# ZIP10 TRANSPORTER: SIGNIFICANCE TO HEAVY METAL TOLERANCE AND METAL ACCUMULATION IN *NOCCAEA CAERULESCENS*

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Kalima Makhmaden: ZIP10 transporter: significance to heavy metal tolerance and metal accumulation in *Noccaea caerulescens*
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## ABSTRACT

Hyperaccumulators are plants that can hyperaccumulate and tolerate excess amounts of heavy metals without any toxicity symptoms and through metal transporter genes they can transport metals from roots to the leaves. They can be also used in the phytoremediation of soils. One of these hyperaccumulator plants is *Noccaea caerulescens.* In this study, four contrasting *N. caerulescens* accessions were used: La Calamine (LC) from Belgium, Ganges (GA) from France, Monte Prinzera (MP) from Italy and Lellingen (LE) from Luxemburg. The accessions show tremendous differences in their capacity to tolerate and accumulate metals.

The aim of this study was to characterize the function of the *ZIP10* transporter gene from *Noccaea caerulescens* and its closest homolog from *Arabidopsis thaliana*. The *ZIP10* gene was chosen because it is differently expressed among *N. caerulescens* accessions, showing highest expression in accession MP. The ZIP10 protein function also was characterized by comparing ZIP10 sequences between *Noccaea* accessions. The *ZIP10* transporter genes from four *Noccaea* accessions were transferred into yeast and they were grown in metal containing plates to determine the effect of the metal transporter to yeast metal tolerance. Also *A.thaliana* RNAi line plants, which show silencing of the *A. thaliana NcZIP10* homolog metal transporter gene expression, were exposed to different metal concentrations and were analyzed for their capacity tolerate heavy metals. The root lengths of RNAi and wild-type plants were measured and compared to each other to determine the effects of *ZIP10* gene on plant metal tolerance. Statistical analyses were done by using two-way ANOVA.

All *ZIP10* yeast transformants transport Co and Ni into cells except LC1 *ZIP10* transformant because of its shortest protein sequence, which most likely results in a nonfunctional protein. The silenced *ZIP10* RNAi lines support a role in transportation of Zn and Cd. The *ZIP10* metal transporter gene may have a role in hyperaccumulation. In this study only a few metals were analyzed, and in further studies the *ZIP10* gene function on other metals should be characterized. The *ZIP10* gene from different *N. caerulescens* accessions could be overexpressed in *A. thaliana.*

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## АБСТРАКТ

Гипераккумуляторы это растения, которые могут гипераккумулировать и переносить избыточное количество тяжелых металлов, без каких-либо симптомов токсичности и через ген транспортер могут транспортировать металлы от корней до листьев. Они также используются в фиторемедиации почв. Одним из этих растений гипераккумуляторов является *Noccaea caerulescens*. В данном исследовании мы использовали четыре контрастных экотипов *N.caerulescens*: La Calamine (LC) из Бельгии, Ganges (GA) из Франции, Monte Prinzera (MP) из Италии и Lellingen (LE) из Люксембурга. Экотипы показывают огромные различия в их способности переносить и накапливать металла.

Цель данного исследования состояла в том, чтобы охарактеризовать функцию *ZIP10* ген транспортера из *Noccaea caerulescens* и его ближайшего гомолога из *Arabisopsis thaliana*. *ZIP10* ген был выбран, так как он среди экотипов *N. caerulescens* выражается по-разному, показывая высшее выражение в экотипе MP. Функция ZIP10 белка также характеризовалась путем сравнения последовательностей ZIP10 между экотипов *Noccaea*. Из четырех экотипов *Noccaea ZIP10* были перенесены в дрожжи и были выращены в металлосодержащих пластинах для определения действия металл транспортера к толерантности дрожжей к металлам. Также линии *A.thaliana* РНК интерференция (РНКи) растений, которые показывают сайленсинг экспрессии *NcZIP10* гена *A.thaliana*, были выращены в различных концентрациях металлов и изучены на их способность переносить тяжелые металлы. Корни РНКи линии и растении дикого типа были измерены и сравнены друг с другом чтобы определить воздействия *ZIP10* гена на терпимость растений. Статистические анализы были проведены с использованием программы «двухсторонная ANOVA».

Все *ZIP10* дрожжевые трансформанты транспортируют Co и Ni в клетки, кроме трансформанта LC1 *ZIP10* из-за его кратчайшей белковой последовательности, которая, скорее всего, приводит к нефункциональности белка. Подавленные линии *ZIP10* РНКи растений поддерживают роль в транспортировке Zn и Cd. В этом исследовании были проанализированы лишь несколько виды металла и в дальнейших исследованиях функций *ZIP10* гена должны быть охарактеризованы с использованием других металлов. *ZIP10* ген из четырех разных экотипов *N. caerulescens* может быть гиперэкспрессирован в *A. thaliana*.

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## ТҮЙІНДЕМЕ

Гипераккумуляторлар бұл ауыр металдарды шектен тыс көп мөлшерде уытты белгілерсіз жинақтай алатын өсімдіктер және олар белгілі бір ген арқылы ауыр металдарды тамырдан жапыраққа дейін тасымалдау қабілетіне ие. Сонымен қатар, бұл өсімдіктер топырақты фитооңалтуда қолданылады. Гипераккумулятор өсімдіктердің бір түрі *Noccaea caerulescens* болып табылады. Бұл зерттеу жұмысында *N.caerulescens*-тың төрт экотүрі: La Calamine (LC) Бельгиядан, Ganges (GA) Франциядан, Monte Prinzera (MP) Италиядан және Lellingen (LE) Люксембургтан қолданылды. Бұл экотүрлер металды қабылдау және жинақтау қабілетіне байланысты үлкен айырмашылықтарды көрсетеді.

Бұл зерттеу жұмысының мақсаты *ZIP10* тасымалдаушы геннің функциясын *N. caerulescens*-тан және оның жақын гомологы *Arabisopsis thaliana*-дан анықтау болып табылады. *ZIP10* генінің таңдалынып алыну себебі, ол *N. caerulescens*-тың экотүрлерінде әр түрлі өрнектелген, соның ішінде MP экотүрінде өрнектелу көрсеткіші жоғары. ZIP10 ақуызының функциясы *Noccaea* экотүрлерінің арасындағы *ZIP10* бірізділіктерін салыстыру арқылы анықталды. ZIP10 гені *Noccaea*-ның төрт экотүрлерінен ашытқыға көшіріліп және метал тасымалдаушының ашытқының металдарға төзімділік қабілетіне әсерін анықтау мақсатында құрамында метал бар табақшаларда өсірілді. Сонымен қатар геннің тежелуін көрсететін РНҚ интерференцияланған (РНҚи) *A.thaliana* тізбекті өсімдіктері әртүрлі концентрациялы металдарда өсіріліп, металдарға төзімділік қабілеті зерттелді. РНҚи тізбектері және жабайы өсімдіктердің тамыр ұзындықтары өлшеніп, *ZIP10* генінің өсімдіктердің төзімділігіне әсері бір-бірімен салыстыру арқылы анықталды. Статистикалық анализ «екіжақты ANOVA» бағдарламасын қолдану арқылы жүргізілді.

LC1 *ZIP10* трансформанты ақуыз бірізділігінің өте қысқа болу себебінен металдарды тасымалдай алмайды және бұл ақуыздың функциясын тоқтатады. Бұл трансформанттан басқа, барлық *ZIP10* ашытқы трансформанттары Co және Ni металдарын жасушаға тасымалдайды. Геннің тежелуін көрсететін РНҚи *A.thaliana* тізбекті өсімдіктері Zn және Cd металдарын тасымалдауда өзіндік рөл атқарады. Бұл зерттеу жұмысында металдардың аз ғана мөлшері зерттелінді, сондықтан келесі зерттеу жұмыстарында *ZIP10* генінің функциясы басқа металдарды қолдану арқылы зерттелуі тиіс. Әр түрлі *N. caerulescens*-тың төрт экотүрінен *ZIP10* гені *A. thaliana* өсімдігіне гиперэкспрестелінуі мүмкін болып табылады.

