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Analysis of the role of *MTP1*, *NRAMP4* and *ZNT1* metal transporters in Ni hypertolerance and hyperaccumulation in *Noccaea caerulescens*

and

Identification of miRNAs involved in response to Zn excess in *Arabidopsis* species

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ABSTRACT

Analysis of the role of *MTP1*, *NRAMP4* and *ZNT1* metal transporters in Ni hypertolerance and hyperaccumulation in *Noccaea caerulescens*

The regulation of nutrient homeostasis in plants represents a fundamental process which is indispensable to avoid metal toxicity caused by over-accumulation of macro and micronutrients. Plants have evolved several strategies to cope with metal excess in order to avoid harmful effects; interestingly, some plant species called hyperaccumulators are characterized by their ability to accumulate and tolerate much higher concentration of toxic elements into the areal part compared to non-accumulator plants (Jaffré *et al.,* 1976). The most represented trait is associated with Ni hyperaccumulation, which comprise almost 400 different plant species, mostly belonging to the families Brassicaceae and Euphorbiaceae (Krämer, 2010); this micronutrient is required in very small amounts in plants (Dalton *et al.,* 1988), since its essential role as constituent of several metallo-enzymes (Brown *et al.,* 1987; Ahmad and Ashraf, 2011). As well as for the other micronutrients, high concentrations of Ni are toxic to plants, which try to avoid metal toxicity in several ways, although the molecular mechanisms regarding these processes are poorly understood. Among hyperaccumulator plants, *Noccaea caerulescens* represents one of the most interesting species, due to the huge variability between ecotypes and populations regarding metal tolerance and accumulation (Lasat *et al.,* 1996; Lombi *et al.,* 2000; Pence *et al.,* 2000; Assunção *et al.,* 2003).

This PhD work was focalized on *N. caerulescens* ecotype Monte Prinzera (MP, Italy), native of a serpentine soil from Italy, which is able to hypertolerate and hyperaccumulate Ni in addition to Zn (Assunção *et al.,* 2008; Richau and Schat, 2009). Molecular mechanisms responsible for Ni tolerance and accumulation are still mostly unknown, although it was suggested that metal transporters, also essential for metal homeostasis, have a fundamental role in Heavy Metal tolerance and accumulation (Krämer *et al.*, 2007). In particular, some genes members of the *NRAMP* (Natural Resistance Associated Macrophage Protein), *ZIP* (ZRT, IRT-like Proteins) and *CDF* (Cation Diffusion Facilitator) family of metal transporters could participate to Ni tolerance and accumulation in Ni-hyperaccumulator plants as previously demonstrated (Persans *et al.,* 2001; Mizuno *et al.,* 2005; Richau *et al.,* 2009; Visioli *et al.,* 2014). Therefore, the possible involvement of the vacuolar transporters *MTP1* and *NRAMP4* and of the plasma membrane transporter *ZNT1* in these processes was investigated by several approaches. The expression of all three genes in *N. caerulescens* MP under Ni excess was initially analyzed and compared with those of *Thlaspi arvense* (non-accumulator), as control, and *N. caerulescens* ecotype Ganges (GA, Zn/Cd hyperaccumulator).

High constitutive levels of all three genes were found in both ecoypes of *N. caerulescens,* under control and Ni excess conditions compared to *T. arvense*, confirming the different regulation of metal transporters in hyperaccumulators/hypertolerant plants (Krämer *et al.*, 2007). Up-regulation of *NRAMP4* and *ZNT1* was observed in *N. caerulescens* MP after different hours of Ni treatment, according to previous data (Visioli *et al.,* 2014). In order to investigate Ni transport properties of both *NcNRAMP4* and *NcZNT1,* yeast complementation assays have been performed; NcNRAMP4 reduced recombinant yeast survival under Ni treatment and the opposite result was found for NcZNT1*,* consistently with the data obtained for the other Ni hyperaccumulator plant *N. japonica* (Mizuno *et al.,* 2005). Transgenic lines of *A. thaliana* overexpressing single *NcNRAMP4* and *NcZNT1* and double *NcNRAMP4/NcZNT1* were also obtained fusing the complete CDS of these genes under the control of CaMV35S promoter and crossing single transgenic lines. A phenotypic characterization was initially performed on these plants to better evaluate the possible impact of the overexpression of these transporters *in planta.* Under standard conditions, single 35S::*NRAMP4* and double 35S::*NRAMP4*/35S::*ZNT1* lines displayed bigger shoot compared to WT plants and the same results were also found for two of the three lines overexpressing *ZNT1,* although with a minor impact compared to the other gene. Ni tolerance *in planta* was also investigated by growing *A. thaliana* WT and transgenic lines under Ni excess on soil and in hydroponic solution. Transgenic lines of *A. thaliana* expressing *NcNRAMP4*, *NcZNT1* or *NcNRAMP4/NcZNT1* in combination displayed higher tolerance to metal excess in comparison to WT plants, thanks to a reduction of Ni accumulation in shoots. *In vitro* analysis has been performed in order to understand the possible role of both genes in Ni tolerance. Single 35S::*NcNRAMP4* and double 35S::*NcNRAMP4*/35S::*ZNT1* transgenic lines displayed longer roots under standard and Ni excess condition compared to the WT, although no significative differences were found regarding 35S::*ZNT1* overexpressing lines.
The possible involvement of the vacuolar transporter MTP1 in Ni hyperaccumulation and hypertolerance in *N. caerulescens* MP was also investigated. Gene expression analysis have initially confirmed the high constitutive levels of MTP1 expression previously found in different hyperaccumulator-hypertolerant plants belonging to *Noccaea* genus (Assunção *et al.*, 2001; van de Mortel *et al.*, 2006) under normal and stressful conditions, although in *N. caerulescens* MP this gene seems to be downregulate upon Ni excess. Interestingly, two different CDSs of *MTP1* characterized by different length were found in cDNA and genomic DNA of both *N. caerulescens* MP and GA ecotypes. Sequences alignment revealed that the shorter form of MTP1, called *MTP1-short,* do not present the highly conserved His-loop region, which has been proposed to act as a Zn sensor for MTP1 activity *in planta* (Kawachi *et al.,* 2008; Tanaka *et al.,* 2015). In order to understand the subcellular localization of both proteins, co-localization experiments have been performed in *N. tabacum* leaves with constructs harbouring the two MTP1 coding sequences fused to eGFP reporter gene. Both forms of NcMTP1 were localized in the vacuolar compartment, suggesting their role in metal detoxification.

To study the metal binding properties of both *NcMTP1-long* and *NcMTP1-short,* yeast complementation assays with *zrc1cot1* double mutant yeast, which lacks these two vacuolar proteins, and WT strain of *S. cerevisiae* were performed, using *AtMTP1* as control for Zn transport ability (Desbrosses-Fonrouge *et al.,* 2005). Differences regarding Zn, Ni and Co transport ability were found comparing the two forms of NcMTP1 in both *N. caerulescens* MP and GA ecotypes, suggesting a possible different role of *MTP1* genes in metal detoxification in these plants, characterized by different metal hyperaccumulation and metal tolerance properties. To clarify their role *in planta,* the complete CDSs of *NcMTP1-long* and *NcMTP1-short* were fused downstream the control of the strong CaMV35S promoter and introduced into *A. thaliana* homozygous *mtp1*+/+ mutants. The transgenic lines will be study in order to investigate the possible involvement of the His-loop in determine the metal binding specificity properties of MTP1, as previously proposed (Kawachi *et al.,* 2008).

Identification of miRNAs involved in response to Zn excess in *Arabidopsis* species

Thanks to advances in high-throughput sequencing technologies and bioinformatics, in last years it has been possible to shed some light on the key roles of small RNAs (sRNAs) in several biological functions of many organisms, including the classes of miRNAs (Axtell, 2013; Shriram *et al.,* 2016; de Vries *et al.,* 2018). In plants these small regulatory molecules seem to be fundamental for the regulation of growth and development (Singh *et al.,* 2018) under normal and stressful conditions, increasing the ability of plants to respond to several environmental signals, including a variety of stressing factors (Zheng *et al.,* 2015; Noman and Aquell, 2017; Pegler *et al.,* 2019). For this reason, miRNAs have emerged as master regulators in plants, also suggesting their possible use as biotechnological tools to improve crop tolerance to biotic and abiotic stresses (Budak and Zhang, 2017; Xu *et al.,* 2019; Wani *et al.,* 2020).
Metal pollution caused by anthropogenic activities is particularly hazardous for agriculture: high concentrations of metals in the soil can reduce crop yield, given that they compete with other essential micronutrients (Dal Corso *et al.,* 2008; Gielen *et al.,* 2012). Much research in recent years has therefore focused on the identification of miRNAs having a potential role in response to metal stress (Yang and Chen, 2013; Jian *et al.,* 2018; Noman *et al.,* 2019; Xu *et al.,* 2019). Most investigations were performed on *A. thaliana* and other plant species, representing an interesting starting point for further studies (Jalmi *et al.,* 2018). Several conserved and non-conserved miRNAs have been identified as key regulators for heavy metal stress response in *A. thaliana* (Ding and Zhu, 2009; Barciszewska-Pacak *et al.,* 2015), rice (Ding *et al.,* 2013), *Medicago truncatula* (Zhou *et al.,* 2012), *Brassica napus* (Jian *et al.,* 2018) maize (Gao *et al.,* 2019), and others, although many aspects concerning miRNA biogenesis, their regulation and interaction with

targets remain to be clarified. In this work, we focused our attention on the identification of miRNAs involved in the response to excess Zn. This essential micronutrient is a cofactor for several enzymes (Broadley *et al.*, 2007; Camak *et al.,* 2007) and it is therefore necessary for plant growth and development. However, high concentrations of Zn in the soil can be toxic: therefore, plants have evolved a variety of strategies to avoid this potentially dangerous situation. The role of metal transporter proteins in response to metal excess in *Arabidopsis* has been investigated, but little is known about the potential involvement of miRNAs in these processes.

We decided to study the Zn/Cd hypertolerant-hyperaccumulator plant *A. halleri* ecotype I16 (Lombardy, Italy; Bert *et al.*, 2000) to verify whether its modulation of miRNA-target interactions differed from that of *A. thaliana* and also elucidate the potential role of miRNAs in Zn hyperaccumulation and hypertolerance. Several approaches were used for this purpose.

First of all, a sRNA-Seq analysis was performed on the sRNAs isolated from shoots of Zn-treated and control *A. thaliana* and *A. halleri* plants; in a later stage, a bioinformatics-based approach was used to analyze the data and potentially identify differently expressed miRNAs. The modulation (down- and up-regulation) of 100 miRNAs in *A. halleri* differed from that of Zn-exposed and control *A. thaliana.* In particular, the number includes several metal-responsive miRNAs with different biological functions, hence suggesting possible differences in the miRNA-target interactions of these plant species.

To confirm the data obtained by sRNA-Seq, Northern Blot analysis and Real Time RT-PCR were performed on some metal-responsive miRNAs that were selected on the ground of two factors, i.e. their different expression in *A. thaliana* and *A. halleri*, as determined by sRNA-Seq analysis, and their possible involvement in response to metals, as suggested in the literature (Yang and Chen, 2013; He *et al.,* 2018; Jalmi *et al.,* 2018; Pegler, 2019). Some of these miRNAs are known to be involved in plant development and morphological adaptation under stressful conditions (including miR157, miR159, miR390), whereas the others have a role in the plant's response to nutrient deficiency and oxidative stress responses (miR395, miR398, miR408). Experiments confirmed that all of them were differently modulated in *A. halleri* and *A. thaliana*, thus indicating a different regulation of gene expression between these two plant species and suggesting the possible involvement of these miRNAs in Zn nutrition (see Zheng *et al.,* 2019).

Subsequently, we focused on miR398b and miR408. These two conserved miRNAs, together with other genes and small RNA regulatory molecules known as Cu-related genes, are mainly involved in the regulation of Cu homeostasis in *A. thaliana* and other plant species, but also play a role in the plant's response to a variety of abiotic stresses, including oxidative stress and the presence of heavy metals (Yamasaki *et al.,* 2009; Pilon, 2017; Jalmi *et al.,* 2018). It has been speculated that a coordinate action of both miRNAs is required for basal Cd tolerance in *Arabidopsis* (Gayomba *et al.,* 2013; Gielen *et al.,* 2016), thus representing an interesting starting point for further analyses.

At first, the expression of both miR398 and miR408 was determined in *A. thaliana* and *A. halleri* exposed to excess Zn: both miRNAs were up-regulated in the two species, especially miR398b, in keeping with the findings of other authors (Remans *et al.,* 2012; Gielen *et al.,* 2016). The promoter sequence of both miRNAs was then amplified from the genomic DNA of *A. thaliana* and *A. halleri* and fused to the *GUS* reporter gene both to study their modulation upon exposure to Zn and better understand their role in the response of *Arabidopsis* subjected to excessive amounts of this metal. A bioinformatic analysis was performed in order to compare the entire sequences of the two miRNAs isolated from *A. thaliana* and *A. halleri*, and several DNA motifs involved in the response were thus identified. GUS assay on *A. thaliana* transgenic lines expressing pAtMIR398::*GUS*, pAhMIR398b::*GUS*, pAtMIR408::*GUS*, pAhMIR408::*GUS* revealed that both miRNAs are expressed in the roots and shoots of *Arabidopsis,* particularly in the vasculature tissues, suggesting their mobility over the entire plant (Buhtz *et al.,* 2010). In addition, GUS expression was also assessed on transgenic plants exposed to Zn and Cu, to clarify whether these two elements compete against each other (Remans *et al.,* 2012; Gao *et al.,* 2019; Pilon and Shahbaz, 2019) and study the response of these two miRNAs to this type of stress.
Finally, we investigated the effect of overexpressing *A. halleri pre-MIR408* in response to Zn treatment, given its conserved role in the plant's ability to adapt to environmental factors, as demonstrated for the *A. thaliana* homolog (Zhang and Li, 2013; Ma *et al.,* 2015; Song *et al.,* 2018).

