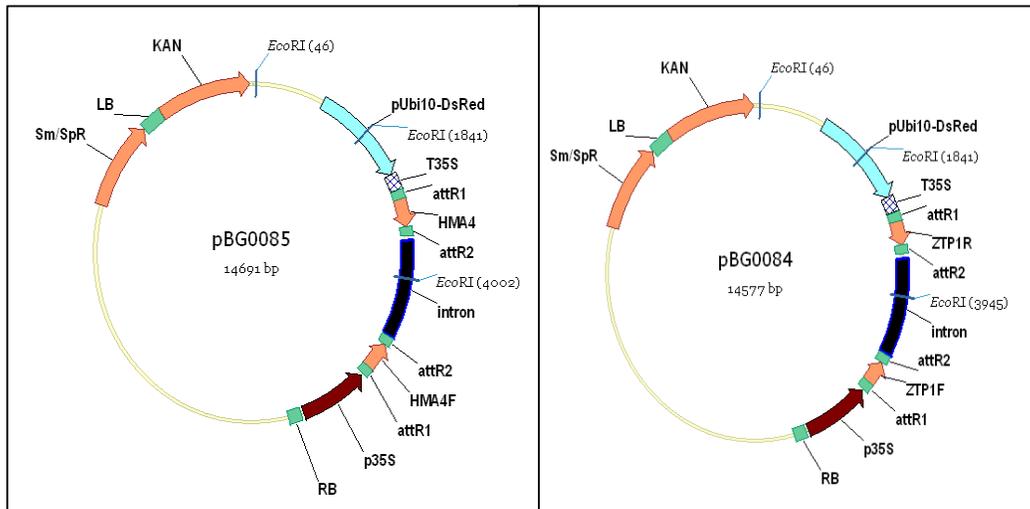
Figure 10. RNAi constructs of HMA4 (left) and ZTP1 (right). Segments of cDNA of each gene are inserted in both forward and reverse directions spiced by an intron, to form a hairpin and trigger the silencing mechanism in the transformed plants. The constructs were named pBG0085 and pBG0084 and will be available for further experiments.

Hairy root transformation

Seeds of *N. caerulescens* were sown in agar plates and seedlings were transformed with RNAi constructs of *NcHMA4* and *NcZTP1* by *Agrobacterium rhizogenes* mediated hairy root transformation. From 100 *N. caerulescens* (ecotype La Calamine) sown seeds, approximately 70 germinated, and 30 plants were transformed with *NcHMA4* and 30 *NcZTP1*. Also, 10 plants had their roots cut but were not transformed, as Control. Plants were visualized at least twice a week in a stereo-macroscope (Leica) under DsRED filter to verify root transformation, and all non-transformed roots were cut after each verification.

After 40 days of checking the roots regularly, the majority of the transformed plants did not show any red root, and were considered as non-transformed. For *NcZTP1*, six plants showed at least one red root, and only two plants showed all roots transformed. For *NcHMA4*, two plants showed all roots transformed, four plants had at least one transformed root and one plant showed a red spot on the base of hypocotyl, but roots did not grow. Transformed plants and roots for each gene are shown in Figure 11.

After 42 days in ½ MS plates, plants were transferred to pots filled with modified ½ Hoagland Solution containing 10µM ZnSO$_4$, that is considered to be the

normal concentration for *N. caerulescens*. Two pots for each construct containing 3 plants each, and two pots containing 3 control plants each were set up. Two weeks after transfer, most of plants showed good growth and root proliferation. Nonetheless, ZTP1 transformants did not grow due to a root fungal contamination, except for one plant that survived the contamination and developed well. No high metal exposure was performed, due to the presence of few non-transformed roots.

Afterwards, a new experiment with 100 seeds was started and seedlings transformed, but after some weeks checking for transformants, a wide-spread fungal contamination was found and the plates were discarded.
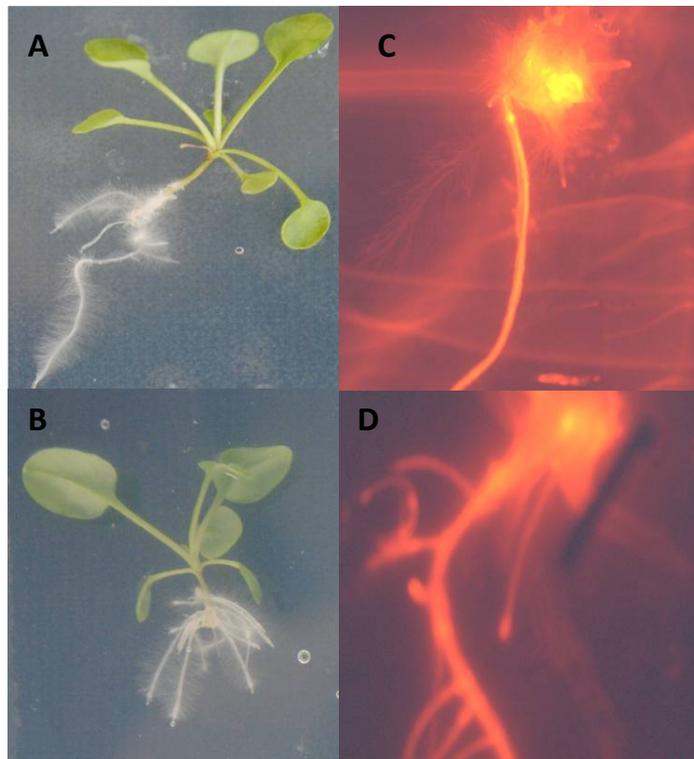

Figure 11. One month old transformed plants with *NcHMA4* (A) and *NcZTP1* (B) RNAi constructs and transformed roots (C and D, respectively *NcHMA4* and *NcZTP1*) shown under DsRED filter in stereo-macroscope.