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# ABBREVIATIONS

| | |
|---|---|
| µ | micro |
| µl | microliter |
| µM | micromolar |
| $^{\circ}C$ | the Celsius degree |
| ANOVA | analysis of variance |
| ATPase | Adenosine triphosphatase |
| BPS | bathophenanthrolinedisulphonate |
| $CaCl_2O_2$ | calcium hypochlorite |
| Cd | Cadmium |
| cDNA | complementary DNA |
| Cr | Chromium |
| Cu | Copper |
| DNA | Deoxyribonucleic acid |
| dsRNA | double stranded RNA |
| DW | dry weight |
| Fe | Iron |
| g | gram |
| GA | Ganges ecotype |
| Hg | Mercury |
| IRT | Iron regulated transporter |
| LC | La Calamine ecotype |
| LE | Lellingen ecotype |
| Mn | Manganese |
| mol | molar |
| MP | Monte Prinzera ecotype |
| mRNA | messenger ribonucleic acid |
| MS medium | Murashige and Skoog medium |
| Nc | *Noccaea caerulescens*, Alpine penny-cress |
| Ni | Nickel |
| Nramp | Natural resistance-associated macrophage protein |
| OD | optical density |

| | |
|---|---|
| oligo-dT | oligodeoxythymidylic acid |
| *p* | probability |
| Pb | Lead |
| PCR | polymerase chain reaction |
| RISC | Ribonucleic acid induced silencing complex |
| RNA | Ribonucleic acid |
| RNAi | Ribonucleic acid interference |
| RT Enchancer | Reverse Transcriptase Enhancer |
| RT-PCR | real time polymerase chain reaction |
| URA | uracil |
| V | volume |
| WT | wild type |
| ZIP | Zinc regulated transporter and iron regulated transporter like proteins |
| Zn | Zinc |
| ZRT | Zinc regulated transporter |

# CONTENTS

# 1. INTRODUCTION

As activities by human and global industrial development processes increase, great masses of heavy metals like zinc (Zn), copper (Cu), lead (Pb), cadmium (Cd) and nickel (Ni), are released into the environment and are major causes of pollution on earth. Soil contamination with heavy metals is considered an important health-threating issue. Remediation of contaminated soils takes a lot of work and time. Su *et al*. (2014) listed that in European countries (France, Spain and Finland), the average concentration of Cd in urban soils is 1.49 $mg/kg^{-1}$ and content of Zn is 224 $mg/kg^{-1}$. In addition, the average contamination of soils with Cd, in these countries, is moderate to strong (Muller, 1969). In a NATO/CCMS report (2002), country surface areas that are contaminated by heavy metals were presented and showed that in particular, Western Europe, these may constitute up to 1 200 000 ha. In general, distribution of heavy metal contamination in Europe was 34.8% to total contamination of land area in 2013 (Panagos *et al*., 2013). Excess amounts of heavy metals in soil can also decrease growth in crops (Lone *et al*., 2008). Furthermore, food crops like maize, potato, bean, tomato, winter rye etc. can transfer heavy metals to their tissues and for example maize has a transfer factor [total concentration in plant (mg/kg)/total concentration in soil (mg/kg)] of 0.30 for Cd and 0.15 for Ni to its cob (Puschenreiter *et al*., 2005). However, the metal transfer factor varies largely between species and varieties, and choosing or breeding plants with low transfer factors may significantly reduce metal concentrations in the edible parts of the plants.

To reclaim contaminated soils, engineering remediation and bioremediation methods are used. Bioremediation includes phytoremediation, which is linked to specific plants. These plants are hyperaccumulators that can accumulate heavy metals in high concentrations in their above ground tissues (Su *et al*., 2014). Recent achievements in biotechnology can lead to opportunities in the transfer of metal transporter genes from hyperaccumulators to other plant species, aiming to improve the removal of heavy metals from contaminated soils (Lone *et al*., 2008). However, limited knowledge of metal transport in plants hinders further use of this method.

ZIP family proteins are included in the accumulation of heavy metals. Currently twenty-five members of the ZIP family have been identified. However most of these protein functions are unknown. ZIP10 metal transporter was selected as a research subject because it is expressed higher in a hyperaccumulator *Arabidopsis halleri* than in *Arabidopsis thaliana*, and because its expression among *Noccaea caerulescens* accessions is highest in Monte Prinzera (MP) and lowest in La Calamine (LC). The *ZIP10* gene function has not been studied before, thus the purpose of this study was to characterize the function of *ZIP10* transporter gene. Its function was studied using *ZIP10* from *N. caerulescens* accessions and *A. thaliana ZIP10* RNAi line plants.

# 2. LITERATURE REVIEW

## 2.1 METAL HYPERACCUMULATORS

Heavy metals are defined conventionally as elements with an atomic number greater than 20 and having metallic characteristics. Chromium (Cr), Cd, mercury (Hg), and Zn are some of the most commonly found heavy metal contaminants in soil. Metals such as Cu, manganese (Mn), Ni, and Zn are micronutrients necessary for the growth of plants while Cd, Hg and lead (Pb) have no known biological function. Heavy metals released in excess amounts in the environment can lead to various serious effects on human and plant health. Heavy metal toxicity is considered as abiotic stress for non-hyperaccumulator plants. Heavy metals have an effect not only on the development and growth of the plant body, but also on different processes, like photosynthesis and can also affect hereditary information (DNA) in plant cells (Li *et al*., 2015; Hu *et al*., 2013). They can inactivate enzyme function, disrupt membrane integrity and damage photosynthetic apparatus.

High concentrations of heavy metals in plants have been taken under study in the last few decades (Barman *et al*., 2000). This led to the advancement in the research of molecular basis for phytoremediation technology. The hyperaccumulation or the concentration of heavy metals depends on factors like plant species, soil conditions and types of heavy metals. Some metal toxicity depends on oxidation state, e.g. Cu and iron (Fe) become toxic in excess concentrations due to participation in the redox process (Sarma, 2011).

There are two different groups of plants: metal excluders, which accumulate metals mainly in the root system, and metal accumulators, which accumulate mainly in their aerial organs. Non-hyperaccumulator plants do not have the ability to tolerate high levels of heavy metals in their shoot biomass and therefore after the uptake of heavy metals, they store them in the root cell wall and vacuole, thus protecting themselves (Rascio and Navari-Izzo, 2011; Milner and Kochian, 2008).

Hyperaccumulation provides high tolerance to specific elements, which are normally phytotoxic, and allows plants to avoid heavy metal poisoning. The high uptake of heavy metals also protects plants from herbivores (Rascio and Navari-Izzo, 2011; Baker and Brooks, 1989). Plants that can hyperaccumulate heavy metals also possess higher potential for

applications in remediation of heavy metals in the environment (Prasad, 2003). Brooks *et al*., (1977) linked the term "hyperaccumulator" with plants that accumulate Ni with values exceeding 1000 mg/g in their above ground tissue. Hyperaccumulators compared to non-hyperaccumulators can store high concentrations of heavy metals in their aerial tissues (0.1% Ni, >1% Zn, 0.01% Cd) without visual symptoms of toxicity (Rascio and Navari-Izzo, 2011). This is considered to be the first hallmark for hyperaccumulators and they have potential uses in food crop biofortification (Hanikenne and Nouet, 2011; Verbruggen *et al*., 2009) and in phytoremediation (Rigola *et al*., 2006). Other hallmarks of hyperaccumulators are efficient transport of metals from root to shoot and great detoxification and sequestration of metals in above ground organs (Rascio and Navari-Izzo, 2011; Papoyan and Kochian, 2004). Possibility of plants to hyperaccumulate heavy metals in their tissues can be defined by high performance of some detoxification mechanisms that provide tolerance to heavy metals (Visioli *et al*., 2014; Ghasemi *et al*., 2009).

Hyperaccumulators (also called metallophytes) can be divided into obligate and facultative hyperaccumulators, according to their occurrence in different soil types. Most hyperaccumulators are obligate or eumetallophytes that are indigenous to metalliferous soils and have the ability to grow on soils that contains metals with high concentrations. Some species grow in soils with low metal concentrations while some species favour soils with high metal concentrations. Facultative hyperaccumulators or pseudometallophytes species occur on metalliferous and also on non-metalliferous soils, and individuals of one species can be either a hyperaccumulator or a non-hyperaccumulator. Facultative hyperaccumulation takes place when a species or a population has a metal accumulation capacity, and access to a sufficient metal is provided by soil factors (Pollard *et al*., 2014; van der Ent *et al*., 2012; Pollard *et al*., 2002).