SOMMARIO

Analisi del ruolo dei trasportatori di metalli *MTP1, NRAMP4* e *ZNT1* nella tolleranza e iperaccumulo di Ni in *Noccaea caerulescens*

La regolazione dell'omeostasi dei nutrienti nelle piante rappresenta un processo fondamentale per evitare effetti nocivi dovuti ad un eccessivo accumulo di macro e micronutrienti. Le piante hanno sviluppato diverse strategie per far fronte all'eccesso di metallo al fine di evitare effetti dannosi; è interessante notare che alcune specie, definite iperaccumulatrici, sono caratterizzate dalla capacità di accumulare e tollerare una concentrazione molto più elevata di metalli nella parte aerea rispetto alle piante non accumulatrici (Jaffré *et al.*, 1976). Il tratto più rappresentato è associato all'iperaccumulo di Ni, che comprende quasi 400 specie vegetali diverse, per lo più appartenenti alle famiglie Brassicaceae ed Euphorbiaceae (Krämer, 2010); questo micronutriente è richiesto in quantità molto limitate nelle piante (Dalton *et al.*, 1988), dato il suo ruolo essenziale come componente richiesto per l'attività di numerosi enzimi (Brown *et al.*, 1987; Ahmad e Ashraf, 2011). Così come per gli altri micronutrienti, alte concentrazioni di Ni sono tossiche

per le piante, che cercano di evitare questi effetti dannosi mediante diverse strategie, sebbene i meccanismi molecolari riguardanti questi processi siano poco compresi.

Tra le diverse piante iperaccumulatrici, *Noccaea caerulescens* rappresenta una delle specie più interessanti, a causa dell'enorme variabilità tra diversi ecotipi per quanto riguarda la tolleranza e l'accumulo di metalli (Lasat *et al.*, 1996; Lombi *et al.,* 2000; Pence *et al.,* 2000; Assunção *et al.,* 2003).

Questo lavoro di ricerca si è focalizzato su *N. caerulescens* ecotipo Monte Prinzera (MP, Italia), nativo di un terreno serpentino, che è in grado di ipertollerare e iperaccumulare Ni oltre a Zn (Assunção *et al.,* 2003; Richau and Schat, 2009). I meccanismi molecolari responsabili della tolleranza e dell'accumulo di Ni sono ancora poco noti, sebbene diversi studi abbiano suggerito che i trasportatori di metalli, anch'essi essenziali per l'omeostasi dei metalli, abbiano un ruolo fondamentale in questo processo (Krämer *et al.,* 2007). Alcuni membri della famiglia di trasportatori di metalli *NRAMP* (Natural Resistance Associated Macrophage Protein), *ZIP* (ZRT, IRT-like Proteins) e *CDF* (Cation Diffusion Facilitator) sembrano essere coinvolti nella tolleranza e accumulo di Ni nelle piante iperaccumulatrici (Persans *et al.,* 2001; Mizuno *et al.,* 2005; Richau *et al.,* 2009; Visioli *et al.,* 2014). Pertanto, il possibile coinvolgimento dei trasportatori vacuolari *MTP1* e *NRAMP4* e del trasportatore di membrana plasmatica *ZNT1* nell'ipertolleranza e/o iperaccumulo di Ni in *N. caerulescens* MP è stato studiato mediante differenti approcci. L'espressione dei tre geni in *N. caerulescens* MP in eccesso di Ni è stata inizialmente analizzata e confrontata con quelle della specie non accumulatrice *Thlaspi arvense*, come controllo, e di *N. caerulescens* ecotipo Ganges (GA) iperaccumulatrice di Zn/Cd. I tre geni sono risultati essere altamente espressi in maniera costitutiva nei due ecotipi di *N. caerulescens*, sia in condizioni standard che con eccesso di Ni, confermando la diversa regolazione dei trasportatori di metalli nelle piante ipertolleranti-iperaccumulatrici (Krämer *et al.,* 2007). In particolare, è stata osservata una modulazione positiva dell'espressione di *NRAMP4* e *ZNT1* in *N. caerulescens* MP dopo 12 e 18 ore di trattamento con il Ni, confermando i risultati precedentemente ottenuti (Visioli *et al.,* 2014) e portando l'attenzione sul loro possibile coinvolgimento nell'iperaccumulo/ipertolleranza di Ni in questa specie. Al fine di studiare le proprietà di legame al Ni sia di *NcNRAMP4* che di *NcZNT1*, sono stati condotti test di complementazione in lievito; è interessante notare che l'espressione eterologa di NcNRAMP4 ha ridotto la sopravvivenza del lievito ricombinante sottoposto a trattamento col Ni, e un risultato opposto si è ottenuto con NcZNT1, in accordo con i dati precedentemente ottenuti nell'altra specie iperaccumulatrice di Ni *N. japonica* (Mizuno *et al.,* 2005). Per studiare l'impatto di questi due trasportatori di metalli in pianta, sono state ottenute linee transgeniche di *A. thaliana* che sovraesprimono singolarmente *NcNRAMP4* e *NcZNT1* ed entrambi i geni *NcNRAMP4/NcZNT1* mediante la fusione della CDS sotto il controllo del promotore CaMV35S e incrocio tra linee overesprimenti.

Inizialmente è stata eseguita una caratterizzazione fenotipica su queste linee transgeniche per valutare il possibile impatto della sovraespressione di questi trasportatori in pianta.

In condizioni standard, le piante di linee transgeniche esprimenti singolarmente 35S::*NRAMP4* e quelle overesprimenti entrambi i geni 35S::*NRAMP4*/35S::*ZNT1* hanno mostrato maggiori dimensioni rispetto alle piante WT e gli stessi risultati sono stati trovati anche per due delle tre linee overesprimenti 35S::*ZNT1*, sebbene con un impatto minore rispetto alle piante overesprimenti *NcNRAMP4*. La tolleranza al Ni in pianta è stata anche studiata facendo crescere in suolo e in soluzione idroponica le piante WT di *A. thaliana* e le diverse linee transgeniche sottoposte ad un eccesso di Ni. È interessante notare che le linee transgeniche di *A. thaliana* hanno mostrato una maggiore tolleranza all'eccesso di metallo rispetto al WT, grazie ad una differente concentrazione di Ni accumulato nella parte aerea. Sono state effettuate anche delle analisi di crescita *in vitro* per comprendere al meglio il possibile ruolo di entrambi i geni nella tolleranza al Ni. Le piante overesprimenti *NcNRAMP4* ed entrambi i geni *NcNRAMP4*/*ZNT1* hanno mostrato radici più lunghe in condizioni standard e in eccesso di Ni rispetto al WT, sebbene non siano state riscontrate differenze significative riguardo alle linee overesprimenti *ZNT1*.

In questo lavoro è stato anche valutato il possibile coinvolgimento del trasportatore vacuolare MTP1 nell'iperaccumulo e ipertolleranza al Ni in *N. caerulescens* MP. L'analisi dell'espressione genica ha inizialmente confermato gli alti livelli di espressione del gene *MTP1* precedentemente osservate in diverse specie di piante iperaccumulatrici-ipertolleranti metalli appartenenti al genere *Noccaea* (Assunção *et al.,* 2001; van de Mortel *et al.,* 2006), sia in condizioni standard che in eccesso di metallo, sebbene in *N. caerulescens MP* l'espressione di questo gene sembra diminuire quando sottoposta al trattamento con il Ni. È interessante notare che due diverse sequenze codificanti MTP1, caratterizzate da una diversa lunghezza, sono presenti nel cDNA e nel DNA genomico di entrambi gli ecotipi MP e GA di *N. caerulescens* presi in esame. L'allineamento nucleotidico ha rivelato che l'MTP1 più corto, chiamato MTP1-short, non presenta la regione altamente conservata His-loop, che è stata proposta fungere da sensore per lo Zn necessario per l'attività di MTP1 in pianta (Kawachi *et al*., 2008; Tanaka *et al.,* 2015). Al fine di studiarne la localizzazione subcellulare, sono stati condotti esperimenti di co-localizzazione in foglie di *N. tabacum* mediante l'utilizzo di costrutti contenenti le due sequenze codificanti MTP1 fuse al gene reporter eGFP. Sia NcMTP1-long che NcMTP1-short sono presenti sul tonoplasto, suggerendo il loro ruolo nella detossificazione dell'eccesso di metallo in pianta. Per studiare le proprietà di legame ai metalli di entrambe le forme di NcMTP1, sono stati eseguiti test di complementazione in lievito utilizzando il doppio mutante *zrc1cot1,* che non possiede queste due proteine vacuolari, e un ceppo WT di *S. cerevisiae*, usando AtMTP1 come controllo per l'abilità di trasporto dello Zn (Desbrosses-Fonrouge *et al.,* 2005). Differenze legate alla capacità di trasportare Zn, Ni e Co sono state riscontrate confrontando NcMTP1-long e NcMTP1-short di entrambi gli ecotipi di *N. caerulescens* MP e GA, suggerendo un possibile diverso ruolo di MTP1 in questi due ecotipi caratterizzati da diverse proprietà di iperaccumulo e ipertolleranza ai metalli.

Per chiarire il loro ruolo in pianta, la CDS completa di entrambi *NcMTP1-long* e *NcMTP1-short* è stata fusa a valle del controllo del promotore forte CaMV35S e introdotto nei mutanti omozigoti di *A. thaliana mtp1* +/+. Le linee transgeniche saranno analizzate per studiare il possibile coinvolgimento dell'His-loop nella determinazione della specificità di legame al metallo in MTP1, come proposto precedentemente (Kawachi *et al.*, 2008).

Progetto 2: Identificazione di miRNAs in risposta all'eccesso di Zn in specie di *Arabidopsis*

Grazie al progresso delle tecnologie di sequenziamento ad alto rendimento e della bioinformatica, negli ultimi anni è stato possibile ottenere più informazioni sui ruoli chiave degli small RNA (sRNAs) in diversi processi biologici di molti organismi, compresa la classe dei miRNA (Axtell, 2013; Shriram *et al.,* 2016; de Vries *et al.,* 2018). Nelle piante, in particolare, queste piccole molecole regolatrici sembrano essere fondamentali per la crescita e lo sviluppo dell'organismo (Singh *et al.,* 2018), migliorando la sua capacità di adattarsi a diversi segnali ambientali, tra cui una varietà di fattori di stress (Zheng *et al.,* 2015; Noman and Aquell, 2017; Pegler *et al.,* 2019). Per questo motivo, i miRNA sono stati posti al centro di molte ricerche, volte a valutare il loro possibile utilizzo come strumenti biotecnologici per migliorare la tolleranza delle colture agli stress biotici e abiotici (Budak e Zhang, 2017; Xu *et al.,* 2019; Wani *et al.,* 2020). L'inquinamento da metalli causato da attività antropogeniche è particolarmente pericoloso per l'agricoltura: alte concentrazioni di metalli nel suolo possono ridurre la resa delle colture, poichè competono con altri micronutrienti essenziali (Dal Corso *et al.,* 2008; Gielen *et al.,* 2012). Molte ricerche negli ultimi anni si sono pertanto concentrate sull'identificazione di miRNAs con un potenziale ruolo nella risposta allo stress da eccesso di metalli (Yang e Chen, 2013; Jian *et al.,* 2018; Noman *et al.,* 2019). Sono stati identificati diversi miRNA, conservati e non conservati, aventi un ruolo nella risposta ai metalli in *A. thaliana* (Ding e Zhu, 2009; Barciszewska-Pacak *et al.*, 2015), riso (Ding *et al.,* 2013), *Medicago truncatula* (Zhou *et al.,* 2012), *Brassica napus* (Jian *et al.,* 2018) mais (Gao *et al.*, 2019) e altre specie, sebbene la maggior parte delle informazioni sulla regolazione dell'interazione tra miRNA e target in condizioni di stress sia stata solo parzialmente chiarita. In questo lavoro, abbiamo focalizzato la nostra attenzione sull'identificazione di miRNA coinvolti nella risposta all'eccesso di Zn. Questo micronutriente è essenziale come cofattore per l'attività di numerosi enzimi (Broadley *et al.,* 2007 Cakmak *et al.,* 2007), ed è richiesto per la crescita e lo sviluppo della pianta; tuttavia, alte concentrazioni di Zn nel suolo possono essere tossiche. Le piante hanno sviluppato una varietà di strategie per evitare questa situazione potenzialmente pericolosa: il ruolo delle proteine trasportatrici di metalli in risposta all'eccesso di metallo in *Arabidopsis* è stato molto studiato, sebbene si sappia poco sul potenziale coinvolgimento dei miRNA in questo processo.