Gene Expression Analysis of RNAi transformed plants

To check whether the targeted genes were knocked down, roots of four plants transformed with HMA4, one ZTP1 and three wild type plants were used for quantitative Real Time PCR. Primers efficiency test was performed for ZTP1 and HMA4 primers and efficiencies of 94.4% and 89.6%, respectively, were found (Figure 12A and 12B). R^2 (R2) values of the regression curves must be higher than 0.95 and ideally higher than 0.985. Slope values of the regression curves were used to calculate primer efficiencies with the following equation: Efficiency = 10(-1/slope) -1. Acceptable primer efficiencies for Real-Time PCR are in the range of 80 – 120% and ideally in the range of 90 – 110%. The values obtained are in the acceptable range of efficiency and therefore were used for gene expression analysis. Melt curves showed for each sample a unique peak, indicating that there was no contamination of cDNA and RNA or primer dimer formation.

The Ct values obtained in expression analysis were normalized with the values of expression of housekeeping genes (ΔCt), ΔΔCt was calculated using the ΔCt value of wild type 1 tested with *HMA4* primers as a calibrator, and the 2-ΔΔCt values were obtained. The expression of transformed plants with the *HMA4* RNAi construct showed that none of the transformants had their *HMA4* expression knocked down (Figure 13A). For *ZTP1*, the gene is clearly down regulated in the transformant compared with any of the wild types (Figure 13B). Furthermore, *HMA4* is 4-fold higher expressed than *ZTP1* in roots of wild plants.
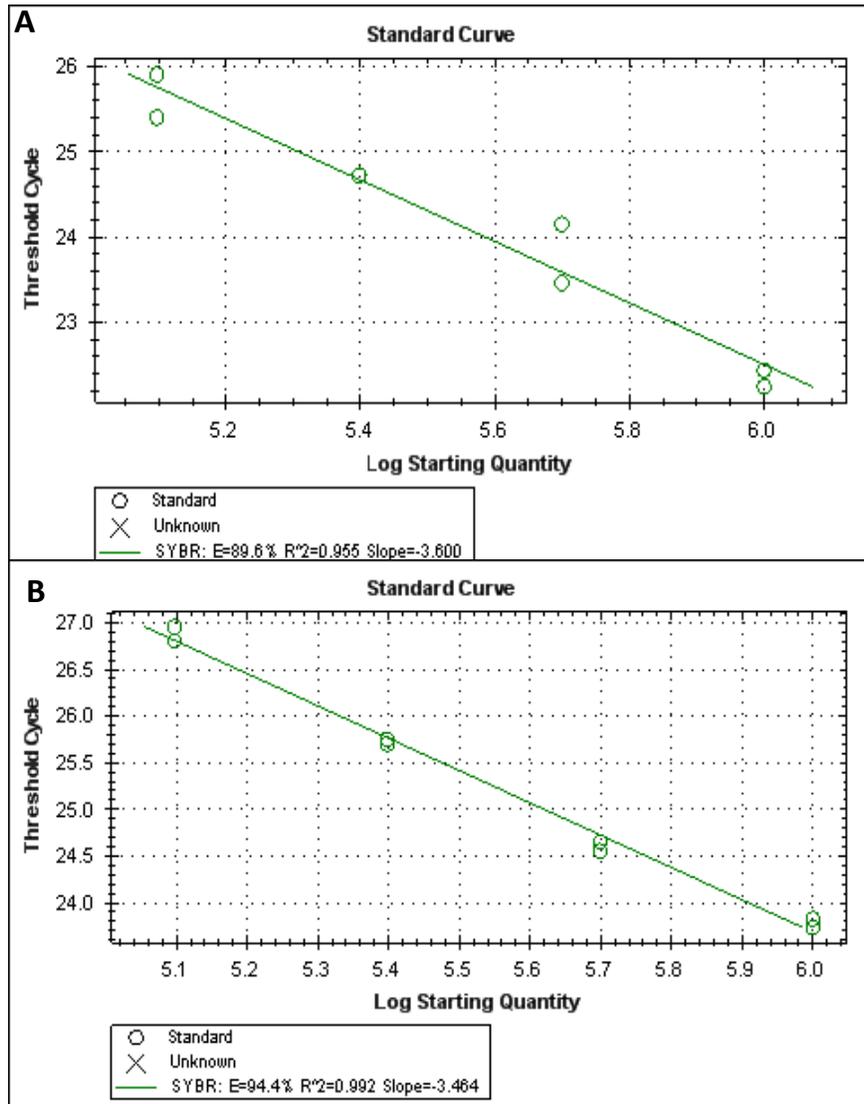
Figure 12. Primers efficiency test. **A:** Standard curve of ZTP1 primers, which have efficiency of 94,4%; **B:** Standard curve of HMA4 primers, which have 89,6% of efficiency.

Figure 13. Expression of *HMA4* and *ZTP1* genes in three wild types (WT), four *HMA4* Transformed Plants (HMA4-TPs) and one *ZTP1* transformed plant (ZTP). 2-ΔΔCt values plotted in the graph shows that any of HMA4-TP had their expression knocked down compared to WT. The *ZTP1* transformed plants showed strong down regulation of the gene. Furthermore, *HMA4* is 4-fold more expressed than ZTP1 in WT.