## 2.2 NOCCAEA CAERULESCENS

Currently ca. 450 species of hyperaccumulators exist and among them about 75% are Ni hyperaccumulators, while only five of them accumulate Cd (Visioli and Marmiroli, 2012). Hyperaccumulator plants belong to the families of *Violaceae, Brassicaceae, Asteraceae, Flacourtiaceae* and others. Most of hyperaccumulator species belong to the *Brassicaceae* family (Seregin *et al*., 2014). One of the members of *Brassicaceae* family is *Noccaea caerulescens* (formerly *Thlaspi caerulescens*), which is recognized as a metal hyperaccumulator of Ni, Zn and Cd and grows on non-metalliferous and metalliferous soils in Europe (France, Spain, Italy), United Kingdom, Slovakia (Mandáková *et al*., 2015; Visioli *et al*., 2014). It is a biennial plant with small flowers, which requires long periods for growth (Guimarães *et al*., 2013). Species of the *Noccaea* genus are model plants largely used to study the ability of plants to hyperaccumulate Cd, Ni and Zn in their shoots at higher concentrations than the others (Gonneau *et al*., 2014; Kozhevnikova *et al*., 2014). Koch and German (2013) reported 32 zinc accumulator and 15 nickel accumulators from the *Noccaea* genus. In the remediation of contaminated soils with toxic metals, *N. caerulescens* plays important role as extremophile plant (Lin *et al*., 2014).

Peer *et al*., (2003) established that all species of *Noccaea* share 87-88% identity with *A. thaliana* in the intergenic transcribed spacer regions. This identity allowed the use of *A. thaliana* as a comparison to *N. caerulescens* in many studies. *A. thaliana* is a model plant which is non-accumulator from Brassicaceae family.

As mentioned above, Ni hyperaccumulators can accumulate 1000 μg/g Ni in the plant dry matter, however in Jaffré *et al*., (2013) studies was reported that some hyperaccumulators accumulate >10 mg/g (1%) Ni and these plants were called like hypernickelophores. For Zn hyperaccumulators van der Ent *et al*., (2012) suggested 3000 mg/g threshold for Zn hyperaccumulation.

*N. caerulescens* has four well-defined accessions that are distinguished by their location and soil properties. They are calamine populations La Calamine (LC) from Belgium and Ganges (GA) from France, serpentine population Monte Prinzera (MP) from Italy and Lellingen (LE) growing in normal soils, from Luxemburg. Accessions are distinguished from each other by their metal transport ability (Guerinot, 2000). GA ecotype is more effective in Cd

hyperaccumulation than other ecotypes, with the LC (in some cases Prayon) ecotype often used as the comparator (Milner and Kochian, 2008). Also, GA shows extreme accumulation of Zn at 60.5 µmol g-1 DW (Table 1). MP, LE and GA are able to accumulate Zn, Cd and Ni in higher concentrations than LC, thus LC among four ecotypes is the weakest hyperaccumulator of those metals (Assunção *et al*., 2003).

Table 1. The concentrations (µmol g-1 DW) of Zn, Cd and Ni that can be accumulated by hyperaccumulator *N. caerulescens* four ecotypes (Assunção *et al*., 2003).

| | **GA** | **MP** | **LE** | **LC** |
|---|---|---|---|---|
| **Zn** | 60.5 | 53.9 | 28.0 | 6.5 |
| **Cd** | 29.9 | nm | 17.3 | 1.8 |
| **Ni** | 16.9 | 48.3 | 17.3 | 1.5 |

nm = not measured, GA=Ganges, MP=Monte Prinzera, LE=Lelingen, LC= La Calamine

## 2.3 ZIP10 TRANSPORTER

To accumulate heavy metals from the soil, plants need metal transporter genes. Some of these genes have been identified using methods such as yeast mutant complementation and degenerate polymerase chain reaction (PCR), based on sequence similarity. P-type ATPases and Nramp protein are examples of metal transporter families. More than 15 years ago a metal transporter family called ZIP family was identified in *Arabidopsis*, and its family members are represented in all eukaryotic plants and animals (Guerinot, 2000).

The ZIP family stands for **Z**RT and **I**RT-like **P**rotein and is comprised of metal transporters identified in many plants. The *IRT*1 (iron regulated transporter) gene of the *A. thaliana*, is the first identified ZIP family member and is expressed in plants under iron deficiency (Varotto *et al*., 2002; Eide *et al*., 1996). The size of the ZIP proteins varies from 309 to 476 amino acid residues and they have eight transmembrane domains (Guerinot, 2000). Members of the ZIP family gene have the ability to transport a variety of divalent cations including Fe, Mn, Zn and Cd (Wu *et al*., 2009; Qin *et al*., 2003). As reported by Guerinot (2000), more than twenty-five ZIP family members have been identified. Eleven were identified from *A. thaliana* and two, *ZRT1* and *ZRT2* (zinc regulated transporter) were identified from yeast. Milner *et al*. (2012) studied eleven members of the ZIP family, and six of them showed Mn transport, six

showed Zn transport and one showed Fe transport. These ZIP family members were tested for their ability to complement yeast mutants (*fet3fet4Δ, ctr1/ctr3Δ, zrt1/zrt2Δ, smf1Δ*) and ten of them showed at least one yeast mutant complementation.

Among the ZIP family members, the *ZIP10* gene has been identified as functioning as a Zn transporter or other metals transporter across membranes in *A. halleri* (Talke *et al*., 2006). The *ZIP10* transcript level in *A. halleri* is much higher compared to that of in *A. thaliana*. The *ZIP10* gene is also the strongest candidate for Ni accumulation in *N. caerulescens,* and its expression among *N. caerulescens* accessions is the highest in MP and lowest in LC, the expression patterns correlating with Ni accumulation (Halimaa *et al*., 2014). In Milner *et al*, (2012) *ZIP10* expression was equal in both roots and shoots of the *Arabidopsis*. Due to its different expression in *N. caerulescens* accessions it is of high interest in research.

## 2.4 YEAST MUTANTS

Yeast (*Saccharomyces cerevisiae*) metal uptake mutants are generally used to determine metal transportation properties of plant transporters. Different yeast mutants exist, for example, *zrt1/zrt2Δ* for zinc uptake, *fet3/fet4Δ* for iron uptake, *ctr1/ctr3Δ* for copper uptake and *smf1Δ* for manganese uptake (Milner *et al*., 2012). Among them, *fet3fet4* double mutant is defective in both low- and high- affinity iron uptake. The yeast *fet3* gene is required for high affinity iron uptake and *fet4* gene is required for low affinity iron uptake. *Fet3fet4* mutant grows very well in undetectable iron uptake activity level. This double mutant gives an opportunity for further analysis of iron uptake pathways. It was suggested that *S. cerevisiae* may have iron uptake pathways in both high and low affinity systems (Dix *et al*., 1994).

## 2.5 RNA INTERFERENCE

RNA interference (RNAi) is used to manipulate gene expression (silencing) in all eukaryotic organisms such as protozoans, invertebrates and vertebrates, plants, fungi and algae (Agrawal *et al*., 2003). This method is triggered by double-stranded RNA (dsRNA), which is divided using an enzyme Dicer into short single-stranded fragments (21-25 strands). These strands of RNA fragments are included into RNA-induced silencing complex (RISC) and guide the specific degradation of single stranded RNA (Mansoor *et al*., 2006). Thus, RNA interference is used to supply double stranded RNA into plant to silence gene.

The method used to deliver dsRNA into plants for inducing RNA interference (RNAi) is genetic transformation of plants with genes engineered to express self-complementary transcripts that can form dsRNA molecules (Kerschen *et al*., 2004). RNA interference was studied in nematode *Caenorhabditis elegans* by Fire *et al*. (1998). From this study it was discovered that a mixture of sense (coding RNA sequence) and antisense (non-coding complementary sequence) RNA could reflect the formation of dsRNA, thus they can silence gene expression. However, dsRNA proved to be more effective in interference than either sense or antisense RNA. Nowadays RNAi is used in the manipulation of gene expression and investigation of gene function in plants.

# 3. OBJECTIVES

The aim of the research work was to characterize the function of the *ZIP10* transporter gene from *Noccaea caerulescens* by transferring. The *ZIP10* metal transporter gene was transferred into yeast and its effects on yeast metal tolerance or accumulation were determined. *ZIP10* RNAi lines of *A. thaliana* were also characterized for their metal tolerance and accumulation. The aim was to determine how RNAi line and wild-type (WT) plants respond to different metals. It is important to find out the significance of ZIP10 transporter in metal hyperaccumulation in *Noccaea caerulescens* because *ZIP10* gene may play role in Ni accumulation. Furthermore, hyperaccumulator *Noccaea caerulescens* can be used to create resistant crops (plants) to heavy metals and create food crops with a high content of nutrients.

# 4. MATERIALS AND METHODS

In this study, the ZIP10 protein function was determined by comparing ZIP10 protein sequences of four *Noccaea* accessions to each other. From the sequences putative metal binding areas were found. The ZIP10 metal transporter function was determined with following ways: firstly the *ZIP10* gene was transferred from four *Noccaea* accessions (LC, LA, GA and MP) to yeast mutant *fet3fet4 S. cerevisae* and yeast growth in metal contents was analyzed. Secondly, *Arabidopsis* RNAi plants with silenced *ZIP10* gene and WT also were analyzed for their capacity to grow in metal-containing plates. *Arabidopsis* RNAi plants also were checked to confirm that the plants were transgenic and that the transgene silences *ZIP10* expression.