In questo lavoro abbiamo anche preso in considerazione la specie ipertollerante e iperaccumulatrice di Zn e Cd *A. halleri* ecotipo I16 (Lombardia, Italia; Bert *et al.,* 2000), con l'obiettivo di identificare eventuali differenze nella modulazione di miRNA e dei loro target rispetto ad *A. thaliana* ed eventualmente chiarire il potenziale ruolo dei miRNA nell'iperaccumulo e nell'ipertoleranza ai metalli. Per questi motivi, sono stati presi in considerazione differenti approcci. Innanzitutto, è stata effettuata un'analisi sRNA-Seq sui sRNA estratti da germogli di piante di *A. thaliana*, trattata e non trattata con Zn, e *A. halleri*; successivamente, utilizzando un approccio bioinformatico, sono stati analizzati i dati ottenuti e identificati i miRNA modulati differenzialmente tra queste specie. 100 miRNAs sono risultati essere modulati in maniera differente (down e up-regolati) in *A. halleri* rispetto a *A. thaliana* trattata e non trattata con Zn, inclusi diversi miRNA che rispondono ai metalli, suggerendo possibili differenze per quanto riguarda la possibile modulazione di alcuni miRNA in queste specie di piante. Per confermare i dati ottenuti dall'analisi di sequenziamento, le tecniche Northern Blot e Real Time RT-PCR sono state utilizzate prendendo in considerazione alcuni miRNA che sono stati selezionati in base alla loro diversa espressione trovata dall'analisi sRNA-Seq in *A. thaliana* e *A. halleri* e il loro possibile coinvolgimento nella risposta ai metalli come proposto dalla letteratura (Yang and Chen, 2013; He *et al.,* 2018; Jalmi *et al.,* 2018; Pegler, 2019). In particolare, alcuni di loro sono noti per essere coinvolti nello sviluppo delle piante e nell'adattamento morfologico in condizioni di stress (tra cui miR157, miR159, miR390) e altri sono coinvolti nella risposta alla carenza di nutrienti e risposta allo stress ossidativo (miR395, miR398, miR408). La diversa espressione di questi miRNA in *A. halleri* rispetto ad *A. thaliana* è stata confermata sperimentalmente, suggerendo una diversa regolazione dell'espressione genica in queste specie vegetali e un possibile coinvolgimento nella regolazione della nutrizione di Zn (vedi Zheng *et al.,* 2019).
Successivamente, l'attenzione è stata focalizzata su miR398b e miR408. Questi due miRNA conservati, assieme ad altri geni e RNA regolatori conosciuti come geni correlati al Cu, sono principalmente coinvolti nella regolazione dell'omeostasi del rame in *A. thaliana* e in altre specie vegetali, sebbene più recentemente sia stato anche chiarito il loro ruolo in risposta a molti stress abiotici, inclusi stress ossidativo e metalli pesanti (Yamasaki *et al.,* 2009; Pilon, 2017; Jalmi *et al.,* 2018). In particolare, l'azione coordinata di entrambi i miRNA in risposta all'eccesso di Cd sembra essere fondamentale per la tolleranza basale in *Arabidopsis* (Gayomba *et al.,* 2013; Gielen *et al.*, 2016), rappresentando un interessante punto di partenza per ulteriori analisi. Per questi motivi, la sequenza promotrice di entrambi i miRNA è stata amplificata dal DNA genomico di *A. thaliana* e *A. halleri* e fusa con il gene reporter GUS, al fine di studiare la loro modulazione in condizioni di eccesso di Zn e chiarirne il ruolo in *Arabidopsis*. Inizialmente è stata effettuata un'analisi bioinformatica confrontando le intere sequenze ottenute sia da *A. thaliana* che da *A. halleri*, identificando diversi motivi di DNA coinvolti nella risposta a stress abiotici. Analisi GUS sulle linee transgeniche di *A. thaliana* che esprimono pAtMIR398::*GUS*, pAhMIR398b::*GUS*, pAtMIR408::*GUS*, pAhMIR408::*GUS* hanno dimostrato che entrambi i miRNA sono espressi sia nelle radici che nei germogli di *Arabidopsis*,

specialmente nei tessuti vascolari, suggerendo una loro mobilità all'interno della pianta (Buhtz *et al.,* 2010). Inoltre, l'espressione del gene *GUS* è stata anche valutata nelle piante transgeniche trattate con eccesso di Zn e Cu, per chiarire al meglio una possibile competizione tra questi due micronutrienti (Remans *et al.,* 2012; Gao *et al.,* 2019; Pilon and Shahbaz, 2019) e studiare la loro risposta all'eccesso di metallo.
Infine, è stato valutato l'effetto della sovraespressione in pianta del precursore di miR408 di *A. halleri* in risposta allo zinco, poiché è riconosciuto il suo ruolo fondamentale nell'adattamento ai segnali ambientali nelle piante, come dimostrato per l'omologo di *A. thaliana* (Zhang and Li, 2013; Ma *et al.,* 2015; Song *et al.,* 2018).

LIST OF ABBREVIATIONS

35S CaMV: 35S Cauliflower Mosaic Virus promoter

ABC: ATP-binding cassette

ABRE: ABA responsive element

ANOVA: Analysis of variance

bp: Base pairs

CaCa: Ca^{2+}/Cation Antiporter

CAX: Cation exchanger

CBC: Cap Binding Complex

CDF: Cation diffusion facilitator

cDNA: complementary deoxyribonucleic acid

CDS: coding sequence

CT: threshold cycle

CURE: Copper responsive element

DNA: deoxyribonucleic acid

DDL: Dawdle

DW: dry weight

EDTA: Ethylenediamine tetraacetic acid

FW: fresh weight

eGFP: enhanced green fluorescent protein

GA: Ganges

GSH: glutathione

GST: glutathione-S-transferase

GUS: β-glucuronidase

HM: Heavy Metal

HMA: Heavy Metal P-type ATPase

HYL1: Hyponastic leaves 1

Hyg: hygromycin

IPTG: isopropyl-β-D-1-thiogalactopyranoside

IRO: Iron responsive element

IRT: Iron Regulated Transporter

Kan: kanamycin

kb: kilobase

LB: left border

LB: Luria Bertani medium

LC: La Calamine ecotype

LiAc: lithium acetate

LR: Lateral root

LTRE: Low Temperature Responsive Element

min: minutes

MP: Monte Prinzera

MRE: Metal Response Element

mRNA: messenger RNA

MS: Murashige & Skoog medium

MT: metallothionein

MTP: Metal Tolerance Protein

NA: nicotianamine

NAS: nicotianamine synthase

NGS: Next-generation sequencing

NRAMP: Natural Resistance-Associated Macrophage Protein

OD: optical density

OPT: Oligopeptide transporter superfamily

ORF: open reading frame

PC: phytochelatin

PCI: phenol:chloroform:isoamyl alcohol

PCR Polymerase Chain Reaction

PEG: Polyethilene glycol

PLACE: Plant cis-acting regulatory DNA elements

PR: Prayon

pre-miRNA: Precursor miRNA

RB: Right border

RNA: ribonucleic acid

RNase: ribonuclease

ROS: reactive oxygen species

rpm: rotation per minute

RT: reverse transcription

SBP: Squamosa promoter-binding-protein

SD: Synthetic Defined (SD) Medium

SDS: sodium dodecyl sulphate

SE: Serrate

SPL7: Squamosa promoter-binding-like protein 7

sRNAs: small RNA

SURE: sulfur-responsive element

Ta: annealing temperature

TA: *Thlaspi arvense*

TAE: Tris-acetate-EDTA

TAIR: The Arabidopsis Information Resource

TE: Trace element

TF: transcription factor

Tm: melting temperature

TMD: trans-membrane domain

Trp: tryptophan

UTR: untraslated region

WT: wild type

X-Gal: 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

X-Gluc: 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid

YEB: Yeast Extract Broth

YSL: Yellow Stripe-like protein

ZIP: ZRT,IRT-like protein

GENERAL OVERVIEW ON METAL HYPERTOLERANCE AND HYPERACCUMULATION IN PLANTS

1. Plant nutrition and metal toxicity

For their growth and development, plants, like all other organisms, require many essential elements in variable amounts (Epstein, 1972), which are mainly present in the soil solution. Metals in particular are the most important molecules, since they are directly involved in plant metabolism. Although the concentration of nutrients in a specific environment can be considerably high, their availability for plant nutrition can vary extensively, since it depends on several soil properties, including pH, redox potential, and aeration (Epstein, 1972). Nevertheless, plants possess various mechanisms to improve the bioavailability of essential elements: the most important are i) the production of metal chelating agents Ma, 2000; Nigam *et al.,* 2001; Abadía *et al.,* 2002) ii) induction of changes in the soil composition and modification of their root architecture (Robinson *et al.,* 2006) and iii) storage functions.

In general, plant nutrients are grouped as follows (Epstein, 1972):

***-Macronutrients*,** which are required in large amounts by plants (0.1% of dry weight) and include N, K, S, Ca, Mg and P;

***-Trace Elements* (TEs).** This group comprises micronutrients, which are necessary in small amounts (less than 0.01% of plant dry weight), and non-essential elements, with no biological function (Tchounwou *et al.,* 2012).

Micronutrients such as Fe, Mn, Zn, Cu, and Ni are essential for plant growth, since they are the cofactors of several enzymes; non-essential elements such as As, Cd, Cr, Hg and Pb, on the other hand, if present above certain threshold levels, can become toxic. In the past, the term “Heavy Metals” was used extensively to define those toxic elements with a density ranging from 3.5 to 7 g cm^{-3} (Duffus, 2002), but more recently the term “Trace Elements (TEs)” is preferred, since it is considered more appropriate (Duffus, 2002; Appenroth, 2010). Some soils are naturally rich in these metals (Wuana and Okieimen, 2011); the renowned calaminar soils, rich in Zn, and serpentine soils, rich in Ni (New Caledonia and in the Mediterranean area; Jaffrè *et al.,* 2013; Anaker, 2014) are just two examples. Environmental pollution brought about by anthropogenic activities has a greater impact, since it has increased enormously the concentration of these elements, especially over the last century (Tchounwou *et al.,* 2012) also reducing crop yield.

2. Metal hyperaccumulators and hypertolerant plants

In 1947, the plant species *Alyssum bertolonii* was reported to accumulate up to 1 mg Ni per g dry weight (Minguzzi and Vergnano, 1948): this was the first description of a plant tolerating and accumulating high levels of metals. The term *hyperaccumulator,* describing a plant with a shoot metal concentration that is several orders of magnitude greater than that of adjacent plants (Jaffré *et al.,* 1976), was only adopted in 1976. Many plant species known as metallicolous or *metallophytes* can tolerate high levels of metals in the ground (Baker *et al.,* 2010); they survive on contaminated soils by limiting the entry and root-to-shoot translocation of trace metals and therefore avoid all toxicity problems. In order to define a plant as a hyperaccumulator, threshold levels of metal concentration have been determined, which take their function and biological effects in plants into account (Fasani *et al.,* 2012). Hyperaccumulators are a special class of hypertolerant plants that combine an extreme tolerance to TEs with high levels of metal accumulation in their leaf tissues. These plants are characterized by i) a high degree of metal uptake by roots, ii) fast and efficient root-to-shoot translocation, and iii) the ability to sequester and detoxify TEs in specific cellular compartments (Cosio *et al.,* 2004; Marquès *et al.,* 2004). Only 0.2% of all angiosperms can be classified as hyperaccumulator/hypertolerant plants (Baker, 2002; Krämer, 2010). The majority of these hyperaccumulate Ni: there are almost 400 different plant species, mostly belonging to the Brassicaceae and Euphorbiaceae families (Krämer, 2010).

3. Models for hyperaccumulation studies: *Arabidopsis halleri* and *Noccaea caerulescens*

The Brassicaceae family contains 3200 species found almost all over the world. Many members of this family are classified as hyperaccumulators of trace elements (As, Cd, Co, Cu, Mn, Ni, Se, Tl, Zn). Two plant species, *Arabidopsis halleri* and *Noccaea caerulescens* are the most important models for hyperaccumulation/hypertolerance studies (Krämer, 2000; Cosio *et al.,* 2004; Papoyan and Kochian, 2004; Verbruggen, 2015) thanks to their high levels of sequence identity, genomic synteny, and molecular tools shared with the model species *Arabidopsis thaliana.*

3.1 *Arabidopsis halleri*

A. halleri is a pseudo-metallophyte, self-incompatible out-crossing plant widespread in both polluted and non-polluted areas. This species is mainly found in Europe, although a subspecies, *A. halleri* spp. Gemmifera, has been identified in Japan (Al-Shehbaz and O'Kane, 2002). Depending on the accession, Zn levels in shoots of *A. halleri* ranges from 3000 to 22,000 mg kg^{-1} DW (Bert *et al.,* 2000); moreover, some populations of this plant species can accumulate high concentration of Cd, i.e. over 100 mg Cd

kg^{-1} leaf DW (Dahmani-Muller *et al.*, 2001; Talke *et al.,* 2006). Some metallicolous populations of *A. halleri*, such as those native to Auby (France) and certain parts of Northern Italy, have been reported to accumulate huge amounts of Cd in their shoots (Bert *et al.,* 2003), particularly in the trichomes and the mesophyll cells of both young and mature leaves.

The ability to tolerate and hyperaccumulate high levels of heavy metals is shared by populations growing both on metalliferous and non-metalliferous soils (Pauwles *et al.,* 2006).

Zn hyperaccumulation in *A. halleri* is constitutive, but there is heritable variation in the degree between its populations. Various investigations have revealed that there is a constitutive expression of genes encoding metal transporters and enzymes for the biosynthesis of metal ligands (Becher *et al.,* 2004; Hammond *et al.,* 2006; van de Mortel *et al.,* 2006): indeed, this specific transcriptional regulation may be essential for the hyperaccumulator trait of *A. halleri*.

3.2 *Noccaea caerulescens*

N. caerulescens is a diploid (2n) biennial or perennial plant native of Europe, characterized by its ability to hypertolerate and hyperaccumulate Zn, Ni and Cd, either alone or in combination (Assunção *et al.,* 2001). Compared to the better characterized hyperaccumulator plant species *A. halleri*, which shares an extremely high sequence identity with the model plant *A. thaliana* (Mitchell-Odds, 2001), *N. caerulescens* shares an estimated 88% homology of its coding regions with it (Rigola *et al.,* 2006).

Although several accessions of *Noccaea* genus are known and characterized, only few members of this family are considered interesting for biological studies on metal hyperaccumulation.

A high variability in Zn, Cd and Ni accumulation and tolerance has been observed in several ecotypes of *N. caerulescens* (Lombi *et al.,* 1995; Schat *et al.,* 2000; Assunção *et al.,* 2003), which appear to be linked to the soil from which they were collected. In particular, this great variability between accessions presumably depends on differences in their metal homeostasis regulation, although many features of hypertolerance/hyperaccumulation in different *Noccaea* species are still unclear.