Stable Transformation

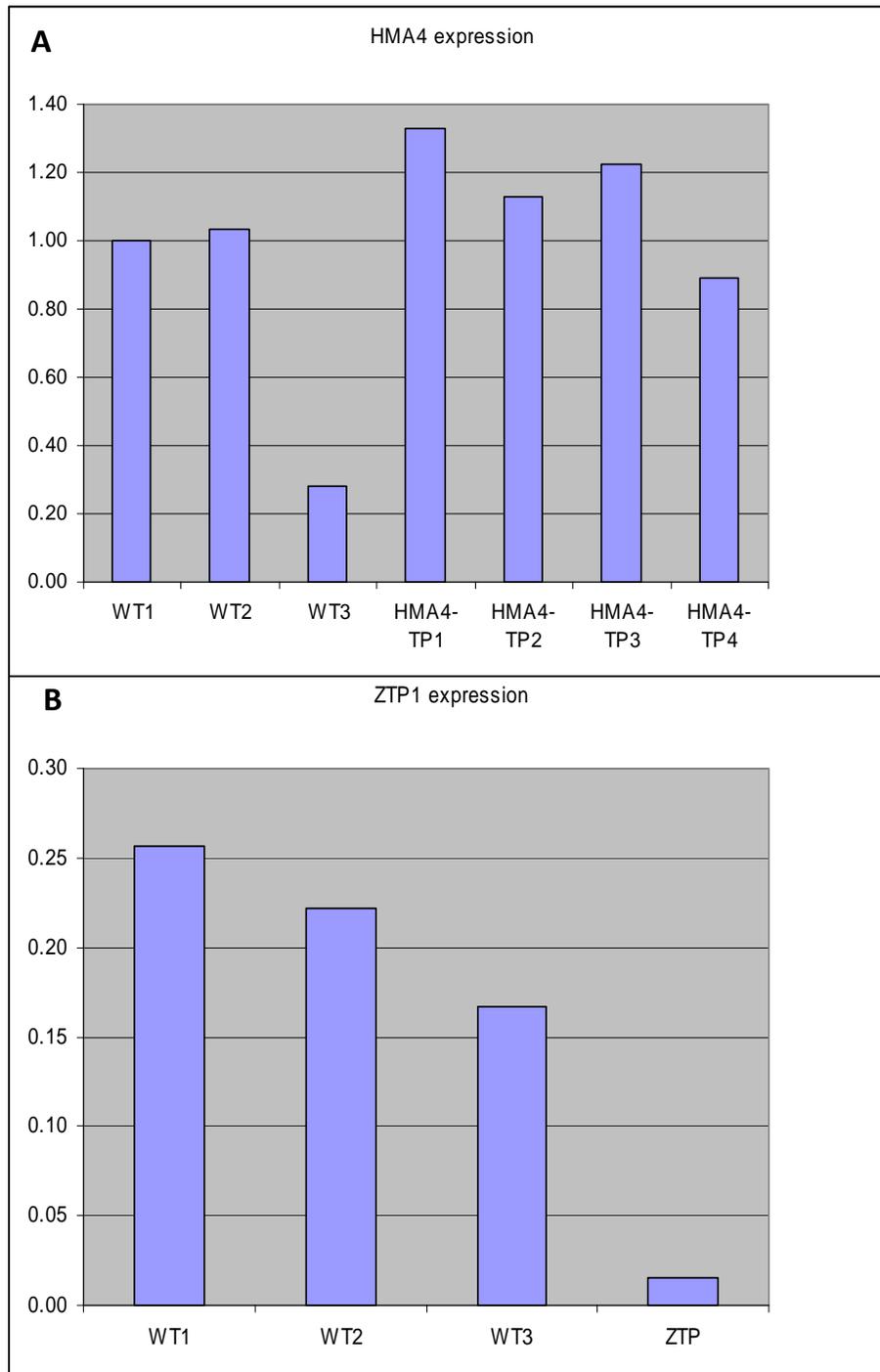Shoots and hypocotyls of *N. caerulescens* ecotypes La Calamine and Ganges were used as explants for transformation and regeneration of new plants, following the method described by Guan *et al* (2008). From the plant tissues that were cultured in BCM media, both La Calamine and Ganges had some of the cultured shoot showing proliferation of new clusters of shoots, but any hypocotyl (entire or half) showed shoot cluster growth. After three weeks in Basic Media, plants were divided into two groups and were transferred to PCM and RCM (Table 5), for pre culture before transformation and regeneration, respectively. After four days in PCM, plants were transformed.

For the group of transformed explants, after co-culture in dark for four days the explants were still showing green shoots. After 4 days, though, most of them were completely white and some did not survive. After one week, all the transformed explants had died. The explants in RCM showed a high proliferation of cells, mainly in Ganges, but differentiation was not observed (Figure 14). The Ganges hypocotyl looked greener than La Calamine, and for both accessions the shoots did not develop anymore, some were already dead after two weeks. The media was changed and the dead parts removed, but no growth was observed anymore. The occurrence of regeneration of new shoots from the different cultured tissues is shown on Table 6.

Both entire hypocotyls and ½ hypocotyls of Ganges showed growth of callus with a visibly increased mass, and some showed some shoots starting but no complete regeneration of shoots or roots was observed. La Calamine ½ and entire hypocotyls of LC 7.8 and LC 7.13 showed mass growth, but entire hypocotyls had cell growth only for few weeks, while ½ hypocotyls kept growing. Some callus of LC ½ hypocotyls showed small roots, but no regeneration of shoots or roots was observed. The growth of cells was observed until approximately 2 months of culture, after this period tissues looked dead or stopped growing. Hypocotyls of Ganges and La Calamine are shown in figure 14.

Table 5. Number of each type of tissue of *N. caerulescens* ecotype La Calamine and Ganges cultured in PCM and RCM.

	PCM			RCM		
	Shoot	Hypocotyl	½ hypocotyl	Shoot	Hypocotyl	½ hypocotyl
LC 7.8	3	3	6	3	3	6
LC 7.13	3	4	8	2	4	8
Ganges	5	11	14	9	10	14

Table 6. Regeneration of new shoots from cultured tissues of *N. caerulescens* in RCM or PCM.