## 4.1 CLONING OF NCZIP10 cDNAs

The *ZIP10* genes from *N. caerulescens* accessions Monte Prinzera (MP, Italy), Lellingen (LE, Luxemburg), Ganges (GA, France) and La Calamine (LC, Belgium) were sequenced and subsequently cloned (Baltzi, 2014) into yeast *fet3fet4* mutant strain, defective in both low- and high- affinity iron uptake. One sequence was obtained for each accession, except two *ZIP10* sequences were cloned from LC (named LC1 and LC3).

## 4.2 COMPARISON OF NCZIP10 (MP, LE, LC, GA) SEQUENCES

Protein alignment was done for the sequences of each *N. caerulescens* ZIP10 sample with "ClustalW" bioinformatics software application. Aligned sequences were compared to each other and differences were identified. The alignment was done to compare ZIP10 sequences between accessions to identify amino acid sequences that could affect the metal transport properties of the ZIP10 protein.

## 4.3 YEAST COMPLEMENTATION

Yeast complementation was done to characterize the *ZIP10* gene function in metal accumulation. Yeast transformants containing different *ZIP10* genes were grown in liquid-URA medium to an optical density of 1 at 600 nm and then serially diluted 10-, 100-, 1000- and 10000- fold. Aliquots of 5 µl of each dilution were pipetted on plates with Co, Zn, Ni, and Mn. Samples were incubated at 30 °C for 2-3 days and photographed. To evaluate the effect of *ZIP10* gene on yeast metal tolerance, different concentrations (Appendix 1A) of Zn, Mn, Ni and Co were used.

## 4.4 CHARACTERIZATION OF TRANSGENIC *A.THALIANA*

*A. thaliana* plants with silenced *ZIP10* gene were used to analyze the significance of ZIP10 for plant metal tolerance. Transgenic *A. thaliana* RNAi line plants were grown in soil, placed in boxes, to prevent pollen distribution. To confirm that *A. thaliana* RNAi lines are transgenic a PCR program was used. The DNA was amplified from plant leaves using the Phire Plant Direct PCR Kit (Thermo Scientific). The presence of the transgene in *A. thaliana* was assessed from the DNA by PCR using the pB7GWIWG2/1 CTCTAGCATGGCCGCGGGA and pB7GWIWG2/4 CCCCCACCCACGAGGAGCAT primer sequences, according to the manufacturer's instructions. For cycling, 2-step protocol was used. PCR initial denaturation was 98°C for 5 min, denaturation 98°C for 5 s, annealing temperature was 75°C, extension was 72°C for 20 s, finagling step was 72°C for 1 min and PCR was extension was 4°C. After electrophoresis the gel was photographed (Figure 2).

## 4.5 STERILIZATION AND PLATING OF *A.THALIANA* SEEDS

To characterize the function of the ZIP10, *A. thaliana* RNAi lines in metal accumulation, seeds of the *A. thaliana* RNAi line plants were sterilized. For sterilization a saturated solution calcium hypochlorite ($CaCl_2O_2$) was prepared and filtrated. After filtration, Tween 0.1% was added. Mature seeds were mixed with prepared $CaCl_2O_2$ solution, and then were washed with 70% EtOH 1 time and with sterilized water 4 times. Sterilized seeds were germinated in MS medium for about 2 weeks in growing room, including vitamins (4.4g MS salts, 0.8% plant agar, 30g sucrose for 1 liter) and metals such as $ZnSO_4$, $MnSO_4$, $NiSO_4$, $CoCl_2$, $CdSO_4$, bathophenanthrolinedisulphonate (BPS). Concentrations of these metals are shown in Appendix 1B. The iron chelator BPS was added to create iron deficiency conditions. The length of plant roots was measured by ImageJ software to evaluate metal tolerance from their root growth.

## 4.6 QUANTIFICATION OF ZIP10 FROM RNAi LINES

### 4.6.1 Digital PCR

The expression of *ZIP10* was measured from *A. thaliana* to confirm gene silencing. First, mature roots were harvested and frozen in liquid nitrogen. Total RNA was isolated using the RNeasy plant mini kit (Qiagen). 200 ng of the purified RNA sample were used to synthesize cDNA with Verso kit using an anchored oligo-dT and RT Enchancer primers in a 20µl reaction. Digital PCR was done to quantify gene expression. ZIP10 *A. halleri* forward 5'-3' TTGCATCCTTCAGGCGGAGTA, and *A. halleri* reverse 5'-3'CGCAAAAAAGAAAGCC ATCACC primers were used. 20 µl PCR-mix and 70 µl oil were added into plate for creation of emulsion. PCR conditions used were 95°C for 5min, 95°C for 30 s, 61°C for 1min, 4°C for 5min, 90°C for 5min and 4°C to hold. After PCR, the plates were analyzed by Droplet Reader.

### 4.6.2 RT-PCR

For normal PCR reaction used PCR-mix volume was 24 µl and contained 2.5 µl of each primer, 1 µl of DNA template and 12.5 µl of Dream Taq Green PCR Master Mix. To 24 µl PCR-mix was added 1 µl cDNA of RNAi lines and WT. Annealing temperature for PCR was 52°C. After PCR was done, 20 µl of PCR mixture was loaded to agarose gel and electrophoresed at 85 V.

## 4.7 ROOT LENGTH MEASUREMENT OF RNAi PLANTS EXPOSED TO METALS

The root length was measured from the *ZIP10 A. thaliana* RNAi9, RNAi11 and RNAi12 lines and WT plants to evaluate the effect of ZIP10 on *A. thaliana* metal tolerance. Measurement was done with twenty-six plants. The plants were grown on plates in MS medium were analyzed using a two way analysis of variance (ANOVA) at a significance level of $\alpha=0.05$.

# 5. RESULTS

## 5.1 COMPARISON OF NCZIP10 (MP, LE, LC, GA) SEQUENCES

From sequence alignment results shown in Figure 1, it can be seen that GA and LC3 protein sequences are identical. Unlike in LC1, LC3 protein sequence shows an earlier stop codon (shown by black arrow) than the other accession sequences and this early termination could affect the protein function and make it non-functional. Also the protein contains a His and Glu amino acids rich repeat (highlighted in a rectangle), and variation among the accessions in this domain.