Analysis of the role of *MTP1*, *NRAMP4* and *ZNT1* metal transporters in Ni hypertolerance and hyperaccumulation in *Noccaea caerulescens*

1. INTRODUCTION

1. Metal hypertolerance and hyperaccumulation in *N. caerulescens*

The hyperaccumulator/hypertolerant trait in *Noccaea caerulescens* depends on several biological and physiological properties, which are only partially known. Like other hyperaccumulator plants, *N. caerulescens* displays enhanced metal uptake and a more efficient root-to-shoot metal translocation rather than non-accumulator species (Lasat *et al.,* 1996; Schat *et al.,* 2000). Interestingly, when compared to *A. thaliana*, both *N. caerulescens* and *A. halleri* (Zn hyperaccumulators) seem to share a common set of alterations in their metal homeostasis networks, including metal uptake by roots and increased rate of root-to-shoot transport, suggesting that hypertolerance/hyperaccumulation depends on specific properties of the plants (Hanikenne and Nouet, 2011). Zn hyperaccumulation in *N. caerulescens* has been extensively studied, since it is a conserved feature among *Noccaea* accessions (Assunção *et al.,* 2001; Dubois *et al.,* 2003; Richau and Schat, 2009). Different works have revealed that *N. caerulescens* has a greater ability to take up Zn from the soil in comparison to the non-accumulator *Thlaspi arvense*, presumably due to a variety of factors, including enhanced capacity to mobilise metals and a different regulation of metal transporters (Lasat *et al.,* 1996; Knight *et al.,* 1997). The roots of *N. caerulescens* were observed to release higher levels of exudates, and thus increase Zn mobilization by rhizosphere acidification (Knight *et al.,* 1997; McGrath *et al.,* 1997; Luo *et al.,* 2000), as well as Ni transport into the plant (Freeman *et al.,* 2004). Organic acids, amino acids, metallothioneins and other compounds are known to be constitutively produced by *N. caerulescens* roots (Krämer *et al.,* 1996; Salt *et al.,* 1999; Cobbett and Goldsbrough, 2002); in the presence of these molecules, the loading of many metals, including Zn, Cd and Ni, into the xylem, is boosted, as well as their root-to-shoot translocation. In addition, higher constitutive levels of different metal transporters, with a major role in response to Zn uptake and homeostasis regulation, were also found in several accessions of *N. caerulescens* (Grotz *et al.,* 1998; Pence *et al.,* 2000; Assunção *et al.,* 2001), suggesting they play a key role in hyperaccumulation-hypertolerance.

2. Molecular mechanisms of Ni tolerance and accumulation in *N. caerulescens*

N. caerulescens ecotype "Monte Prinzera" (MP, Italy) is an interesting model for Ni hyperaccumulation studies, although poor information is currently available on Ni tolerance and accumulation. Native to a serpentine soil found in the Natural Park of the Tuscan-Emilian Apennines (Italy), this ecotype is able to tolerate and accumulate not only high concentrations of Zn – a constitutive trait shared by all members of this plant species – but also Ni (Assunção *et al.,* 2001; Richau and Schat, 2009; Seregin *et al.,* 2014), although in a different amount considering the populations that grow in the same field.

Compared to the calaminar population of *N. caerulescens* LC, known to hyperaccumulate Zn, the MP ecotype has been observed to accumulate higher concentration of Ni (Richau and Schat*,* 2009); moreover, Zn accumulation is also greater in MP than LC, although both ecotypes accumulate more Zn than Ni (Richau and Schat*,* 2009). As in *Alyssum*, Ni hyperaccumulation in *N. caerulescens* seems to mainly depend on the activity of low-affinity transporters, probably encoded by genes involved in the regulation of Zn homeostasis (Krämer *et al.,* 2000). High levels of several trace elements, including Zn, Fe, Mn and Co, are able to inhibit Ni uptake and accumulation in both non-accumulating and hyperaccumulator plants (Assunção *et al.*, 2001; Taylor and Macnair, 2006). A positive correlation between foliar Zn and Ni content was observed in *N. caerulescens* MP and LC, although Ni tolerance and accumulation appear to be two independent phenomena (Richau and Schat*,* 2009). Interestingly, Zn and Ni seem to strongly compete with each other during the xylem-loading processes in *N. caerulescens,* presumably for some metal transporters with a role in root-shoot translocation (Assunção *et al.*, 2001; Deng *et al.*, 2019). In addition to low-affinity Zn transporters, other genes involved in Fe and Mn homeostasis regulation *in planta* have been observed to take part in Ni transport in non-accumulating and Ni-hyperaccumulator plants (Nishida *et al.,* 2012; Deng *et al.,* 2019). It is notable that Co, normally available at low concentrations in plant cells, strongly competes with Ni uptake in *N. caerulescens*, suggesting that Ni and Co homeostasis involve low-affinity transporters (Deng *et al.,* 2019).

N. goesingense and *N. japonica* are other two species belonging to the *Noccaea* genus of the Brassicaceae family that are classified as Ni hyperaccumulators (Krämer *et al.,* 1997; Mizuno *et al.,* 2003; Freeman and Salt, 2007). In *N. goesingense* in particular, high levels of Ni seem to be stored in the vacuolar compartment of leaf cells, thanks to an efficient system of Ni transport in the plant mediated by metal chelating agents such as Nicotianamine and Histidine (Krämer *et al.,* 2000; Küpper *et al.,* 2001; Freeman *et al.,* 2005), which seem to increase root-to-shoot transport, thus reducing Ni accumulation in the roots.

3. Role of metal transporters in hyperaccumulator plants

Physiological studies on hyperaccumulators have revealed high metal concentrations in their xylem sap brought about by an enhanced xylem loading (Lasat *et al.,* 1996; Lombi *et al.,* 2001; Verbruggen *et al.*, 2009) involving several types of metal transporters. These proteins, which belong to a wide range of gene families, are necessary for the acquisition and distribution of micronutrients, and more precisely i) uptake of metals by the roots, ii) xylem loading and translocation into the shoot, iii) cell uptake and distribution and iv) storage and detoxification in the vacuolar compartment (Verbruggen *et al.,* 2009). The role of many families of metal transporters in hyperaccumulator plants have been studied (Krämer *et al.*, 2007; **Fig. 1**). Their biological function appears to be highly conserved among all plant species, although many differences exist regarding their regulation (Verbruggen *et al.,* 2009).

Ecological and physiological studies have revealed that hyperaccumulator species regulate metal homeostasis and cell distribution differently from non-accumulator plants, thus confirming the existence of a complex network that coordinates metal uptake and its efficient detoxification (see Krämer, 2007). The most important classes of metal transporters are listed below:

- ***Oligopeptide transporter superfamily (OPT).*** A superfamily of oligopeptide transporters involved in the loading and unloading of nicotianamine-metal chelates from the vascular tissues (DiDonato *et al.,* 2004). These proteins play an important role in the uptake of Fe (III) chelated by phytosiderophore complexes but may also be able to transport other metals. The family includes the yellow-stripe 1-like (YSL) subfamily, involved in the hyperaccumulation of Zn, Cd and Ni in *N. caerulescens* (Schaaf *et al.,* 2005; Gendre *et al.,* 2006);

- ***ZIP family (ZRT, IRT-like Proteins).*** Although only few members have been well characterized, these proteins are mainly involved in metal uptake by roots. Pioneering investigations revealed their key role in Fe (Vert *et al.,* 2002; Lin *et al.,* 2009) and Zn (Grotz *et al.,* 1998; Lin *et al.,* 2009; Milner *et al.,* 2013) homeostasis regulation by root uptake, even though more recent studies suggest a broader metal-binding specificity (Mizuno *et al.,* 2005; Nishida *et al.,* 2011);

- ***P-type ATPase-HMA***. Also known as heavy metal transporting ATPases (HMAs), these proteins play an important role in transporting transition metal ions against their electrochemical gradient by means of ATP hydrolysis (Baxter *et al.,* 2003). HMA4, found in both hyperaccumulator and non-hyperaccumulating plants, is one of the best characterized transporters of this family. In *A. thaliana,* the HMA4 protein is localized at the plasma membrane and is essential for Zn homeostasis, Cd detoxification and root-to-shoot translocation (Mills *et al.,* 2003; Wong and Cobbett, 2009). The constitutive levels of *HMA4* are higher in both *A. halleri* (Talke *et al.,* 2006; Hanikenne *et al.,* 2013) and *N. caerulescens* (Hammond *et al.,* 2006; van de Mortel *et al.,* 2006) than non-accumulating plants, suggesting its involvement in the hyperaccumulation/hypertolerance of heavy metals;

- ***Cation Diffusion Facilitator (CDF).*** These transporters are ubiquitously conserved among prokaryotes and eukaryotes, where they are mainly involved in metal detoxification by vacuolar compartmentalization (Gustin *et al.,* 2011). Results of several research works indicate their role in Zn/Cd hyperaccumulation/hypertolerance, but evidence suggests they may have a broader metal-binding specificity (Ricachenevsky *et al.,* 2013);

- ***ATP-binding cassette (ABC).*** Plant genome encodes hundreds of ABC transporters, which form one of the largest protein families found in all living organisms (Kang *et al.,* 2011). These proteins were first found to be involved in the final step of the detoxification process, i.e. the storage of metals in the vacuole (Martinoia *et al.,* 1993); however, although several works have also revealed their important role in organ growth, plant nutrition, plant development, response to abiotic stress, and interaction with the environment, to date, only few members of the ABC transporter family have been fully characterized;

- ***NRAMP (Natural Resistance Associated Macrophage Protein) family***. Involved in maintaining metal homeostasis in plants, this large family of metal transporters is composed of several members with a broad metal binding specificity (Thomine and Schroeder, 2004); NRAMP proteins in particular seem to be mainly involved in Fe homeostasis, with a role in Fe uptake (*NRAMP1*: Curie *et al.,* 2000) and remobilization from the vacuole (*NRAMP3* and *NRAMP4*: Lanquar *et al.,* 2005);

- ***CaCA (Ca^{2+}/Cation Antiporter).*** This superfamily of metal transporters is composed of several members playing an active role in the vacuolar sequestration of heavy metals, including the MHX (Mg^{2+}/H^+ exchanger) and CAX (Cation exchanger) transporters, involved in Mg and Zn translocation (Shaul *et al.,* 1999). *A. halleri* was found to possess higher constitutive levels of MHX proteins than *A. thaliana* (Elbaz *et al.,* 2006). CAX transporters play a role in the vacuolar sequestration of several metals, including Ca, Mn, Cd and Zn, suggesting their possible involvement in metal tolerance (Schaaf *et al.,* 2002; Mei *et al.,* 2009).

Although the molecular mechanisms responsible for Ni tolerance and accumulation in plants are mostly unknown, several metal transporters in *N. caerulescens* MP have been observed to be modulated in the presence of high concentration of Ni (Halimaa *et al.,* 2014; Visioli *et al.,* 2014). The most interesting families are described in greater detail in the following pages.

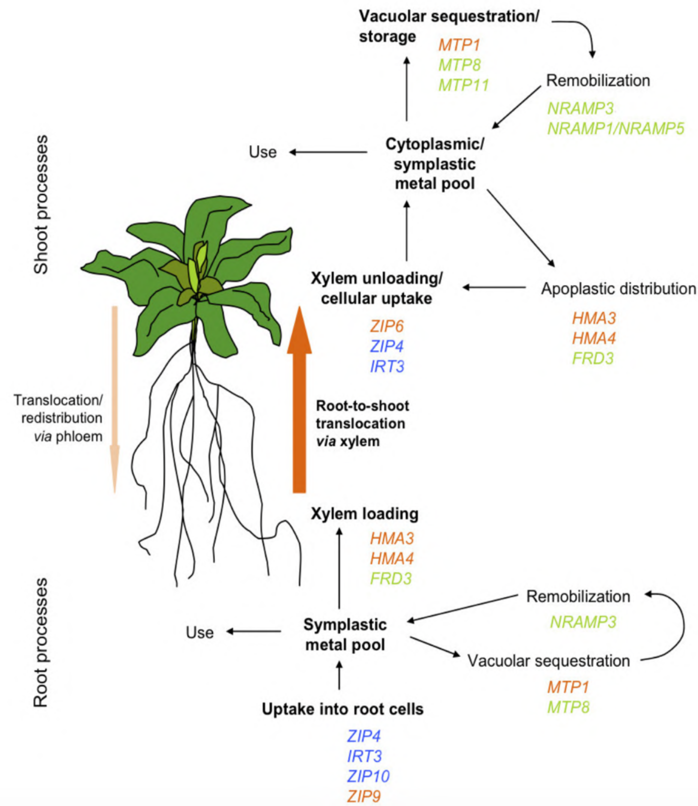

Figure 1: Diagram of the most important metal transporters in the hyperaccumulating plant species *A. halleri* and *N. caerulescens* (Krämer *et al.*, 2007).

4. The ZIP family of metal transporters

The ZIP (ZRT-IRT-like Protein) family of transporters was initially named based on the two founding members: the high affinity yeast plasma membrane Zn uptake transporter, ZRT1, and the high affinity *A. thaliana* plasma membrane Fe uptake transporter, IRT1 (Zhao *et al.,* 1996). These proteins are highly conserved across all living kingdoms, including plants, fungi, animals and protists (Guerinot, 2000), where they play a role in metal homeostasis regulation.

Many works on ZIP transporter proteins in plants and animals have revealed their ability to transport a variety of divalent cations, including Zn^{2+}, Fe^{2+}, Mn^{2+} and Cd^{2+}, suggesting their fundamental role in regulating metal homeostasis (Guerinot, 2000; Milner *et al.*, 2013).