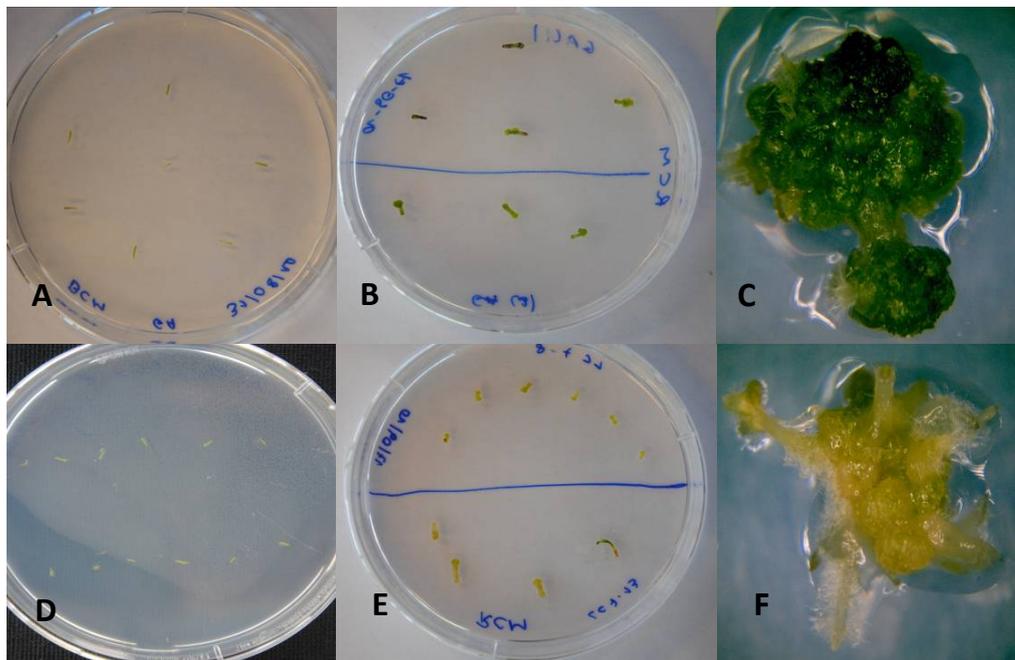|	LC 7.8	LC 7.13	Ganges
Shoots	4	2	4
½ Hypocotyls	0	0	0
Entire Hypocotyls	0	0	0

Figure 14. Cultured hypocotyls of accession Ganges (A, B, C) and La Calamine (D, E, F). **A** and **D** represents hypocotyls with 18 days old cultured in BCM media; **B** and **E** represents hypocotyls one month old cultured in PCM media and **C** and **F** represents hypocotyls of 2 months old visualized in stereomacroscope.

Discussion

In this work I aimed to study the function of the *NcHMA4* and *NcZTP1* genes, that are, respectively, important genes in metal transport to the shoots (Papoyan and Kochian, 2004) and accumulation into vacuoles (Kobae *et al.*, 2004). To accomplish this goal, we focused on the creation of RNAi and promoter + reporter gene constructs. Also, a parallel work with regeneration of plants in vitro and stable transformation was performed to permit further studies with stable transformation system, which is the main limiting step in understanding mechanisms related to metal homeostasis in *N. caerulescens*.

Promoter Constructs

Sequences of *NcZTP1* promoter isolated from ecotype La Calamine showed homology with an intron between the coding sequence and the promoter region of the *AtMTP1* gene, the orthologue gene of *NcZTP1* in *Arabidopsis thaliana* (Assunção *et al*, 2001). The complete genome sequence in *N. caerulescens* is not yet known, but analysis of the expression profile in *N. caerulescens* showed high similarity with the *A. thaliana* genome, approximately 88% (Rigola et al, 2006), and therefore the genome of *A. thaliana* is used as a reference to find non-coding regions on *N. caerulescens*.

Furthermore, two contigs were assembled from the sequences obtained and BLAST2 between these contigs showed that they are highly similar, but not the same sequence. This can be result of isolation of two alleles in the plant, but, because the DNA used was from a La Calamine plant that is result of several self-crossing, this hypothesis is very unlikely. Also, these two sequences can indicate the presence of a paralogue within the genome of *N. caerulescens.* Previous southern blot analysis of *N. caerulescens* and *A. thaliana* showed that there appear to be other *MTP1/ZTP1* homologous DNA sequence present in both *N. caerulescens* and *A. thaliana* (Assunção *et al*, 2001), but a corresponding cDNA was not found and expression has not been tested. In *A. halleri* zinc tolerance was attributed to an increase in copy number of MTP1 gene (Dräger *et al*, 2004). Further analysis will allow confirming whether these are paralogous genes and characterize them.

The sequencing of the *NcZTP1* promoter region is close to be completed and further studies will be possible by making constructs with this promoter leading to the

expression of reporter genes. This kind of construct can be an important tool to study tissue specific activity of a gene, tracking where and whether it is active under different metal conditions *in vivo*.

A construct was made by LR reaction between an entry vector containing the promoter region of *NcHMA4* gene and a destination vector harbouring GFP/GUS as reporter genes. The activity of the promoter, however, could not be tested by transforming plants, since the final construct showed an unexpected profile when digested with different enzymes and thus was not confirmed to be correct. Digestion of the entry vector showed the expected EcoRI site in the sequence of the promoter (Figure 5), therefore the missing site in the construct can not be a PCR error. One possibility could be that some contamination of vectors occurred at some step of the experiment, causing the unexpected pattern in digestions. Sequencing of the constructs was conducted to check whether the sequence is correct or not in both entry vector and the final construct, and sequences will be further analyzed. Afterwards, some plants can be transformed with the construct to check if the promoter can be tracked or not with this construct, but this approach was not tested so far.

In Arabidopsis, a tissue-specific expression analysis showed that the HMA4 gene is localized primarily to the vascular tissue in roots, leaves and stems (Hussain *et al*, 2004) and other analysis showed that it functions delivering metals into the xylem for transfer to the roots (Mills *et al*, 2004). In *N. caerulescens*, *HMA4* showed to be expressed strongly and almost exclusively in the roots, and its expression was up-regulated by exposure to high Cd and Zn (Papoyan and Kochian, 2004). By comparison with the homologue in Arabidopsis, *NcHMA4* was hypothesized to play a similar role (Papoyan and Kochian, 2004). However, no tissue-specific expression analysis was done yet to check if the localization of the gene is the same as in Arabidopsis, and such study of tissue and cellular localization will help to unravel gene function in metal homeostasis. Therefore, to have a good construct it is very important to further study the *NcHMA4* promoter activity *in vivo*.