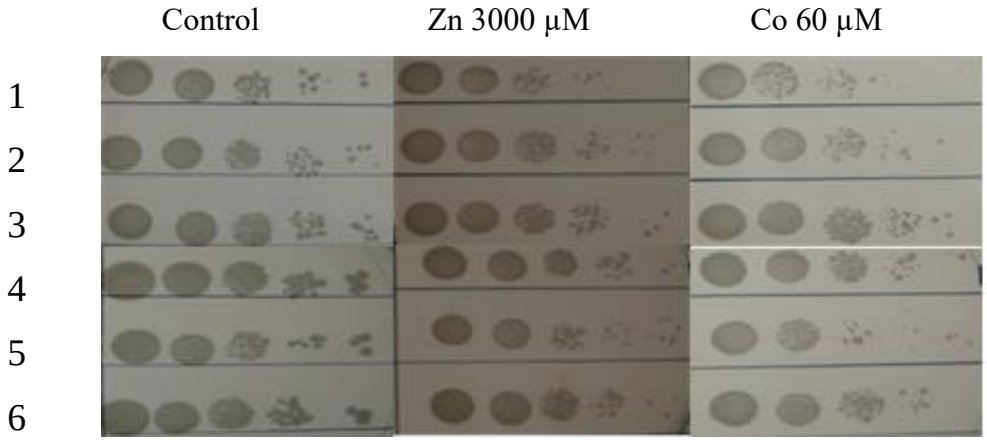
```
#LC1    MTKSPLISSAAVTVILLLAISHFPGARSQSLTCEPDS-NSCNDKTKALQLKLIGIFAILI        59
#LC3    MTKSPLISSAAVTVILLLAISHFPGARSESLTCETDSDDSCTDKTKALQLKLIGIFAILI        60
#GA     MTKSPLISSAAVTVILLLAISHFPGARSESLTCETDSDDSCTDKTKALQLKLIGIFAILI        60
#MP     MTKSPLISSTAVTVILLLAISHFPGARSQSLTCETNS-NSCTDKTKAIQLKLIGIFAILI        59
#LE     MTKSPLISSAAVTVILLLAISHFPGARSQSLTCEPDS-NSCNDKTKALQLKLIGIFAILI        59
        *********:******************:***** :* :**.*****:************

#LC1    SSIIGVFLPLFARSVPAFQPDRSPFFIVKAFASGIILSTAFMHVLPDSFHMLSSPCLAEN        119
#LC3    GSIIGVFLPLFARSVPAFQPDKSPFFIVKAFASGIILATAFMHVLPDSFHMLSSPCLAEN        120
#GA     GSIIGVFLPLFARSVPAFQPDKSPFFIVKAFASGIILATAFMHVLPDSFHMLSSPCLAEN        120
#MP     SSIIGVFLPLFARSVPAFQPNKSPFFIVKAFASGIILSTAFMHVLPDSFHMLSSPCLAKN        119
#LE     SSIIGVFLPLFARSVPAFQPDRSPFFIVKAFASGIILSTAFMHVLPDSFHMLSSPCLAEN        119
        .******************::***************:********************:*

#LC1    PWRKFPFSGFLAMTSAIFTLMVDSITTSVFTKSARKDMRPEVGTDKADVASAETPDQETG        179
#LC3    PWHKFPFSGFLAMIAAVFTLMVDSITTSVFTKSARKDMRPEVGTEKADVASAETPDQETG        180
#GA     PWHKFPFSGFLAMIAAVFTLMVDSITTSVFTKSARKDMRPEVGTEKADVASAETPDQETG        180
#MP     PWHKFPFSGFLAMISAVFTLMVDSITTSVFTKSARKDMRPEVGTDKADVASAETPDQETG        179
#LE     PWRKFPFSGFLAMTSAIFTLMVDSITTSVFTKSARKDMRPEVGTDKADVASAETPDQETG        179
        **:********** :*:****************************:***************

#LC1    RGQVPVTHYGHSHGHGHGHGHGHELSTGLQLVRYRVIAIVLEIGIVTHSVVIGLAVGASN        239
#LC3    RGQVPMIHH------GHGHGHGHELSTGLQLVRYRVIAIVLELGIVVHSVVIGLAVGASN        234
#GA     RGQVPMIHH------GHGHGHGHELSTGLQLVRYRVIAIVLELGIVVHSVVIGLAVGASN        234
#MP     RGQVPVTHYGHSHG------HGHELSPGLQLVRYRVIAIVLELGIVVHSVVIGLAVGATN        233
#LE     RGQVPVTHYGHSHGHGHGHGHGHELSTGLQLVRYRVIAIVLEIGIVTHSVVIGLAVGASN        239
        *****: *:           ****** ***************:***.***********:*

#LC1    NTCTIRVLVAALCFHQMFEGMSLGGSILQAEYTWMKKSVMAFFFAVTTPGGLALGMGINS        299
#LC3    NTCTIRVLVAALCFHQMFEGMSLGGCFLQAEYTWMKKSVMALFFAVTTPGGVALGMVINK        294
#GA     NTCTIRVLVAALCFHQMFEGMSLGGCFLQAEYTWMKKSVMALFFAVTTPGGVALGMVINK        294
#MP     NICTIRVMVAALCFHQMFEGMSLGGCILQAEYTWMKKSVMAFFFAVTTPGGVALGMGINK        293
#LE     NTCTIRVLVAALCFHQMFEGMSLGGSILQAEYTWMKKSVMAFFFAVTTPGGLALGMGINS        299
        * *****:*****************.:**************:*********:**** **.

#LC1    TYKENSPSSLITIGLLNGASAGLLNRPTWL←                                     330
#LC3    TYKENSPSSLITIGLLNGASAGLLIYMALVDLLAADFMGQKMQRSIKLQLKSYAAV----        350
#GA     TYKENSPSSLITIGLLNGASAGLLIYMALVDLLAADFMGQKMQRSIKLQLKSYAAV----        350
#MP     TYKENSPSSLITIGLLNGASAGLLIYMALVDLLAADFMGQKMQRSIKLQLKSYAAV----        349
#LE     TYKENSPSSLITIGLLNGASAGLLIYMALVDLLAADFMGKKMQRSIKLQLKAYAAV----        355
        ************************   : :         ..: * *    :. .*

#LC3    ---FLGAAGMSLMARWA--- 364
#GA     ---FLGAAGMSLMARWA--- 364
#MP     ---LLGAAGMSLMARWA--- 363
#LE     ---MLGAAGMSLMARWA--- 369
           :* .: :     *
```

Figure 1. Protein sequence alignment of ZIP10 from *N. caerulescens* accessions (LC, GA, MP, LE). Alignment was done by Clustal Omega software.

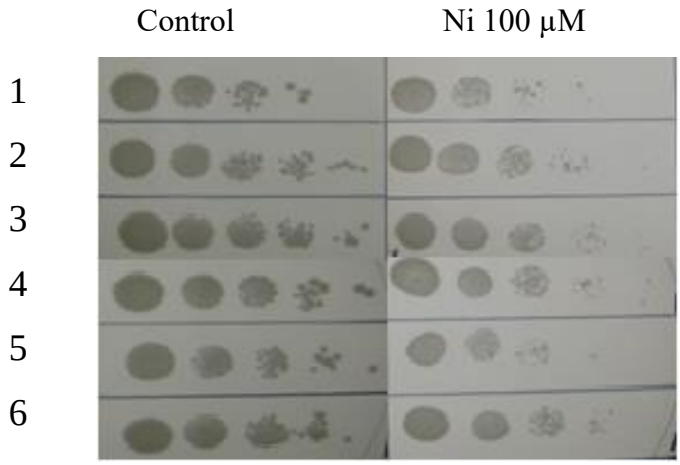
## 5.2 YEAST COMPLEMENTATION

To determine the metal tolerance of yeasts containing *ZIP10* gene from different accessions, yeasts were tested for growth in metal containing plates. Grown yeasts were compared to control plates that were grown simultaneously with each metal plate (Figure 2). Yeast complementation studies showed that yeasts were able to grow in Mn, Co, Ni and Zn containing mediums. Yeasts grew very well in (Figure 2 a) Zn containing plates compared to the control. There are some differences in 10000-fold dilution; the ZIP10 transformants, except for LC1, grew slightly less than the control yeast.

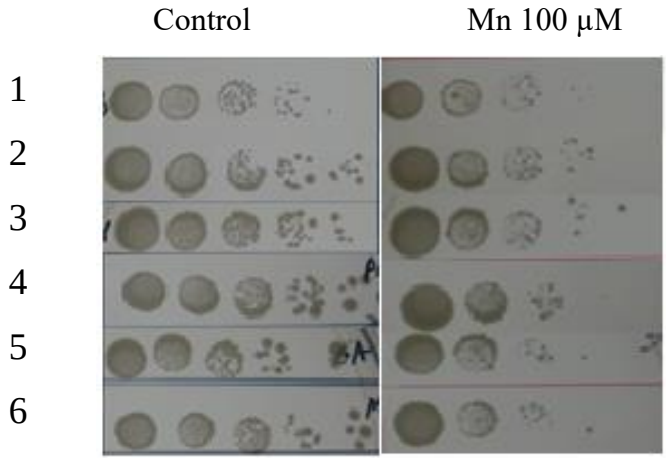
Yeasts were also able to grow in (Figure 2 b) Ni-containing plates, but in dilution series from 100- fold, growth was significantly lower for all ZIP10 transformants but LC1 ZIP10 growth was better compared to empty vector control. This means that ZIP10 is possibly able to transport Ni. The *ZIP10* transformants (except LC1 *ZIP10*) made the yeast also more sensitive to Co, indicating Co transport. Yeasts in Mn-containing plates (Figure 2 c) grew very well in optical density 1, but growth decreased with the increase in dilution.




(a)




(b)




(c)

Figure 2. Complementation of *fet3fet4* yeast metal uptake mutant with 6 different *ZIP10* genes from *N. caerulescens* accessions: 1- LC3; 2-LE, 3-LC1; 4-pAG426 (empty vector control); 5-GA; 6-MP.

## 5.3 CHARACTERIZATION OF *A. THALIANA* ZIP10 RNAI LINES BY PCR

T-DNA was not amplified from WT *A. thaliana* plants (Figure 3), which means it is not transgenic. A PCR product was obtained from RNAi9, RNAi11 and RNAi12 showing that they are transgenic.