The modulation of ZIP genes, required for a correct balance between nutrients, depends on the plant's metal status and their concentration in the surrounding environment (Grotz *et al.,* 1998; Milner *et al.,* 2013).

ZIP proteins range from 309 to 476 aa in length, with eight putative membrane-spanning domains; the His-rich "variable region" between domains III and IV contains the specific, metal-binding domain involved in its uptake, and therefore differs among the ZIP family members (Guerinot, 2000).

The genome of *Arabidopsis* contains 15 members of the ZIP family, with different roles in metal homeostasis (Milner *et al.,* 2013). Most of these transporters are located at the plasma membrane, where they play a key role in micronutrient uptake (Grotz *et al.,* 1998), but there are several other ZIP members with a different subcellular localization (Milner *et al.,* 2013).

Gene expression analyses have also revealed a positive modulation of *ZIP1*, *ZIP5*, *ZIP9* and *IRT3* expression in the roots and shoots of Zn-starved *A. thaliana* (Wintz *et al.,* 2003), suggesting their involvement in the homeostasis regulation of this element. ZIP transporter proteins have also been identified in several other plant species, including soybean, *M. truncatula, N. caerulescens* and *N. japonica* (Assunção *et al.,* 2001; López-Millán *et al.,* 2003; Plaza *et al.,* 2007), but only few members of these families have been fully characterized. Many *A. thaliana* ZIP transporters were able to complement yeast mutants defective for the uptake of Fe, Zn, Mn and Cu (Milner *et al.,* 2013; **Fig. 2**) suggesting that they may be responsible for their uptake in plants.

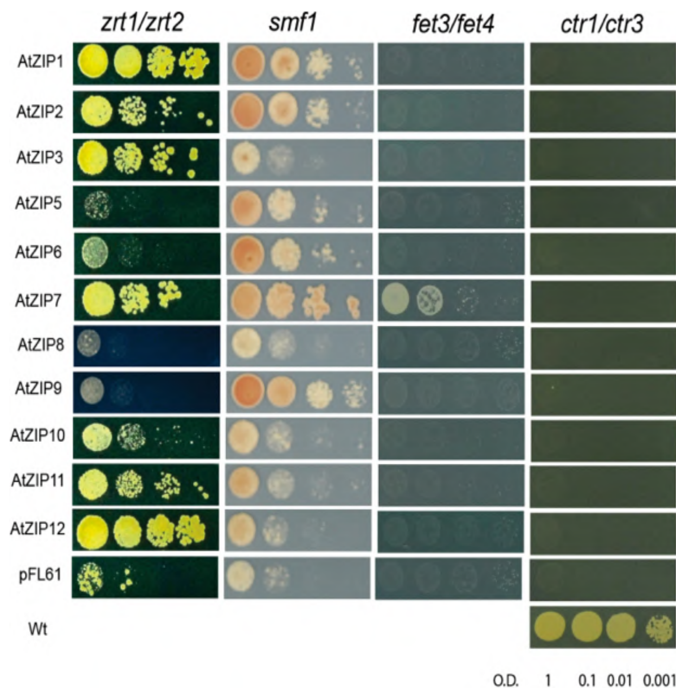

Figure 2: Complementation of yeast metal uptake mutants with *AtZIP* genes.
Legend: (*zrt1/zrt2*Δ) yeast mutants defective for the uptake of Zn; (*smf1*Δ) yeast mutants defective for the uptake of Mn; (*fet3/fet4*Δ) yeast mutants defective for the uptake of Fe; (*ctr1/ctr3*Δ) yeast mutants defective for the uptake of Cu (Milner *et al.,* 2013).

4.1 Regulation of ZIP genes in hyperaccumulator plants

The potential involvement of *ZIP* transporters in the metal tolerance and accumulation in hyperaccumulator plants have been studied in different scientific works (Becher *et al.,* 2004; Weber *et al.,* 2004; van de Mortel *et al.,* 2006; Talke *et al.,* 2006). As compared to *A. thaliana,* the hyperaccumulator species *A. halleri* and *N. caerulescens* constitutively express higher levels of several *ZIP* genes, independently of their Zn status (Grotz *et al.*, 1998). In particular, *ZIP4* and *IRT3* are positively modulated by conditions of Zn deficiency in the roots of both *A. thaliana* and *A. halleri,* but to a different extent (Talke *et al.,* 2006). Similar modulation and expression levels were confirmed for *ZIP5* and *ZIP6* by global transcriptomic analyses performed on *A. halleri* and *A. thaliana* (Becher *et al.,* 2004; Weber *et al.*, 2006), with higher constitutive levels of *ZIP6* detected in the shoots of *A. halleri*.

As well as in *A. halleri,* several studies on *ZIP* genes have been performed on *N. caerulescens* to understand their possible involvement in the hyperaccumulation of metals such as Zn, Cd and Ni. The roots of *N. caerulescens* display higher constitutive levels of the *NcZNT1* and *NcZNT*2 Zn transporters (orthologues of the *A. thaliana ZIP4* and *IRT3* genes, respectively) than those of non-accumulating plants (Pence *et al.*, 2000; Assunção *et al.*, 2001); moreover, since many investigations suggest that both proteins might have a broader metal binding specificity (Mizuno *et al.,* 2005; Lin *et al.,* 2016), they can be considered an interesting target for Ni hyperaccumulation analyses. A similar modulation pattern was also observed for *NcZNT5* and *NcZNT6,* which unlike *A. thaliana,* appeared to be constitutively expressed in several *N. caerulescens* accessions (Plaza *et al.*, 2007; Wu *et al.,* 2013). Interestingly, gene copy analyses on the Ni hyperaccumulator plant *N. japonica* also revealed the possible presence of multiple copies of *ZNT5,* suggesting the importance of *ZIP* genes in metal hyperaccumulation/hypertolerance (Wu *et al.,* 2013).

4.2 The biological role of *ZNT1* in *N. caerulescens*

NcZNT1 in *N. caerulescens* is the orthologous of *ZIP4* in *A. thaliana;* this gene is a member of the ZIP family of metal transporter genes and plays an important role in the regulation of Zn homeostasis in plant cells (Assunção *et al.*, 2010; Castro *et al.,* 2017).

AtZIP4 in *A. thaliana* shares a 90% cDNA and 87% amino acid identity, respectively, with the *ZNT1* transporter in the Zn hyperaccumulator *N. caerulescens* (Lin *et al.,* 2016). *ZNT1* was isolated from this latter species (Pence *et al.,* 2000) with the complementation strategy previously used to isolate and characterize Zn transporters in *Arabidopsis* (Grotz *et al.,* 1998). *ZNT1* is mainly expressed in the root and shoot vasculature of *N. caerulescens*, suggesting a role in long-distance transport of Zn (Milner *et al.,* 2013). Unlike *AtZIP4,* the *ZNT1* promoter is active when either Zn is lacking or is present at adequate levels (Lin *et al.,* 2016).

Interestingly, *N. caerulescens* and *A. halleri* are also able to maintain a greater Zn influx into the roots under Zn-sufficient conditions, with high constitutive levels of *ZNT1* expression, suggesting the existence of a different conserved mechanism regulating *ZNT1/ZIP4* expression in hyperaccumulator plants (Milner and Kochian, 2008).

4.3 ZNT1 may have a role in Ni tolerance and accumulation

Although *NcZNT1* seems to be expressed at higher levels in the Zn/Cd-adapted *N. caerulescens* accessions GA and LC than in the Ni-adapted serpentine accession MP (Lin *et al.,* 2016), expression analyses on *ZNT1* in the Ni hyperaccumulator plant *N. caerulescens* MP have revealed its positive modulation in shoots in the presence of excess Ni (Visioli *et al.,* 2014). In addition, heterologous expression tests on the same gene in *N. japonica* have also shown improved cell growth in a medium supplemented with excess Ni (Mizuno *et al.,* 2005), suggesting its possible involvement in Ni transport and accumulation *in planta.*

5. The NRAMP family of metal transporters

NRAMP genes are a ubiquitous family of metal transporter proteins involved in metal homeostasis (Sebastien and Schroeder, 2004). Like ZIP, NRAMP proteins seem to display scarce selectivity towards divalent cations, suggesting a broad range of activities in cells (Thomine and Schroeder, 2004; Victoria *et al*., 2012). The SMF1 protein in yeast, for instance, has been found to transport several metal cations, including Mn, Fe and Zn (Liu *et al.,* 1997; Illing *et al.,* 2012), although two *NRAMP* genes in rice, *OsNRAMP3* and *OsNRAT1*, are selective for Mn and Al (Xia *et al.,* 2010; Yamaji *et al.,* 2013). Generally, most NRAMP proteins transport multiple divalent ions, including Mn, Zn, Cu, Fe, Cd, Ni and Co (Thomine and Schroeder, 2004; Nevo and Nelson, 2006).

The AtNRAMP family in *A. thaliana* is composed of six members, as well as *EIN2* (Alonso *et al.,* 1999; Maser *et al.,* 2001), which shares a 40-50% amino acid composition similarity with mouse NRAMP1.

The first studies carried out on plants indicated they were involved in preserving Fe homeostasis (Curie *et al.,* 2000; Thomine *et al.,* 2003; Lanquar *et al.,* 2005), but NRAMPs are able to transport other metals too.

5.1 Functional role of NRAMP4 in *A. thaliana* and *N. caerulescens*

The broad metal binding specificity of *NRAMP* genes in plants and their regulation under different conditions of nutrient availability have suggested they play a role in determining the presence of the hyperaccumulation/hypertolerance trait.

Most investigations on Fe and Zn transport in *N caerulescens* and in *A. thaliana* have been focused on two members of the *NRAMP* gene family, i.e. *NRAMP4* and its homologous *NRAMP3* (Oomen *et al*., 2009). At amino acid level, *NcNRAMP3* and *NcNRAMP4* in *N. caerulescens* have a 96.5% and 95.1% similarity with their *A. thaliana* homologues, *AtNRAMP3* and *AtNRAMP4* (Oomen *et al.,* 2009). Although *NcNRAMP4* is functionally similar to *AtNRAMP4* with respect to metal transport, higher expression levels of *NRAMP3* and *NRAMP4* are present in several *N. caerulescens* accessions, including the Ni hyperaccumulator ecotype MP (Oomen *et al*., 2009; Visioli *et al*., 2014). AtNRAMP4 and NcNRAMP4 are both localized in the vacuolar compartment of the plant cell, where they play a key role in Fe homeostasis (Thomine, 2003; Carter 2004). Like *NRAMP3*, the expression of *AtNRAMP4* is up-regulated under Fe-deficient conditions, as demonstrated in *nramp3nramp4* double knockout mutants (Lanquar *et al.,* 2005). Other experiments on mutant yeasts have shown that *NcNRAMP4* is involved in the transport of Cd and Zn, like its *A. thaliana* homologous gene (Oomen *et al.,* 2009), hence suggesting its role in preserving metal balance.
Many other transporters have also been identified, the regulation of which differs between Zn/Cd hyperaccumulators and non-accumulating plants, suggesting that both *NRAMP3* and *NRAMP4* belong to a common set of genes involved in metal homeostasis (Oomen *et al*., 2009).

5.2 NRAMP4 may have a role in Ni tolerance and accumulation

Given that Zn is quite similar to Ni, many transporter proteins involved in Zn homeostasis may also have a role in Ni uptake and transport, although the mechanisms regulating these processes are still unclear (Mizuno *et al*., 2005; Halimaa *et al.,* 2014; Visioli *et al*., 2014).
The low metal specificity of *N. caerulescens NRAMP4* and, more in general, of the NRAMP family members suggests a putative role for these vacuolar transporters in Ni transport, especially in Ni hyperaccumulator plants. Several transporters with a broad metal-binding specificity, including members of ZIP family *ZNT1* and *ZNT2,* as well as *NRAMP* genes, were found to be modulated by Ni treatment in the Ni hyperaccumulator/hypertolerant *N. caerulescens* MP (Visioli *et al*., 2014). Moreover, works on the Ni hyperaccumulator plant *N. japonica* have confirmed the possible involvement of NRAMP4 in Ni homeostasis regulation (Mizuno *et al*., 2005; **Fig. 3**).
In particular, *NjNRAMP4* resulted to be incapable of transporting metals other than Ni, suggesting that its coding sequence may be important in determining the amino acid residues required for Ni transport specificity (Mizuno *et al.,* 2005).

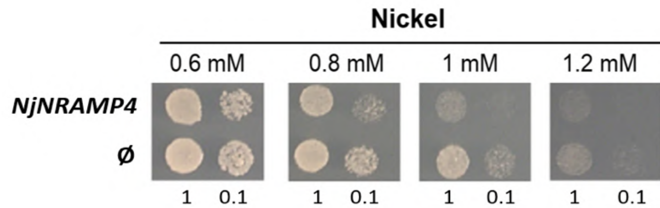

Figure 3: Recombinant yeasts grown (30 °C for 36 h) at different Ni concentrations. *NjNRAMP4* impaired yeast growth, as can be seen comparing it with the empty vector (Ø) (adapted from Mizuno *et al.*, 2005).

6. Cation Diffusion Facilitator (CDF) family in plants

The CDF gene family of metal transporters has been extensively studied in plants and other organisms (Paulsen and Sajer, 1997). The bacterial metal sensor CzcD (Nies, 1992) and the two yeast transporters ZRC1 (Kamizono *et al.,* 1989) and COT1 (Conklin *et al.,* 1992) have been linked to Zn and Co transport respectively and were the first CDFs proteins to be characterized. In general, CDF transporters are localized at different membranes and are principally involved in metal transport and detoxification (Haney *et al.,* 2005; Peiter *et al.*, 2007).