RNAi constructs

Two RNAi constructs were made, one of *NcHMA4* and one of *NcZTP1*, and plants were transformed to obtain a knocked down phenotype. The study of knockout mutants in different organisms is a common approach that has being used to discover the function of genes by observing the phenotype in the absence of the targeted gene. In Arabidopsis, T-DNA knockout plants for several genes are available and mutant seeds are commercially available for study purposes. RNAi technology is a promising tool to study the function of targeted genes, especially in species for which the complete genome is not sequenced and annotated.

After co-culture of 30 seedlings with each construct, the frequency of transformation for both constructs was found to be low. Additionally, even if plants show the presence of DsRED on roots, the marker gene for the transformation, the genes can be not knocked down, as discussed below. Beside that, non-transformed roots were still growing along with transformed ones. This shows how laborious it can be to obtain fully transformed roots, and have plants that can be tested with high and low metal exposure. Despite the low transformants frequency, roots of four HMA4, one *ZTP1* transformed plants and three wild type plants were used for gene expression analysis by Real-Time PCR, in order to check whether the constructs were able to knock down *HMA4* and *ZTP1* on the tested roots.

For *ZTP1*, the gene was much lower expressed, around ten times in the transformed roots compared to the wild-type. This shows that the *NcZTP1* RNAi construct is efficient . In addition, *NcHMA4* was found to be 4-fold more expressed in roots of wild type plants than *ZTP1* (Figure 13). The *NcHMA4* is mainly and highly expressed in roots (Papoyan and Kochian, 2004), while *NcZTP1* is expressed in roots and shoots (Assunção *et al*, 2001), mainly in shoots. The efficiency of the construct targeting *ZTP1* in *N. caerulescens* roots could be due to this gene be constitutively lower expressed than *HMA4* in roots and therefore easier to be silenced, but generally highly expressed genes are easier to be knocked down, although the expression of *HMA4* genes was not decreased in the transformed plants. Nevertheless, only few plants were tested and a bigger experiment would be needed to affirm whether the constructs for *HMA4* could not decrease the expression because of the higher expression of this gene in roots or due to inefficiency of the construct for some reason.

Furthermore, the transformed plants with *NcZTP1* construct did not show any visible difference of phenotype in normal concentration of Zn (10μM) compared to the wild type plants after 4 weeks in ½ modified Hoagland's hydroponics media. Although this concentration could be already enough to cause stress in the plants due to ZTP1 silencing, the presence of more than one copy of *NcZTP1* gene in *N. caerulescens* could lead to a redundant activity of the genes, and therefore when one copy is silenced, other copies could replace the activity provided the copies are not very similar. Though not tested yet, the two different sequences found in promoter isolation of *NcZTP1* suggest that there is more than one copy of this gene in *N. caerulescens*, but the level of similarity between the coding sequences need to be tested. In *A. halleri*, a hyperaccumulator and closely related species to *N. caerulescens,* it was found that there are more than one copies of *MTP1* gene (Dräger *et al.*, 2004). For *HMA4*, this gene is triplicated within the genome of *A. halleri* (Hanikenne *et al*, 2008). If the same variation of copies occurs in *N. caerulescens*, this could also explain the difficulty in knocking down the expression of this gene. An unpublished recent data indicates that there are at least four copies of *NcHMA4* in *N. caerulescens* (Iqbal and Schat, unpublished)

Further experiments with more transformants subjected to different metal exposure conditions will allow to understand the function and essentiality of *NcHMA4* and *NcZTP1* in *N. caerulescens*. Moreover, further southern blot and other analysis would be needed in order to identify different copies of *NcZTP1* and their sequences. Also, expression analysis on shoots together with mineral content analysis could also be tested to see how the low expression in roots affects the metal homeostasis and gene expression in the whole plant.

Stable Transformation

Stable transformation of *N. caerulescens* was attempted by repeating the method described by Guan *et al* (2008). The cited authors have tested the regeneration from shoots, cotyledons and hypocotyls, and afterward transformed shoots, while only shoots and hypocotyls were used in this study for transformation and regeneration. The same media composition, conditions of transformation, selection and regeneration, and periods of culture were applied, however the same results were not obtained.

Regarding the transformation of explants, none of transformed explants survived within one week after transformation. According to Guan *et al* (2008), putative transformed shoots should be readily obtained in large amounts. Despite all the conditions were the same, the bacteria strain used by the authors was EHA105, while the strain tested in this work was AGLO, therefore it is possible that the bacteria strain used in our work have caused the death of explants overgrowing and causing irreversible damage. However, it is not possible to conclude that the bacteria had caused the death of tissues, and further tests with different strains, their concentrations and periods of co-culture are needed.

The regeneration of explants was tested. From shoots clusters as explants, growth of new shoots was observed for some of the cultured plants in both La Calamine and Ganges accessions, however, not as many as described by Guan et al (2008). In addition, after two weeks in RCM media, shoots stopped growing or died, while Guan *et al* (2008) described a complete regeneration of shoots into new plants after regeneration cycle. It was observed that the Micropore® tape used to seal the plates, despite very advantageous permitting a better gas exchange with the environment, also causes a quicker drought of the media, and consequentially, of the explants. This finding indicates that the shoots can have died due to the drying of the media. Therefore, to transfer the plants more often to new plates is advised, in order to avoid drying.