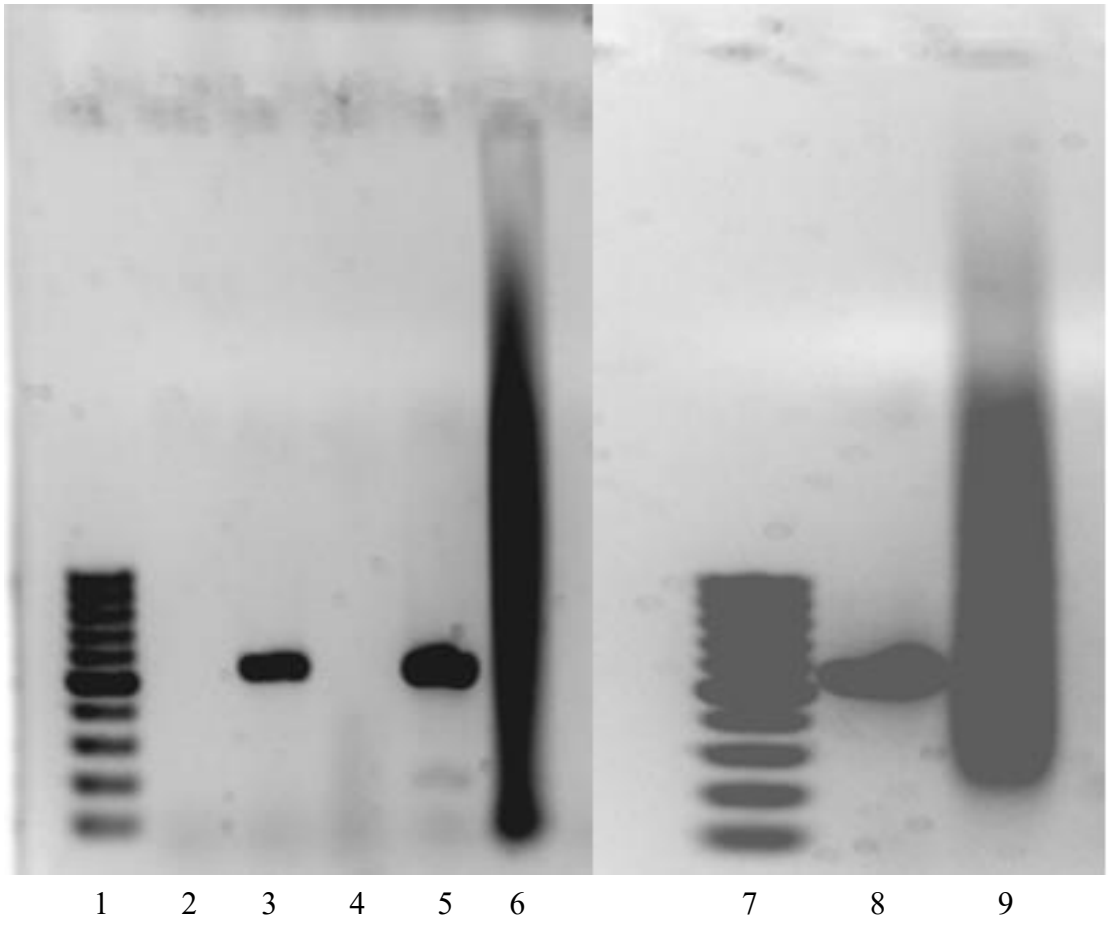



Figure 3. PCR results from *A. thaliana* RNAi line and wild type (WT) plants to confirm that they are transgenic. O'Gene Ruler 100bp (1), (7) was used as control, WT(2), RNAi12(3), RNAi11(5), RNAi9(8) and water (6), (9).

## 5.4 QUANTIFICATION OF ZIP10 FROM RNAi LINES

### 5.4.1 Digital PCR

Digital PCR results illustrate that ZIP10 gene was silenced in RNAi lines (Figure 4). This also confirms that they are transgenic.

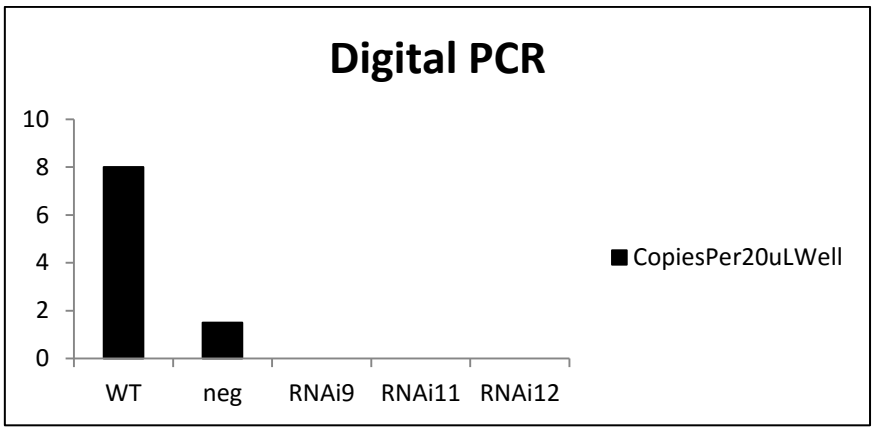



Figure 4. The *ZIP10* gene expression in ZIP10 RNAi lines and WT using Digital PCR

### 5.4.2 RT-PCR

RT-PCR results (Figure 5) were inconclusive in determining the expression of *ZIP10* gene in RNAi lines and WT. There lighter black bands illustrate wild type and RNAi's ZIP10 expression.

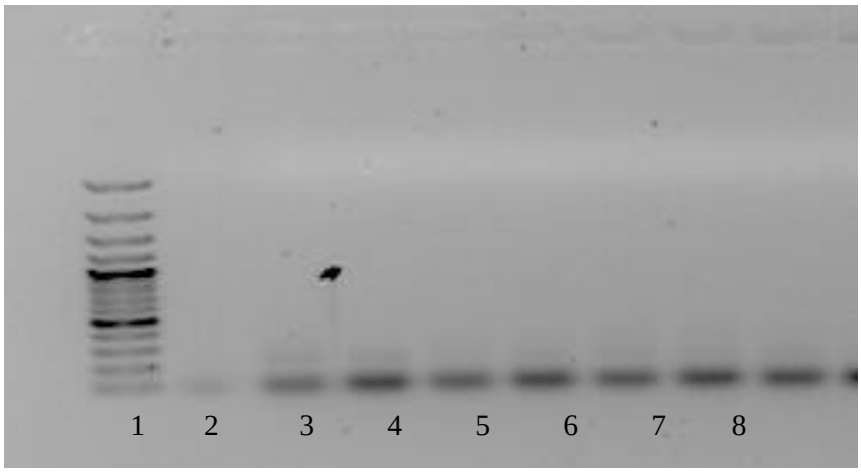



Figure 5. RT-PCR results from *A. thaliana* RNAi's and wild type plants. In gel were loaded Gene Ruler 100bp Plus (1), WTa(2), WTb(3), RNAi9a(4), RNAi9b(5), RNAi11a(6), RNAi11b(7), RNAi12a(8) and RNAi12b(9) samples.