CDF proteins act as Metal^{2+} (Me^{2+})/H^+ antiporters and are generally characterized by six transmembrane domains (TMD), although several exceptions exist (Guffanti *et al.,* 2002; Chao and Fu, 2004; Grass *et al.,* 2005). Investigations on plants have revealed the involvement of CDF proteins in the transport of a variety of metals, including Fe^{+2}, Cu^{+2}, Cd^{+2}, Mn^{+2}, Co^{+2} and probably Ni^{+2} (Gustin *et al.,* 2009; Ricachenevsky *et al.,* 2013). CDFs transporters in plants were renamed MTPs (Metal Tolerance Proteins), given their key role in vacuolar Zn compartmentalization and detoxification in both hyperaccumulators and non-hyperaccumulating species (Montanini *et al.,* 2007). Plants MTP proteins can be phylogenetically classified into seven groups (Gustin *et al.,* 2011), belonging to the three main clusters also found in other organisms (Montanini *et al.,* 2007). Members of CDF group 1 have been characterized, including the Zn vacuolar transporters *AtMTP1* and *AtMTP3* (Kobae *et al.,* 2004; Desbrosses-Fonrouge *et al.,* 2005), which share a 68% sequence identity.

6.1 MTP1 is involved in metal detoxification

Several studies on many species, including *A. thaliana* (Kawachi *et al.,* 2008; Tanaka *et al.,* 2013), *Nicotiana* spp. (Shingu *et al.,* 2005), poplar (Blaudez *et al.,* 2003), *M. truncatula* (Chen *et al.,* 2009), rice (Yuan *et al.,* 2012; Menguer *et al.,* 2013) and barley (Podar *et al.,* 2012) have focused on the involvement of MTP1 in metal transport. Subcellular localization experiments with AtMTP1 have shown that it is found at tonoplast level (Desbrosses Fonrouge *et al.,* 2005), confirming its role as a vacuolar transporter; in addition, heterologous expression in yeast and overexpression *in planta* revealed that AtMTP1 is only

involved in the transport of Zn (Arrivault *et al.,* 2006; Ricachenevsky *et al.,* 2013). Similar experiments were carried out with MTP1 orthologues in other plant species, including barley (Podar *et al.,* 2012), rice (Yuan *et al.,* 2012; Menguer *et al.,* 2013) and poplar (Blaudez, 2003; Montanini *et al.,* 2007). The histidine-rich cytoplasmic loop between TMD IV and V (**Fig. 4**) seems to be essential for transporter specificity, and acts as a sensor for Zn (Shingu *et al.,* 2005; Kawachi *et al.,* 2008; Podar *et al.,* 2012; Tanaka *et al.,* 2015). In fact, in the Ni/Zn hyperaccumulator plant *N. goesingense*, CDF members NgMTP1t1 and NgMTP1t2, derived from a single-copy genomic sequence, seem to be involved in Ni detoxification through vacuole accumulation (Persans *et al.,* 2001), confirming the importance of the His-loop region in metal transport.

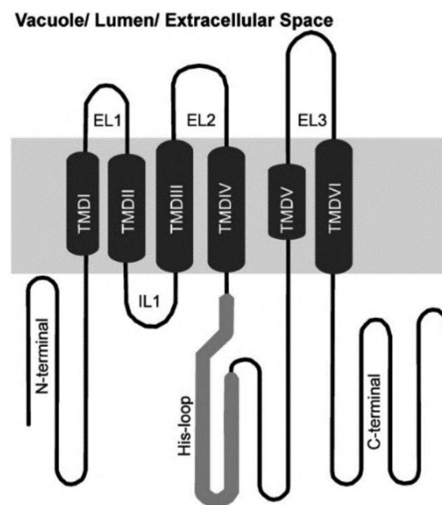

Figure 4: Structure of MTP1 proteins (Ricachenevsky *et al.,* 2013). The transmembrane domains (TMDs) are predicted; the histidine loop (His-loop) involved in metal binding is more extended than in ZIP transporters. IL, intracytosol loop; EL, extracytosol loop.

6.2 MTP1 is fundamental for metal hypertolerance and hyperaccumulation in plants

The conserved role of MTP1 in Zn detoxification has been studied in both hyperaccumulator and non-accumulating plant species, and it was found that this gene is required for Zn hypertolerance in *A. halleri* (Dräger *et al.*, 2004; Gustin *et al.,* 2009).

Unlike *A. thaliana*, MTP1 is constitutively expressed in this species (Becher *et al.,* 2004; Dräger *et al.,* 2004; Talke *et al.,* 2006); moreover, *A. halleri* genome has 4–5 MTP1 paralogs, with *AhMTP1-A1*/*AhMTP1-A2* and *AhMTP1-B* loci that are linked to Zn hypertolerance. MTP1 transcripts are particularly abundant in shoots (Dräger *et al.,* 2004; Shahzad *et al.,* 2010), suggesting that duplication of MTP1 loci could be fundamental for Zn tolerance in these plants.

MTP1 in *N. caerulescens* is constitutively overexpressed in leaves in comparison with *T. arvense* and *A. thaliana* (Assunção *et al.,* 2001) and similar results were obtained with *N. goesingense*; in particular, NgMTP1

was able to complement Zn, Cd, and Co-sensitivity in yeast (Persans *et al.,* 2001; Kim *et al.,* 2004). *MTP1* in *N. caerulescens* is constitutively overexpressed in leaves in comparison with *T. arvense* and *A. thaliana* (Assunção *et al.,* 2001) and similar results were obtained with *N. goesingense*; in particular, NgMTP1 was able to complement Zn, Cd, and Co-sensitivity in yeast (Persans *et al.,* 2001; Kim *et al.,* 2004).

6.3 Physiological role of the conserved His-loop region in MTP1

The highly conserved His-loop domain present in the MTP1 protein is composed of 21 His residues in *A. thaliana*; however, *Escherichia coli* YiiP, a member of the CDF family (Lu and Fu, 2007, Lu *et al.,* 2009), does not possess this region. Interestingly, the Zn/Ni hyperaccumulator *N. goesingense* displays two spliced versions of *MTP1* (Persans *et al.,* 2001), which differ for the presence or absence of this His-loop. Experiments with yeast mutants were carried out to study the affinity for the metal substrate of these two forms (Persans *et al.,* 2001). The shorter version of MTP1 displays different binding specificity for various metals, including Ni (Persans *et al.,* 2001). In order to better elucidate the physiological role of the His-loop region, Kawachi and colleagues (2008) produced a mutated version of AtMTP1 and performed several experiments. The removal of the entire His-loop from AtMTP1 led to the production of a non-functional protein (Kawachi *et al.,* 2008) confirming that this region is probably required for the Zn detoxification activity of MTP1 (Kawachi *et al.,* 2008). Another version of the AtMTP1 mutated protein, called His-half AtMTP1, lacked the first 32 residues of the His-loop (185-216). When expressed in yeast, this His-half MTP1 protein displayed the same Zn transport activity found for MTP1, but also a broader metal binding specificity for other divalent ions, including Co (Kawachi *et al.,* 2008). The Zn transport activity of both AtMTP1 and His-half-AtMTP1 was assessed through yeast complementation assays and ectopic expression *in planta* (Kawachi *et al.,* 2012; Tanaka *et al.,* 2015), but no particular differences were found in the transport of other metals.

These data suggest that the His-loop plays a role in sensing Zn in the plant cell, but a possible variation of its metal binding properties cannot be excluded.

2. AIM OF THE WORK

Metal homeostasis regulation in plants is essential for the growth and development. This process is characterized by different and complex mechanisms, which are only partially known. Micronutrients and non-essential elements can be actively or passively absorbed by roots and translocated all over the plants by several transporters. Although the majority of these elements can be naturally present in high concentration in soils and water, anthropogenic sources have increased the concentration of Trace Elements, with toxic effects on organisms (Epstein, 1972). Plants have evolved several mechanisms to reduced metal absorption from the soil or by increasing their detoxification; interestingly, hyperaccumulator and hypertolerant plants are characterized by the ability to accumulate and tolerate extremely high concentration of heavy metals in the areal part, without negative effects on plant growth and development. These plants have been identified for a long time (Minguzzi and Vergnano, 1948) but the determinants of hyperaccumulation/hypertolerant traits are actually only partially known. Several genes have been shown to participate in metal absorption by roots, translocation into the shoots via xylem and storage into specific plant tissues (Brooks, 1998; Verbruggen *et al.,* 2009; Krämer, 2010).

A. halleri and *N. caerulescens* represent two interesting species for molecular studies on hyperaccumulation/hypertolerance, thanks to their close relationship to the well-known model species *A. thaliana*. Most of *A. halleri* ecotypes are known to be Zn hyperaccumulator, although some of them can also hyperaccumulate the toxic element Cd (Reeves *et al.,* 2001). On the contrary, different ecotypes of *N. caerulescens*, as well as different accessions belonging to *Noccaea* genus, have shown substantial variation in Zn, Cd or/and Ni accumulation/tolerance capacity (Assunção *et al.*, 2001; Dubois *et al.,* 2003; Richau and Schat, 2009). The diverse expression of metal transporter proteins, which have an essential role in metal homeostasis regulation, seems to be the crucial factor in metal hyperaccumulation/hypertolerance variability between species of the same genus (Krämer *et al.,* 2007; Halimaa *et al.,* 2014).

This work focused on the Italian ecotype of *N. caerulescens* "Monte Prinzera" (MP, Italy), which is native from a serpentine Ni-enriched soil in Italy. This species is able to accumulate and tolerate high concentration of Ni in addition to Zn (Assunção *et al.,* 2001; Richau *et al.,* 2009), sharing these properties with two other species of the same genus: *N. goesingense* and *N. japonica* (Krämer *et al.,* 1997; Mizuno *et al.,* 2003). Low-affinity transporters for Fe, Zn and probably other metals could be directly or indirectly involved in Ni transport in accumulators and Ni-hyperaccumulator plant species (Mizuno *et al.,* 2005; Richau *et al.,* 2009; Nishida *et al.,* 2012). In this research work the response of *N. caerulescens* MP to different Ni concentrations was compared to *T. arvense* (control) and *N. caerulescens* ecotype Ganges (GA, Zn/Cd hyperaccumulator). Three genes encoding metal transporters, the vacuolar *NRAMP4* (member of NRAMP family of transporters) and *MTP1* (members of the CDF proteins family) proteins and the

plasma membrane *ZNT1* (belonging to ZIP family of metal transporters) protein were selected for further analysis, since previous studies have indicated their potential role in the Ni hyperaccumulation and hypertolerance (Persans *et al.,* 2001; Mizuno *et al.,* 2005; Milner *et al.,* 2013). The modulation of these genes under Ni treatment was initially analyzed by Real Time RT-PCR performed on plants of the Ni hyperaccumulator *N. caerulescens* MP, the Zn/Cd hyperaccumulator *N. caerulescens* GA and *T. arvense* (as control). Yeast complementation assays have also been performed in order to elucidate Ni transport properties of *NRAMP4* and *ZNT1 in planta*. To study the effect of the overexpression of *NcNRAMP4* and *NcZNT1*, the CDS of these two genes was amplified and fused downstream the strong promoter CaMV35S to generate *A. thaliana* transgenic lines. Double 35S::*NRAMP4*/35S::*ZNT1* transgenic lines were also obtained by crossing plants carrying single 35S::*NRAMP4* and 35S::*ZNT1* to study the impact of both genes on Ni tolerance and its accumulation. *In vitro* and *in vivo* analysis have been performed on transgenic lines, to study the role of both genes in Ni tolerance and accumulation in *N. caerulescens* MP.
Regarding MTP1, two different CDSs characterized by different length were found in both cDNA and genomic DNA of *N. caerulescens* MP and GA. Co-localization experiments with *N. tabacum* leaves have shown that both NcMTP1-long and NcMTP1-short are localized in the vacuolar compartment, suggesting their role in metal excess detoxification. In order to elucidate putative differences in metal transport properties, both CDSs were fused into a yeast-expressing vector and complementation assays with *zrc1cot1* double mutant and WT strain of *S. cerevisiae* have been performed considering Zn, Ni and Co as metals of interest. Moreover, both *NcMTP1-long* and *NcMTP1-short*, in addition to *A. thaliana* MTP1, were fused downstream the control of CaMV35S promoter and introduced by floral dip transformation into *A. thaliana* homozygous *mtp*1+/+ mutants, in order to investigate the metal binding specificity of both NcMTP1 proteins compared to the highly characterized AtMTP1.
The results obtained in this work suggest a possible role of all three genes in Ni accumulation-tolerance in *N. caerulescens* MP, although further analysis will be required to better understand the mechanisms responsible for the Ni hyperaccumulation trait in *N. caerulescens*.

3. MATERIALS AND METHODS

3.1 Plant material and growth conditions

Different species belonging to the Brassicaceae family were used in this PhD work:

- *Arabidopsis thaliana* ecotype Col-0 (non-accumulator);
- *Noccaea caerulescens* ecotype Monte Prinzera (MP, from Italy; Ni hyperaccumulator);
- *Noccaea caerulescens* ecotype Ganges (GA, from France; Zn/Cd hyperaccumulator);
- *Thlaspi arvense* (non-accumulator).

Seeds was sterilized for 1 min with 70% ethanol and for 11 min with 20% sodium hypochlorite and 0.03% Triton X-100, then rinsed three times with sterile water. For *in vitro* experiment, plants were cultured on standard 1X MS medium (Murashige and Skoog, 1962) under controlled conditions (16-h light/8h dark regime at 22 °C/18 °C; light intensity of 80 to 120 μmol m^{-2} s^{-1}). For *in vivo* experiment, plants were grown in hydroponics, using Hoagland's medium (1 mM KH_2PO_4, 2 mM $MgSO_4 \cdot 7 H_2O$, 5 mM $Ca(NO_3)_2 \cdot 4 H_2O$, 5 mM KNO_3, 46 μM H_3BO_3, 0.3 μM $CuSO_4$, 0.011 μM $NH_4MoO_4 \cdot 4 H_2O$, 1%, pH 5.7) or in the soil, and kept in greenhouse, under controlled conditions.