Furthermore, no regeneration from hypocotyls cultured as explants in BCM media was found by Guan et al (2008) and therefore RCM media was not tested for these tissues. Indeed regeneration of shoots was not found from the hypocotyls of Ganges and La Calamine cultured in BCM media tested in this work, however, the explants were transferred and kept in RCM. These explants showed a visible increased cell division and a disordered growth of very small roots (La Calamine) and shoots

(Ganges). Since hypocotyls explants on RCM are not described in the reference work, it is not possible to confirm whether the same phenotype was found. It is possible that the balance of hormones used by the Guan *et al* (2008) in RCM media is not the ideal to induce regeneration of shoots from hypocotyls. Different combinations of hormones were tested by the authors before indicating the best one, but all combinations were tested for shoots. The high proliferation of cells in the hypocotyls, mainly ½ hypocotyls, suggests that these tissues are capable to regenerate if the ideal balance of hormones is supplied, and, therefore, further experiments should test different combinations of BA, KT and NAA in order to develop a suitable media for regeneration of *N. caerulescens* hypocotyls.

Finally, testing all the adjustments suggested and also include other further improvements can enable to develop a reproducible protocol for transformation and regeneration of *N. caerulescens*. A well established and reproducible method would provide a huge contribution for the metal hypperaccumulation field, for instance permitting to test constructs like the ones developed in this work not only in roots, but also at the whole plant level.

References:

Arrivault, S., Senger, T. and Kramer, U. (2006) The Arabidopsis metal tolerance protein AtMTP3 maintains metal homeostasis by mediating Zn exclusion from the shoot under Fe deficiency and Zn oversupply. *Plant Journal*, **46**, 861-879.

Assuncao, A.G.L., Bookum, W.M., Nelissen, H.J.M., Vooijs, R., Schat, H. and Ernst, W.H.O. (2003b) Differential metal-specific tolerance and accumulation patterns among *Thlaspi caerulescens* populations originating from different soil types. *New Phytologist*, **159**, 411-419.

Assuncao, A.G.L., Martins, P.D., De Folter, S., Vooijs, R., Schat, H. and Aarts, M.G.M. (2001) Elevated expression of metal transporter genes in three accessions of the metal hyperaccumulator Thlaspi caerulescens. *Plant Cell and Environment*, **24**, 217-226.

Assuncao, A.G.L., Schat, H. and Aarts, M.G.M. (2003a) Thlaspi caerulescens, an attractive model species to study heavy metal hyperaccumulation in plants. *New Phytologist*, **159**, 351-360.

Becher M., Talke I.N., Krall L., Krämer U. (2004) Cross-species microarray transcripts profiling reveals hight constitutive expression of metal homeostasis genes in shoots of the zinc hyperaccumulator *Arabidopsis halleri*. *Plant Journal*, **37**, 251-268. in Verbruggen, N., Hermans, C. and Schat, H. (2009) Molecular mechanisms of metal hyperaccumulation in plants. *New Phytologist*, **181**, 759-776.

Bell, R.W., Dell, B. (2008) Micronutrients for Sustainable Food, Feed, Fibre and Bioenergy Production. IFA, Paris.

Blaudez, D., Kohler, A., Martin, F., Sanders, D. and Chalot, M. (2003) Poplar metal tolerance protein 1 confers zinc tolerance and is an oligomeric vacuolar zinc transporter with an essential leucine zipper motif. *Plant Cell*, **15**, 2911-2928.

Desbrosses-Fonrouge, A.G., Voigt, K., Schroder, A., Arrivault, S., Thomine, S. and Kramer, U. (2005) Arabidopsis thaliana MTP1 is a Zn transporter in the vacuolar membrane which mediates Zn detoxification and drives leaf Zn accumulation. *Febs Letters*, **579**, 4165-4174.

Dräger, D.B., Desbrosses-Fonrouge, A.G, Krach, C., Chardonnens, A.N., Meyer, R.C., Saumitou-Laprade, P. and Krämer, U (2007) Two genes encoding Arabidopsis halleri MTP1 metal transport proteins co-segregate with zinc tolerance and account for high MTP1 transcript levels. *The Plant Journal*, **39**, 425–439.

Epstein, E., Bloom, A. (2005) Mineral Nutrition of Plants: Principles and Perspectives, 2nd ed. Sinauer Associates, Sunderland, Maryland.

Filatov V., Dowdle J., Smirnoff N., Ford-Lloyd B., Newbury H.J., Macnair M.M (2006) Comparison of gene expression in segregating families identifies genes and genomic regions involved in a novel adaptation, zinc hyperaccumulation. Molecular Ecology, **15,** 3045-3059. in Verbruggen, N., Hermans, C. and Schat, H. (2009) Molecular mechanisms of metal hyperaccumulation in plants. *New Phytologist*, **181**, 759-776.

Freeman, J.L., Persans, M.W., Nieman, K., Albrecht, C., Peer, W., Pickering, I.J., Salt, D.E. (2004). Increased glutathione biosynthesis plays a role in nickel tolerance in Thlaspi nickel hyperaccumulators. Plant Cell **16**, 2176−2191.

Gendre D., Czernic P., Conéjéro G., Pianelli K., Briat J-F., Lebrun M., Mari S. (2007) TcYSL3, a member of the YSL gene family from the hyperaccumulator specie *Thlaspi caerulescens*, encodes a nicotinamine-Ni/Fe transporter. *Plant Journal*, **49**, 1-15. in Verbruggen, N., Hermans, C. and Schat, H. (2009) Molecular mechanisms of metal hyperaccumulation in plants. *New Phytologist*, **181**, 759-776.

Gerendas, J., Polacco, J.C., Freyermuth, S.K., Sattelmacher, B. (1999). Significance of nickel for plant growth and metabolism. Journal of Plant Nutrition and Soil Science **162**, 241−256.

Guan Z.K., Chai, T. Y.,Zhang, Y. X., Xu, J., Wei, W., Han, L., Cong, L. (2008) Gene manipulation of a heavy metal hyperaccumulator species Thlaspi caerulescens L. via Agrobacterium-mediated transformation. *Molecular Biotechnology*, **40,** 77-86.