## 5.5 *A. THALIANA* PLANTS IN METAL CONTAINING PLATES

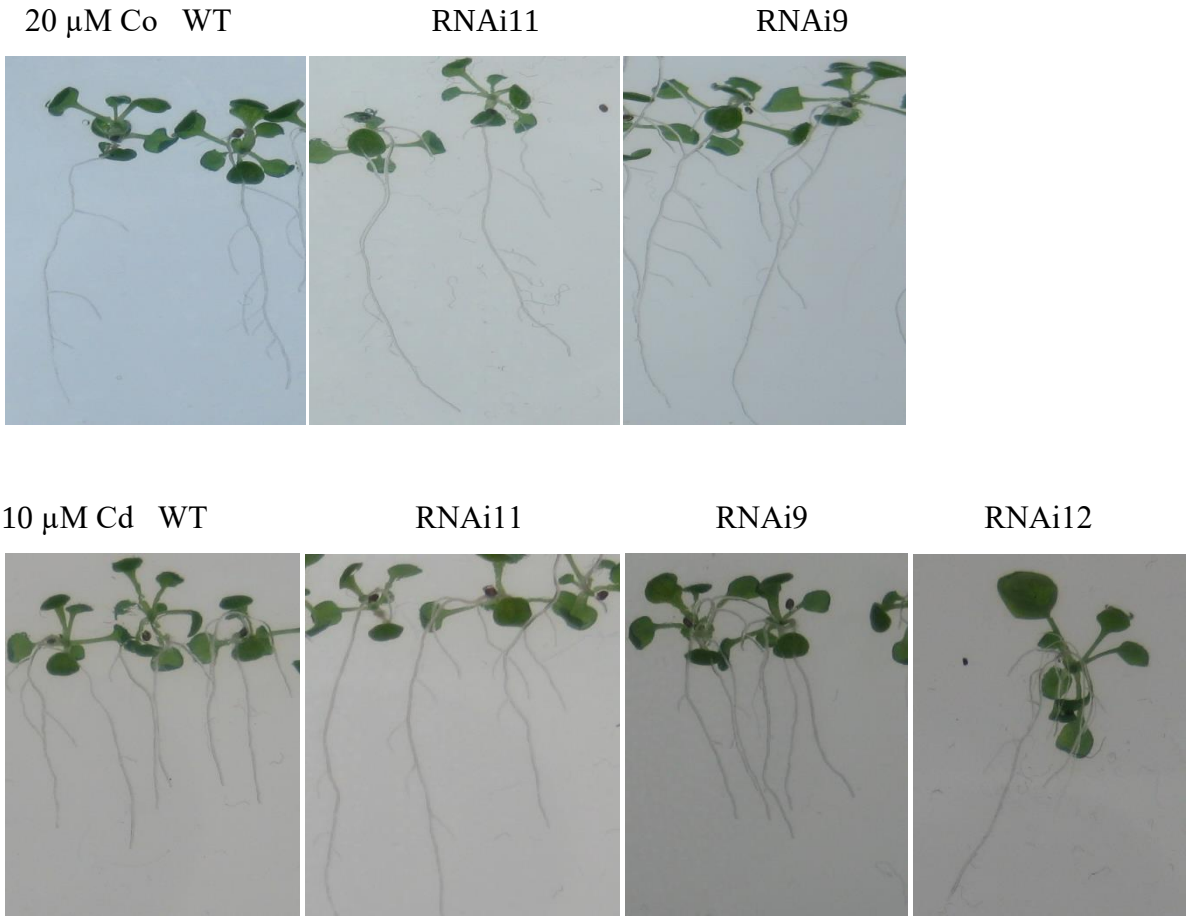



Figure 6. ZIP10 *A. thaliana* RNAi line and wild type plants which were grown in metal containing plates

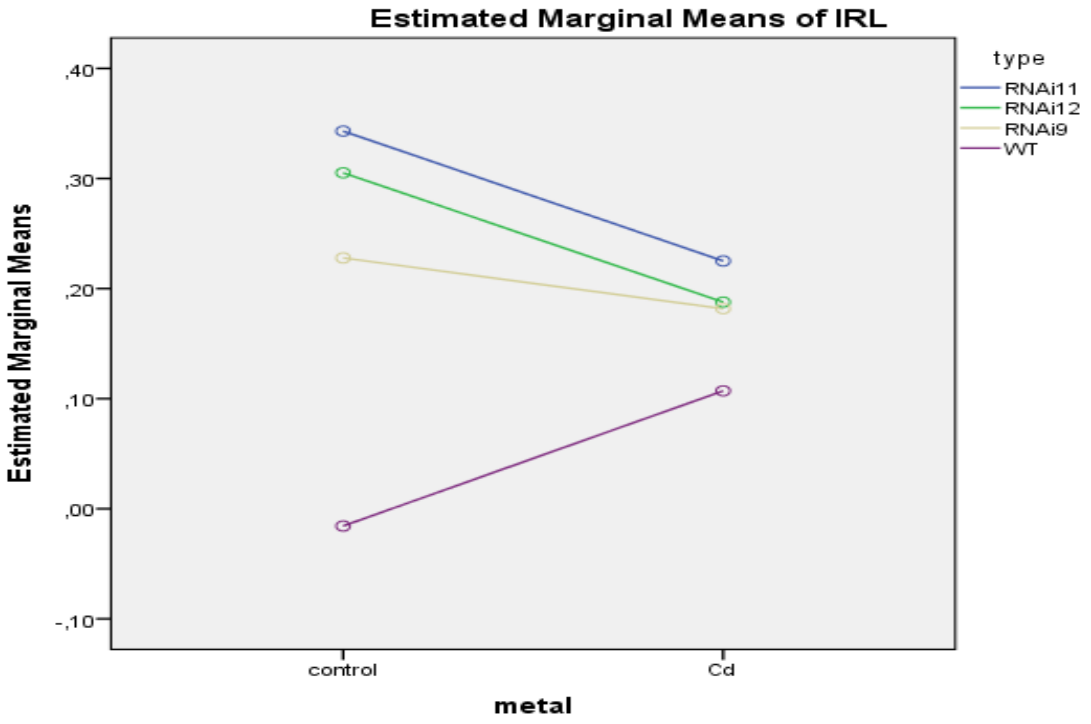
From Figure 6, it can be seen that the root length of RNAi9 line was longer than WT or RNAi11 root length. In 10 µM Cd RNAi11 has the longest root compared to WT, RNAi9 and RNAi12.

Root lengths were measured from photos taken of the plants growing on different metals. From root length measurements of *A. thaliana* RNAi line and WT plants (Table 2) it can be said that root length of the RNAi11 is much longer than the WT, also seen in Figure 6. There are not big differences in root lengths of RNAi9 and RNAi11, also RNAi12 in Cd, Zn and control.

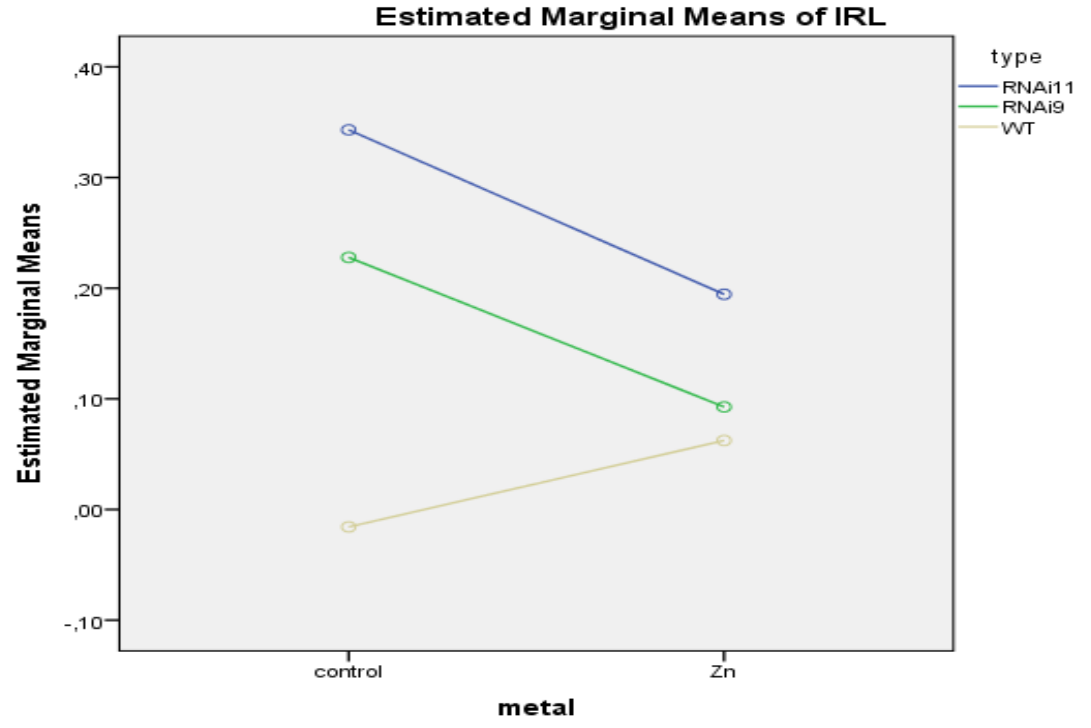
Table 2. Mean values of the root length measurements of the RNAi line and WT plants. (α=0.05, One-way Anova)

| lines / metals | WT | RNAi9 | RNAi11 | RNAi12 | *p* value |
|---|---|---|---|---|---|
| 20 µM $MnSO_4$ | 1.33 | 1.57 | 2.37 | | .000 |
| 35 µM Ni $SO_4$ | 1.09 | 2.16 | 2.25 | | .184 |
| 20 µM $CoCl_2$ | 1,05 | 2.58 | 2.21 | | .003 |
| 400 µM Zn | 1.17 | 1.27 | 1.61 | | .000 |
| 10 µM Cd $SO_4$ | 1.30 | 1.56 | 1.72 | 1.61 | .000 |
| 10 µM BPS | 1.17 | 2.36 | 2.45 | | .057 |
| Cd $SO_4$+BPS | 1.23 | 1.87 | 2.12 | | .059 |
| control | 1.35 | 1.78 | 2.25 | 2.05 | |

Since the control plants grew differently compared to the RNAi lines, a two-way ANOVA was done to evaluate if the RNAi lines respond differently to metals than the WT plants. Root length was used as a measure of plant response to metals. The RNAi's and WT plants respond differently to Cd exposure (Figure 7(a)). Also in case of Zn, RNAi's and WT plants respond differently (Figure 7(b)). There were no differences in the responses of the RNAi lines and WT plants exposed to other metals. Thus, ZIP10 may have a function in Cd or Zn transport.