3.2 Genomic DNA extraction

Genomic DNA was extracted from plants by grinding 100 mg of fresh material in Extraction Buffer (200 mM Tris pH 7.5, 250 mM NaCl, 25 mM EDTA pH 8.0, 1% SDS) adding PCI at the sample (UltraPure™ Phenol:Chloroform:isoamyl Alchol, from Life Technologies, Löhne, Germany) and using precipitation in isopropanol (Sambrook and Russell, 2001).

3.3 RNA extraction and cDNA synthesis

Total RNA was isolated from tissues using TRIzol® reagent (Life Technologies, Carlsbad, CA, USA) following the manufacturer's instructions and treated with RQ1 RNase-Free DNase (Promega, Madison, WI, USA) to remove genomic DNA contaminations. To quantify gene expression, first-strand cDNA synthesis was performed by means of the Superscript® III Reverse Transcriptase (Life Technologies), following the instructions. In addition, a RNase H (Life Technologies) treatment was performed to remove RNA from the hybrid cDNA-RNA filament.

3.4 PCR (Polymerase chain reaction)

The screening of transformed plants and colony PCR on transformed bacteria was performed using GoTaq® DNA Polymerase (Promega), whereas high-fidelity amplification was performed to amplify the complete CDSs of the genes considered in this work using the High-Fidelity Platinum® *Pfx* DNA Polymerase (Thermo Fisher Scientific), following the manufacturer's instructions.

3.5 Expression analysis: Real Time RT-PCR

The relative gene expression of *NRAMP4, ZNT1* and *MTP1* in *N. caerulescens* MP, GA and *T. arvense* and *A. thaliana* transformed plants was evaluated with Real Time RT-PCR. RNA was extracted as previously described, and then reverse transcribed to cDNA by the Superscript® III Reverse Transcriptase Kit (Life Technologies) according to the manufacturer's instructions.

Real Time RT-PCR analysis was performed on cDNA with the KAPA SYBR® FAST ABI Prism® 2X qPCR Master Mix (Kapa Biosystems, Wilmington, MA, USA); each reaction was performed in triplicate. ABI PRISM® 7000 Sequence Detection System (Life Technologies) was used for the analysis. In detail, PCR amplification was performed for 40 cycles (95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s for each cycle). *ACT2/8* (*ACTIN2*: At3g18780; *ACTIN8*: At1g49240) were used as reference genes, according to the MIQE guidelines (Bustin *et al.,* 2009). The expression of *NRAMP4, ZNT1* and *MTP1* in *N. caerulescens* (GA and MP ecotypes), *T. arvense* and *A. thaliana* transgenic lines (#F_NRAMP4_OE and #R_NRAMP4_OE for *NcNRAMP4;* #F_ZNT1_OE and #R_ZNT1_OE for *NcZNT1*) was checked using the primers listed in **Table 3.1**. The $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) was applied to organize the expression data.

Table 3.1

Gene	Primer name	Primer sequence (5'-3)
NRAMP4	#F_NRAMP4_RT	TTCCCGATACTCTACATATGG
	#R_NRAMP4_RT	CCATCGCATGTACCATGAGC
	#F_NRAMP4_OE	CCTTTTGCAGTCATTCCTCTT
	#R_NRAMP4_OE	CCATCAGATACCCGTTAATAGA
ZNT1	#F_ZNT1_RT	TCAACTCGCATAGCCCTGG
	#R_ZNT1_RT	AGCCTCACATTACAACTCATC
	#F_ZNT1_OE	TGGTCGTGGAAAGAGGGAAT
	#R_ZNT1_OE	CCATCGCATGTACCATGAGC
MTP1	#F_MTP1_RT	CGCCTAGAGAGATTGACGC
	#R_MTP1_RT	ACCTTTCCCACTGTGATAGC
ACTIN (2/8)	#F_ACT2	GAACTACGAGCTACCTGATG
	#R_ACT2	CTTCCATTCCGATGAGCGAT

3.6 Constructs preparation and *A. thaliana* transformation

3.6.1 Generation of *A. thaliana* transgenic lines overexpressing *NcNRAMP4* and *NcZNT1*

To analyze the role in Ni tolerance and accumulation of NcNRAMP4 and NcZNT1 *in planta,* the CDS of both genes was amplified and cloned downstream the control of the CaMV35S promoter (**Table 3.2**). For *NcNRAMP4,* primers #F_attNRAMP4 and #R_attNRAMP4 were designed to amplify and cloned the complete CDS of *NRAMP4* of *N. caerulescens* MP ecotype downstream the constitutive CaMV35S promoter. The amplicon obtained was then cloned in the final pGEM®-T easy vector and sequenced.
The complete CDS of *NcNRAMP4* was cloned in the Gateway pDONR201 vector (Thermo Fisher Scientific, Waltham, MA, USA) and then transferred to the expression vector pH2GW7 by LR recombination (The Gateway® LR Clonase ™ enzyme mix kit, Thermo Fisher Scientific).
The construct pH2GW7::*NcNRAMP4* was then introduced by electroporation into competent *Agrobacterium tumefaciens* cells, strain GV3101.
For *NcZNT1,* primers #F_ZNT1 and #R_ZNT1 were designed to amplify and cloned the complete CDS of *ZNT1* of *N. caerulescens* MP ecotype downstream the constitutive CaMV35S promoter.
The amplicon obtained was cut from pGEM®-T easy vector with *BamHI* and *XhoI* restriction enzymes and ligated under control of CaMV35S in the final expression vector pMD1 previously prepared and then introduced by electroporation into competent *A. tumefaciens* cells, strain EHA105.

The *A. tumefaciens* strains transformed with pH2GW7::*NcNRAMP4* and pMD1::*NcZNT1* were used for *A. thaliana* transformation by floral dip (Clough and Bent, 1998).

Transformed plants with pH2GW7::*NcNRAMP4* and pMD1::*NcZNT1* were selected *in vitro* for their resistance to hygromycin (15 mg/L) and kanamycin (50 mg/L) respectively and the integration of the transgenes in the genome of *A. thaliana* plants was confirmed by PCR. Transformed plants were tested for *NcNRAMP4* and *NcZNT1* expression by Real time RT-PCR (Section 3.5) and at least two lines for each construct were propagated until stable homozygous T_3 lines were obtained, which were used for experiments.

To obtain double 35S::*NRAMP4*/ 35S::*ZNT1* transgenic *A. thaliana* lines, plants transformed individually with *NcNRAMP4* and *NcZNT1* were crossed in order to obtain plants carrying both genes. The presence of both *NcNRAMP4* and *NcZNT1* were confirmed with PCR on genomic DNA of *A. thaliana* 35S::*NRAMP4*/35S::*ZNT1* plants.

Table 3.2

Gene	Primer name	Primer sequence (5'-3)
NcNRAMP4	#F_attNRAMP4	*GGGGACAAGTTTGTACAAAAAGCAGGCT*ATGTCGGAGACGGAGAGAGA
	#R_attNRAMP4	*GGGGACCACTTTGTACAAGAAAGCTGGGT*CTAATTGCAAGGAGTGTACGT
NcZNT1	#F_BamHIZNT1	GGATCCATGATCATCGCCGATCTTCTTTG
	#R_XhoIZNT1	CTCGAGCTAAGCCCAAATGGCGAGTG

3.6.2 Transformation of *A. thaliana mtp1*+/+ homozygous mutants with 35S::*MTP1* constructs

The CDSs of *NcMTP1-long* and *NcMTP1-short* were separately amplified from cDNA and gDNA of *N. caerulescens* MP; *AtMTP1* was amplified from cDNA of *A. thaliana* Col-0 (**Table 3.3**).

NcMTP1-long, NcMTP1-short and *AtMTP1* were cloned in the Gateway pDONR201 vector (Thermo Fisher Scientific, Waltham, MA, USA) and then transferred to the expression vector pH2GW7 by LR recombination (The Gateway® LR Clonase ™ enzyme mix kit, Thermo Fisher Scientific).

The constructs pH2GW7::*NcMTP1-long,* pH2GW7::*NcMTP1-short* and pH2GW7::*AtMTP1* were introduced by electroporation into competent *A. tumefaciens* cells (GV3101) and used for *A. thaliana mtp1*+/+ homozigous mutants transformation by floral dip (Clough and Bent, 1998). The integration of the transgenes in the genome of *A. thaliana* plants was confirmed by PCR using a combination of specific primers.

Table 3.3

Gene	Primer name	Primer sequence (5'-3)
NcMTP1	#F_attNcMTP1	*GGGGACAAGTTTGTACAAAAAAGCAGGCT*ATGGAGTCTTCAAGTCACATCA
	#R_attNcMTP1	*GGGGACCACTTTGTACAAGAAGCTGGGT*TCAGCGCTCGATTTGTACGG
AtMTP1	#F_attAtMTP1	*GGGGACAAGTTTGTACAAAAAAGCAGGCT*ATGGAGTCTTCAAGTCCCA
	#R_attAtMTP1	*GGGGACCACTTTGTACAAGAAGCTGGGT*TTAGCGCTCGATTTGTATCGT

3.7 Analysis of intracellular protein localization: MTP1

3.7.1 Preparation of the *NcMTP1-long*::eGFP and *NcMTP1-short*::eGFP constructs

The coding sequences of *NcMTP1-long* and *NcMTP1-short*, lacking the stop codon, were fused separately to the coding sequence of the green fluorescence protein (eGFP) deprived of the start codon. Specific restriction enzymes were introduced in the sequences to allow the cloning of the fusion genes in the pMD1 expression vector downstream the CaMV35S (**Table 3.4**). The final constructs pMD1-*NcMTP1-long::eGFP* and pMD1-*NcMTP1-short::eGFP* were then used for subcellular localization experiments.

Table 3.4

Gene	Primer name	Primer sequence (5'-3)
MTP1	#F_NcMTP1_XbaI	TCTAGAATGGAGTCTTCAAGTCACATCA
	#R_NcMTP1_SalI	GTCGACGCGCTCGATTTGTACGGTTAC
eGFP	#F_eGFP_SalI	GTCGACGTGAGCAAGGGCGAGGAGC
	#R_eGFP_SacI	GAGCTCTTACTTGTACAGCTCGTCCATG

3.7.2 *N. tabacum* protoplasts isolation and transfection

Leaves were collected from 3-4 week-old *N. tabacum* plants grown *in vitro* in standard MS medium under controlled condition (20-25 °C, light intensity of 250 µmol $m^{-2}s^{-1}$).
Young leaves were cut in 20 mL of TEX solution (3.1 g/L Gamborg's B5 salts, 500 mg/L MES, 750 mg/L $CaCl_2 \cdot 2H_2O$, 250 mg/L NH_4NO_3, 136.9 g/L sucrose, pH 5.7) and then incubated overnight adding an enzymatic solution (1.2% Cellulase Onozuka R10, 0.4% macerozyme R10 prepared in TEX

buffer). The enzyme/protoplast solution was then filtered with a 75 µm and 300 µm nylon mesh and mixed with W5 solution (154 mM NaCl, 125 mM $CaCl_2 \cdot 2H_2O$, 5 mM KCl, 0.1% glucose). After a centrifugation at 600 rpm for 10 min, the pellet was re-suspended in W5 at 2 X 10^5 mL^{-1} and centrifugated at 600 rpm for 5 min. The pellet was re-suspended in W5 at 2 X 10^5 mL^{-1}, stored on ice for 120 min and the pellet obtained from precipitation was re-suspended at 2 X 10^5 mL^{-1} in MMM (0,1% MES, 0.4 M Mannitol and 15 mM $MgCl_2 \cdot 6H_2O$). The transfection was performed adding 300 µL of PEG (40% PEG4000, 0.4 M Mannitol and 0.1 M $CaCl_2$) solution to 15 µL (10-20 µg of plasmid DNA) and 300 µL of protoplast suspension leaving it to incubate for 2 min and then transferred 18 h at room temperature (20-25 °C, light intensity of 250 µmol $m^{-2}s^{-1}$). Images were taken with a Leica DM RB microscope (Leica Microsystem GmbH) using a 488-nm laser for the detection of GFP in the lambda range of 496- to 555-nm.

3.7.3 Co-localization experiment in *N. tabacum* leaves

Competent cells of *A. tumefaciens* (strain GV3101) were transformed by heat shock using 500-1000 ng of plasmid (pMD1::*MTP1-eGFP*) and grown in a selective LB solid medium supplemented with gentamicin (25 mg/L), rifampicin (50 mg/L) and kanamycin (50 mg/L) for 2 days at 28°C. The transformed cells were then inoculated in 5 mL of LB with the addition of gentamicin (25 mg/L), rifampicin (50 mg/L) and kanamycin (50 mg/L), placed at 28°C and stirred at 220 rpm overnight. The next day 1 mL of bacterial culture was placed in a 1.5 mL tube and centrifuged at 4500 rpm for 5 minutes. The supernatant has been discarded and the bacteria were resuspended in 1 mL of Infiltration Buffer (IF) (2 mM Na_3PO_4 2 mM, 50 mM MES, 0.2 mM Acetosyringone, Glucose 0.5%, H_2O up to 1 mL) with vortex. These steps were repeated two times. A dilution (1:5) of the bacterial solution was performed in a 1.5 mL tube with 500 µL of IF with a final OD_{600nm} of 0.05. The contents of the tube were taken with a syringe equipped with a needle and a slight point-like abrasion was made with the needle in the lobes of the lower page of the *N. tabacum* leaf to be infiltrated. The needle has been removed and the syringe was placed in correspondence with the point abrasion for the agro-infiltration. The plants were returned to the growth chamber for 48 hours for observation of the fluorescence. The confocal microscope Zeiss LSM710 was used for this experiment, equipped with two lasers capable of producing a wavelength radiation of 488 nm (used to excite the GFP and YFP proteins) and 543 nm (used to excite the RFP protein) respectively. As sub-cellular markers, RFP-SYP51 (tonoplast; Barozzi *et al.,* 2019), RFP-KDEL (endoplasmatic reticulum; Ariani *et al.,* 2019), RFP-SYP122 (plasma membrane; Barozzi *et al.,* 2019) and ST-RFP (Golgi apparatus; Ariani *et al.,* 2019) were used. The confocal microscopy analyses were performed at laboratory of Botany (Di.S.Te.B.A. Campus Ecotekne, Lecce 73100 Italy) in collaboration with Dr. Gian Pietro Di Sansebastiano.