Guerinot, M.L., Yi, Y. (1994). Iron: nutritious, noxious, and not readily available. Plant Physiology **104**, 815–820.

Gustin, J.L., Loureiro, M.E., Kim, D., Na, G., Tikhonova, M. and Salt, D.E. (2009) MTP1-dependent Zn sequestration into shoot vacuoles suggests dual roles in Zn tolerance and accumulation in Zn-hyperaccumulating plants. *Plant Journal*, **57**, 1116-1127.

Grusak, M.A. (2002) Enhancing mineral content in plant food products. *Journal of the Americam College of Nutrition*, **21,** 178S-183S

Hammond, J.P., Bowen, H.C., White, P.J., Mills, V., Pyke, K.A., Baker, A.J.M., Whiting, S.N., May, S.T. and Broadley, M.R. (2006) A comparison of the Thlaspi caerulescens and Thlaspi arvense shoot transcriptomes. *New Phytologist*, **170**, 239-260.

Hassan, Z. and Aarts, M.G.M. (2010) Opportunities and feasibilities for biotechnological improvement of Zn, Cd or Ni tolerance and accumulation in plants. Environmental and Experimental Botany.

Hanikenne, M., Talke, I.N., Haydon, M.J., Lanz, C., Nolte, A., Motte, P., Kroymann, J., Weigel, D. and Kramer, U. (2008) Evolution of metal hyperaccumulation required cis-regulatory changes and triplication of HMA4. *Nature*, **453**, 391-U344.

Hong-Bo, S., Li-Ye, C., Cheng-Jiang, R., Hua, L., Dong-Gang, G. and Wei-Xiang, L. (2010) Understanding molecular mechanisms for improving phytoremediation of heavy metal-contaminated soils. *Critical Reviews in Biotechnology*, **30**, 23-30.

Hussain, D., Haydon, M.J., Wang, Y., Wong, E., Sherson, S.M., Young, J., Camakaris, J., Harper, J.F. and Cobbett, C.S. (2004) P-type ATPase heavy metal transporters with roles in essential zinc homeostasis in Arabidopsis. *Plant Cell*, **16**, 1327-1339.

Huttly, A. (2009) Reporter genes. *Methods Mol Biol*, **478**, 39-69.

Klucas R.V., Hanus F.J., Russell S.A., Evans H.J. (1983) Nickel: a micronutrient element for hydrogen-dependent growth of Rhizobium japonicum and for expression of urease activity in soybean leaves. Proc Natl Acad Sci USA, **80**, 2253-3357. in Plessl, M., Rigola, D., Hassinen, V.H., Tervahauta, A., Karenlampi, S., Schat, H., Aarts, M.G.M. and Ernst, D. (2009) Comparison of two ecotypes of the metal hyperaccumulator Thlaspi caerulescens (J. & C. PRESL) at the transcriptional level. *Protoplasma*, **239**, 81-93.

Kobae, Y., Uemura, T., Sato, M.H., Ohnishi, M., Mimura, T., Nakagawa, T. and Maeshima, M. (2004) Zinc transporter of Arabidopsis thaliana AtMTP1 is localized to vacuolar membranes and implicated in zinc homeostasis. *Plant and Cell Physiology*, **45**, 1749-1758.

Kupper, H. and Kochian, L.V. (2009) Transcriptional regulation of metal transport genes and mineral nutrition during acclimatization to cadmium and zinc in the Cd/Zn hyperaccumulator, Thlaspi caerulescens (Ganges population). *New Phytologist*, **185**, 114-129.

Lasat, M.M., Baker, A.J.M. and Kochian, L.V. (1998) Altered Zn compartmentation in the root symplasm and stimulated Zn absorption into the leaf as mechanisms involved in Zn hyperaccumulation in Thlaspi caerulescens. *Plant Physiology*, **118**, 875-883.

Limpens, E., Ramos, J., Franken, C., Raz, V., Compaan, B., Franssen, H., Bisseling, T. and Geurts, R. (2004) RNA interference in Agrobacterium rhizogenestransformed roots of Arabidopsis and Medicago truncatula. *Journal of Experimental Botany*, Vol. 55, **399**, pp. 983±992.

Livak, K.J., Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ CT method. Methods **25**: 402-408.

Maestri, E., Marmiroli, M., Visioli, G. and Marmiroli, N. (2010) Metal tolerance and hyperaccumulation: Costs and trade-offs between traits and environment. *Environmental and Experimental Botany*, **68,** 1-13.

Marschner H. (1995) Mineral nutrition of higher plants, 2nd edn. Academic, London. in Plessl, M., Rigola, D., Hassinen, V.H., Tervahauta, A., Karenlampi, S., Schat, H., Aarts, M.G.M. and Ernst, D. (2009) Comparison of two ecotypes of the metal hyperaccumulator Thlaspi caerulescens (J. & C. PRESL) at the transcriptional level. *Protoplasma*, **239**, 81-93.

Maser, P., Thomine, S., Schroeder, J.I., Ward, J.M., Hirschi, K., Sze, H., Talke, I.N., Amtmann, A., Maathuis, F.J.M., Sanders, D. (2001) Phylogenetic relationships within cation transporter families of Arabidopsis. *Plant Physiology*, **126**, 1646-1667.

Mills, R.F., Francini, A., da Rocha, P., Baccarini, P.J., Aylett, M., Krijger, G.C. and Williams, L.E. (2005) The plant P-1B-type ATPase AtHMA4 transports Zn and Cd and plays a role in detoxification of transition metals supplied at elevated levels. *Febs Letters*, **579**, 783-791.

Mills, R.F., Krijger, G.C., Baccarini, P.J., Hall, J.L. and Williams, L.E. (2003) Functional expression of AtHMA4, a P-1B-type ATPase of the Zn/Co/Cd/Pb subclass. *Plant Journal*, **35**, 164-176.