(a)




(b)

Figure 7. Responses of RNAis and WT plants to Cd and Zn exposure (a) WT and RNAi lines in Cd and control plates (Two-way Anova p= 0.000) and (b) WT and RNAi lines in Z and control plates (Two-way Anova p= 0.000).

# 6. DISCUSSION

The *ZIP10* gene has been proposed to function as a Zn or Ni transporter based on its expression in hyperaccumulators *A. halleri* and *N. caerulescens* (Talke *et al*., 2006; Halimaa *et al*., 2014). However, knowledge about *ZIP10* gene function in metal accumulation is limited.

The amino acid sequences of ZIP10 protein from four accessions of *N. caerulescens* were aligned. These sequence analysis deduced, in line with Guerinot's (2000) description, 330-369 amino acid residues in ZIP10 from *Noccaea* accessions. His and Glu rich repeat region in proteins (Figure 1 in a black rectangle) has been suggested as the metal binding site in ZIP transporters. These amino acids are potential ligands of metals, and they may play an important role in binding the substrate (Rogers *et al*., 2000). The ZIP10 translated protein sequences showed some variance among *N. caerulescens* accessions. LC3 ZIP10 and GA ZIP10 had 100% same protein sequence with HHGHGHGHGH putative metal binding region. In protein sequences of LC1 ZIP10 and LE ZIP10 the metal binding site was HYGHSHGHGHGHGHGH region, and in MP ZIP10 HYGHSHGHGH region was presented. These differences may affect protein function.

From results of yeast complementation, it can be noted that all ZIP10 transformants from the *N. caerulescens* accessions grew in metal exposures, however some of them grew very well and some of them weakly. However, only a limited number of metals were tested. In further studies other metals need to be included.

A gene ontology analysis, made on the basis of gene expression differences of the *N. caerulescens* accessions, has pointed that the accessions are distinguished from each other by the expression of metal transporter genes (Halimaa *et al*., 2014). Halimaa *et al*., (2014) mention that all accessions of *N. caerulescens* are more or less effective Zn hyperaccumulators, and LE is most sensitive to Zn. In the results of this study, growth of all ZIP10 transformants from *N. caerulescens* accessions, and empty vector control on Zn were distinguished from each other by only minor differences. *ZIP10* gene function corresponds to the characteristics of the accessions (Zn tolerance is a species-wide trait in *N. caerulescens*), for example yeast with LC1 ZIP10 grew well on Zn, which is consistent with the observation

that LC has the weakest accumulation of Zn (Assuncao *et al*., 2003). The growth of LC1 ZIP10 yeast transformant does not differ from the growth of the control yeast with empty vector in Co exposure. This means that the LC1 ZIP10 yeast probably does not absorb Co in the cells.

The yeasts that do not grow as well probably transport Co in cells using the ZIP10 transporter that has been transferred into them. Thus, LC1 ZIP10 is probably non-functional both in Zn and Co transport. In addition, yeast containing LC1 ZIP10 grew much better in Ni and Mn containing plates compared to empty vector control and other accessions besides MP thereby suggesting that LC1 ZIP10 is not a Ni or Mn transporter either. Even though the LE ZIP10 metal binding amino acid sequence in protein sequence was identical with to the LC1 ZIP10 sequence, their metal uptake capacity was not similar. Yeast containing LE *ZIP10* gene had the weakest growth in all metal different concentrations except Mn compared to other accessions and empty vector control.

Accession MP shows extreme accumulation of Ni compared to the other accessions (Assuncao et al., 2003). In Halimaa *et al*., (2014) it was suggested that MP ZIP10 may have a role in accumulation of Ni. In the results of this study, this is supported by the finding that the growth of the empty vector control yeast is better than the growth of MP ZIP10 transformant on Ni exposure. In case of Co, MP growth is slightly weaker than the growth of the control yeast, so MP could uptake Co. MP was not able to complement the Mn uptake.

The growth of GA and LC3 *ZIP10* yeast transformants on metal plates is similar, inasmuch as their protein sequence showed 100% identity. As demonstrated in Table 1, GA accession had a moderate accumulation of Ni. Halimaa *et al*, (2014) also reported that GA had a moderate Ni accumulation. In our results, the growth of the ZIP10 GA transformant in Ni and Co is the weakest compared to the control yeast and other accessions. This would indicate that GA ZIP10 transports Ni and Co. In this study *fet3fet4* iron uptake yeast mutant was used whereas in further studies *N. caerulescens* ZIP10 transporter gene can be characterized by using *zrt1zrt2* Zn uptake mutant, because in Milner *et al*, (2013) studies of the *A. thaliana ZIP10* gene grew well in Zn uptake mutant, indicating that the protein has a role in Zn transport. ZIP10 protein localization also needs to be analyzed, because it is not known whether ZIP10 was located in the plasma membrane or not.

The *ZIP10* gene was analyzed from each *Noccaea* accessions in yeast. Then to characterize the function of the *ZIP10* gene in plants, previously constructed *Arabidopsis* RNAi line plants with silenced *ZIP10* gene were grown in metal containing plates with different concentrations. These RNAi line plants were transgenic as was expected and *ZIP10* gene was silenced in RNAi lines. The metal transport properties of the ZIP10 could not be conclusively determined from root measurements of the RNAi line and WT plants. The root measurements only indicate that RNAi9 root length was longest on 35 μM Ni, 20 μM Co and 10 μM BPS plates, while on other metals root length was comparable to the control. The root length of line RNAi11 was shortest on 400 μM Zn and 10 μM Cd, while on other metals the root length of RNAi11 was comparable to the control. A two-way ANOVA illustrated that the *ZIP10* RNAi line plant roots responded differently to Cd and Zn than the WT. This indicates that ZIP10 may have a role in Cd and Zn transport.

The *ZIP10* gene in both yeast transformants and RNAi plants grew in all metal containing plates. However, it does not confirm that the ZIP10 can transport all metals. In yeast complementation studies it was determined that the LC1 ZIP10 is not functional and the ZIP10 from other accessions transports possibly Co and Ni. There is some inconsistency between plant and yeast experiments, which could be explained by the yeast mutant used in the experiments. Furthermore, yeasts were not tested for Cd tolerance.

Contaminated soils with heavy metals should be cleaned to avoid contamination of food crops. If the ZIP10 protein can transport Zn, Cd and possibly other metals to the above ground parts of plants, it can be used for the phytoremediation of Cd and possibly Ni from the soil and in the biofortification of food crops by transporting essential micronutrients (Zn) to edible plants. Also, this gene could be transferred to the non-hyperaccumulator plants to make them hyperaccumulators and help them to accumulate metals from contaminated soils. In this study transgenic *ZIP10* RNAi *A. thaliana* lines were used to show the role of ZIP10 in metal transport. In further studies the *ZIP10* gene can be overexpressed in plants to create metal hyperaccumulating plants.

# 7. CONCLUSION

The function of the ZIP10 metal transporter protein has not been studied before. In this study its function was characterized. Yeast tolerance tests indicated that all NcZIP10 proteins, except LC1 ZIP10, transport Co and Ni into cells. The LC1 ZIP10 transformant has the shortest protein sequence and the results demonstrated that it could not transport metals. None of the yeast transformants transported Mn. The ZIP10 RNAi line plants indicated a role for ZIP10 in Zn and Cd transport. Thus, ZIP10 is possibly a metal transporter with a broad substrate range. The ZIP10 proteins' metal transport capacity may have significance for hyperaccumulation. It can be used in the phytoremediation of soils.

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# APPENDIX 1

A. Metals and their concentrations that were used to evaluate ZIP10 effects on yeast metal tolerance

| Metal | concentration, μM |
|---|---|
| Manganese | 100 |
| Nickel | 80 |
| | 100 |
| Cobalt | 50 |
| | 60 |
| Zinc | 1000 |
| | 2000 |
| | 3000 |

B. Metals and their concentrations that were used to evaluate ZIP10 effects on RNAi line plant metal tolerance

| Metal | concentration, μM |
|---|---|
| Manganese (II) sulfate | 20 |
| Nickel (II) sulfate | 35 |
| Cobalt (II) chloride | 20 |
| Zinc | 400 |
| Cadmium sulfate | 10 |
| bathophenanthrolinedisulphonate | 10 |
| Cadmium sulfate + bathophenanthrolinedisulphonate | 10+10 |