Papoyan, A. and Kochian, L.V. (2004) Identification of Thlaspi caerulescens genes that may be involved in heavy metal hyperaccumulation and tolerance. Characterization of a novel heavy metal transporting ATPase. *Plant Physiology*, **136**, 3814-3823.

Peer W.A., Mamoudian M., Lahner B., Reeves R.D., Murphy A.S., Salt D.E. (2003) Identifying model metal hyperaccumulating plans: germplasm analysis of 20 *Brassicaceae* accessions from a wide geographical area. *New Phytologist*, **159**, 421-430.

Pence, N.S., Larsen, P.B., Ebbs, S.D., Letham, D.L.D., Lasat, M.M., Garvin, D.F., Eide, D. and Kochian, L.V. (2000) The molecular physiology of heavy metal transport in the Zn/Cd hyperaccumulator Thlaspi caerulescens. *Proceedings of the National Academy of Sciences of the United States of America*, **97**, 4956-4960.

Persans, M.W., Nieman, K. and Salt, D.E. (2001) Functional activity and role of cation-efflux family members in Ni hyperaccumulation in Thlaspi goesingense. *Proceedings of the National Academy of Sciences of the United States of America*, **98**, 9995-10000.

Plaza, S., Tearall, K.L., Zhao, F.J, Buchner, P., McGrath, S.P. and Hawkesford, M.J. (2007) Expression and functional analysis of metal transporter genes in two contrasting ecotypes of the hyperaccumulator Thlaspi caerulescens. *Journal of Experimental Botany*, Vol. 58, **7**, pp. 1717–1728

Plessl, M., Rigola, D., Hassinen, V.H., Tervahauta, A., Karenlampi, S., Schat, H., Aarts, M.G.M. and Ernst, D. (2009) Comparison of two ecotypes of the metal hyperaccumulator Thlaspi caerulescens (J. & C. PRESL) at the transcriptional level. *Protoplasma*, **239**, 81-93.

Reddy, P.S., Mahanty, S., Kaul, T., Nair, S., Sopory, S. K., Reddy, M. K. (2008) A hihg-thrpughtput genome-walking method and its use for cloning unknown flanking sequences. *Analytical Biochemistry*, **381**, 248-253.

Rensing, C., Ghosh, M. and Rosen, B.P. (1999) Families of soft-metal-ion-transporting ATPases. *Journal of Bacteriology*, **181**, 5891-5897.

Rigola, D., Fiers, M., Vurro, E. and Aarts, M.G.M. (2006) The heavy metal hyperaccumulator Thlaspi caerulescens expresses many species-specific genes, as identified by comparative expressed sequence tag analysis. *New Phytologist*, **170**, 753-765.

Talke I., Hanikene M., Krämer U. (2006) Zinc dependent global transcriptional control, transcriptional de-regulation and higher gene copy number for genes in metal homeostasis of the hyperaccumulator *Arabidopsis halleri*. *Plant Physiology*, **142**, 148-167. in Verbruggen, N., Hermans, C. and Schat, H. (2009) Molecular mechanisms of metal hyperaccumulation in plants. *New Phytologist*, **181**, 759-776.

van de Mortel, J.E., Schat, H., Moerland, P.D., van Themaat, E.V.L., Ven der Ent S., Blankestijn H., Ghabdilyan A., Tsiatsiani S., Aarts, M.G.M. (2008) Expression differences for genes involved in lignin, glutathione and sulphate metabolism in response to cadmium in *Arabidopsis thaliana* and the related Zn/Cd hyperaccumulator *Thlaspi caerulescens*. *Plant Cell Environment*, **31**, 301-324. in Verbruggen, N.,

Hermans, C. and Schat, H. (2009) Molecular mechanisms of metal hyperaccumulation in plants. *New Phytologist*, **181**, 759-776.

van de Mortel, J.E., Villanueva, L.A., Schat, H., Kwekkeboom, J., Coughlan, S., Moerland, P.D., van Themaat, E.V.L., Koornneef, M. and Aarts, M.G.M. (2006) Large expression differences in genes for iron and zinc homeostasis, stress response, and lignin biosynthesis distinguish roots of Arabidopsis thaliana and the related metal hyperaccumulator Thlaspi caerulescens. *Plant Physiology*, **142**, 1127-1147.

van der Zaal, B.J., Neuteboom, L.W., Pinas, J.E., Chardonnens, A.N., Schat, H., Verkleij, J.A.C. and Hooykaas, P.J.J. (1999) Overexpression of a novel Arabidopsis gene related to putative zinc-transporter genes from animals can lead to enhanced zinc resistance and accumulation. *Plant Physiology*, **119**, 1047-1055.

Verbruggen, N., Hermans, C. and Schat, H. (2009) Molecular mechanisms of metal hyperaccumulation in plants. *New Phytologist*, **181**, 759-776.

Verret, F., Gravot, A., Auroy, P., Leonhardt, N., David, P., Nussaume, L., Vavasseur, A. and Richaud, P. (2004) Overexpression of AtHMA4 enhances root-to-shoot translocation of zinc and cadmium and plant metal tolerance. *Febs Letters*, **576**, 306-312.

Williams, L.E. and Mills, R.F. (2005) P-1B-ATPases - an ancient family of transition metal pumps with diverse functions in plants. *Trends in Plant Science*, **10**, 491-502.

Weber M., Harada E., Vess C., von Roepenack-Lahaye E., Clemens S. (2004) Comparative microarray analysis of *Arabidopsis thaliana* and *Arabidopsis halleri* roots identifies nicotinamine synthase, a ZIP transporter and other genes as potential metal hyperaccumulation factors. *Plant Journal,* **37,** 269-281. in Verbruggen, N., Hermans, C. and Schat, H. (2009) Molecular mechanisms of metal hyperaccumulation in plants. *New Phytologist*, **181**, 759-776.