3.8 Yeast complementation assay

3.8.1 Preparation of the pADSL::*NcNRAMP4* and pADSL::*NcZNT1* constructs

To investigate the Ni transport properties of *NcNRAMP4* and *NcZNT1,* the complete CDSs of all genes were separately amplified from cDNA of *N. caerulescens* and cloned in pGEM®-T easy vector (Promega) for the sequencing. Specific restriction enzymes were introduced in the sequences (**Table 3.5**) to allow the cloning of the fusion genes in the pADSL expression vector, downstream the ADH promoter.

Table 3.5

Gene	Primer name	Primer sequence (5'-3)
NcNRAMP4	#F_BamHI_NcNRAMP4	GGATCCATGTCGGGAGACTGATAGAGAG
	#_EcoRI_NcNRAMP4	GAATTCCTAATTGCAAGGAGTGTACGT
NcZNT1	#F_BamHI_ZNT1	GGATTCATGATCATCGCCGATCTTCTT
	#R_EcoRI_ZNT1	GAATTCCTAAGCCCCAAATGGCGAGTG

3.8.2 Preparation of the pADSL::*MTP1* constructs

NcMTP1-long and NcMTP1-short were tested for their ability to complement vacuolar metal uptake-deficient yeast mutants and determine their different transport specificity. The complete CDSs of *N. caerulescens NcMTP1-long* and *NcMTP1-short* were separately amplified from cDNA of *N. caerulescens* MP and GA, whereas *AtMTP1* was amplified from cDNA of *A. thaliana* Col-0 WT. Specific restriction enzymes were introduced in the sequences to allow the cloning of the fusion genes in the pADSL expression vector, downstream the ADH promoter. *SpeI* and *SalI* restriction enzymes were used to directionally cloned *MTP1* sequences in pADSL vector. The primers used for the cloning are listed in **Table 3.6.**

Table 3.6

Gene	Primer name	Primer sequence (5'-3)
NcMTP1	#F_SpeI_NcMTP1	ACTAGTATGGAGTCTTCAAGTCACATCA
	#R_SalI_NcMTP1	GTCGACTCAGCGCTCGATTTGTACGG
AtMTP1	#F_SpeI_AtMTP1	ACTAGTATGGAGTCTTCAAGTCCCA
	#_SalI_AtMTP1	GTCGACTTAGCGCTCGATTTGTATCGT

3.8.3 Yeast transformation and complementation

For NRAMP4 and ZNT1*,* a *Saccharomyces cerevisiae* wild-type strain DY1457 (MATalfa, *ade6*, *can1*, *his3, leu2*, *trp1, ura3*) was transformed with the empty pADSL vector and pADSL vector expressing *NcNRAMP4* and *NcZNT1*, using the lithium acetate/single-stranded carrier DNA/polyethylene glycol method (Agatep *et al*., 1998). The same strategy was used also for MTP1, using *zrc1cot1* mutant (MATα *his3*, *leu2, trp1, ura3-52, kan-* zrc1::*HIS3* cot1::*URA3*) in addition to DY1457 WT. The selection of transformants yeasts was done on selective medium with 0.7% of yeast nitrogen base (YNB; Sigma-Aldrich®), 1.92 g/L of all aminoacids except Trp (Sigma-Aldrich®) and 2% of glucose was add to the medium after sterilization as carbon source necessary for growth. For drop assays, transformed yeast strains were grown overnight in 5 ml selective liquid SD-trp medium at 28°C to early stationary phase. Yeast cells were then diluted to OD_{600} nm = 1, 0.1, 0.01 and 0.001 and drop assays were performed on selective SD-trp medium modified with the addiction of specific metals considered for all experiments. Minimum inhibitory concentration (MIC) was determined for each metal used for yeast complementation assay and this concentration was considered for the experiments. Liquid culture assays were also performed to evaluate metal sensitivity (Ni for NRAMP4 and ZNT1; Ni, Zn and Co for MTP1) for all recombinant yeasts. Briefly, pre-cultures of *S. cerevisiae* DY1457 or *zrc1cot1* strains were grown in 5 ml of selective medium SD-trp overnight at 28°C with shaking and then diluted OD_{600} nm = 0.01 as starting point. Metals were added to the selective medium and the growth of recombinant yeasts was tested measuring OD_{600} nm for several hours. Yeast complementation assays were performed in collaboration with Dr. Giovanna Visioli and Dott.ssa Caterina Agrimonti (University of Parma).

3.9 Nickel treatment on *A. thaliana* WT and transgenic lines

3.9.1 *In vitro* analysis

Seeds of *A. thaliana* WT and transgenic lines overexpressing *NRAMP4* and *ZNT1*, alone or in combination, were sterilized and germinated as described in Section 3.1 on standard 1X MS medium (Murashige and Skoog, 1962) and then transferred in solid 1X MS medium modified with the addition of 50 µM of $NiSO_4$ (Ni excess). The primary root length was measured after 10 days.

3.9.2 Ni tolerance and accumulation on soil

Seeds of *A. thaliana* Col-0 WT, 35S::*NRAMP4,* 35S*::ZNT1*, 35S::*NRAMP4*/35S::*ZNT1* plants were vernalized for two days at 4°C to break seed dormancy and grown in soil in the greenhouse in controlled conditions (16 h light/8 h dark, illumination 100–120 µmol $m^{-2}s^{-1}$, day/night temperature 22°C/18°C) for five weeks. Plants were then supplied for 3 weeks with a solution of $NiSO_4$ (350 ppm). To determine the levels of Ni, Fe and Zn in *Arabidopsis* plants, the shoots of all genotypes were collected and separate pools were prepared. Samples were oven-dried at 60°C for 48 hours and then homogenized before processing. Ni content was determined after microwave-assisted acid digestion (EPA 3052, 1996) by means of ICP-MS analysis (EPA 3051A 2007+EPA 6010C 2007). Phenotypical analysis on *A. thaliana* was also performed in order to evaluate the effect of the overexpression of *NRAMP4, ZNT1* and *NRAMP4*/*ZNT1* together on Ni tolerance.

3.9.3 Ni tolerance and accumulation in hydroponic solution

To determine the metal tolerance and accumulation of transgenic 35S*::NRAMP4*, 35S::*ZNT1,* and 35S::*NRAMP4*/35S::*ZNT1* *A. thaliana* lines, five plants for each of two independent transgenic lines and *A. thaliana* WT were grown hydroponically in half strength Hoagland's nutrient solution for two weeks under controlled conditions (16 h light/8 h dark, illumination 100–120 µmol $m^{-2}s^{-1}$, day/night temperature 22°C/18°C) and then treated with 20 µM of $NiSO_4$. Shoot tissues were collected for each sample and Ni concentration was determined by means of ICP-MS analysis following the protocol described in Gullì *et al.* (2018).

3.10 Statistical analysis

Statistical analysis of data was analyzed using the one-way analysis of variance (ANOVA) followed by a post hoc Tukey's test performed with the GraphPad Prism 7 (GraphPad Software). Statistical significant variations are marked by asterisks (*$P<0.05$; **$P<0.01$; ***$P<0.001$).

4. RESULTS

4.1 Nickel tolerance in *N. caerulescens* MP

Ni is normally required in small amounts by the plants (Cempel and Nikel, 2005) and excess levels can disturb metal homeostasis, hence reducing plant growth (Freeman *et al.,* 2004). It is therefore clear that establishing the Ni threshold level beyond which the growth of non-accumulating plants is affected represents the first step in investigating and understanding Ni hyperaccumulation and hypertolerance.
The impact of excess Ni on plant development was first analysed by comparing the growth of the non-accumulating species *T. arvense* with that of *N. caerulescens* MP, in the presence or absence of Ni. After 2 weeks of adaptation in a hydroponic medium consisting of half-strength Hoagland's solution (control condition), *T. arvense* and *N. caerulescens* MP plants were transferred in a modified medium containing 10 μM $NiSO_4$ (excess Ni) for further 2 weeks. This concentration was chosen after preliminary experiments on these ecotypes revealed differences in their ability to accumulate and tolerate Ni (Richau *et al.,* 2009; Visioli *et al.,* 2014).
After 2 weeks, Ni was observed to strongly inhibit the growth on the non-accumulating *T. arvense* plants, which also displayed chlorotic shoots; moreover, there was also a significant reduction in both total fresh weight and root length in comparison to the controls (**Fig. 4.1-A**), confirming that 10 μM $NiSO_4$ is sufficient to inhibit plant growth. On the other hand, no differences were found between control and Ni-grown *N. caerulescens* MP (**Fig.4.1-B**), thus confirming the Ni-hypertolerant trait of this species.

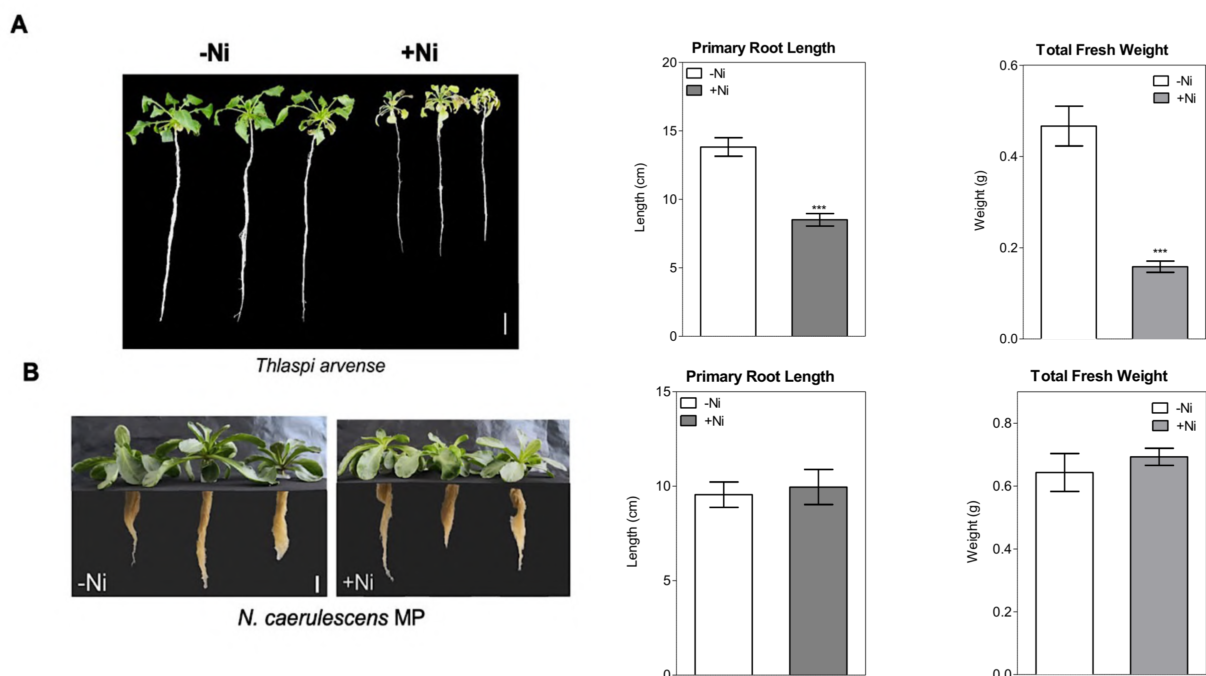

Figure 4.1: Plants of *T. arvense* (A) and *N. caerulescens* MP (B) grown in standard 0.5X Hoagland's solution (-Ni) and modified adding 10 μM of $NiSO_4$ (+Ni) for 2 weeks. Bars correspond to SD (n = 6). Scale bar=2 cm.

4.2 Expression analysis of ***NRAMP4*** and ***ZNT1***

Although various authors have investigated the role of metal transporter proteins in hyperaccumulators, there is no evidence to date indicating the existence of Ni-specific transporters in these plants (van der Pas *et al.,* 2019). It has been suggested that Ni uptake in *N. caerulescens* may depend on low-affinity cation transporters (Richau *et al.,* 2009).

Previous research works on Ni hyperaccumulators *N. caerulescens* MP and *N. goesingense* revealed that some members of the *ZIP* and *NRAMP* families of metal transporters present in plants, including *ZNT1*, *ZNT2*, *NRAMP3* and *NRAMP4,* may be involved in Ni transport (Mizuno *et al.,* 2005; Visioli *et al.,* 2014). Therefore, the modulation of *NRAMP4* and *ZNT1* upon exposure to Ni has been investigated in both non-accumulating (*T. arvense*) and hyperaccumulating (*N. caerulescens* GA and MP) plant species. Although this research was mainly focused on *N. caerulescens* MP, ecotype GA (a Zn/Cd hyperaccumulator) was introduced in this work to elucidate possible differences in the modulation of these two genes in hyperaccumulator plants characterized by different affinity for the metal.

For this experiment, Real Time RT-PCR was performed on samples from *N. caerulescens* (GA and MP ecotype) and *T. arvense* (TA) plants grown in hydroponics at different Ni concentrations for 1 day to quantify *NRAMP4* and *ZNT1* expression upon exposure to Ni.

As shown in **Fig. 4.2,** the vacuolar transporter *NRAMP4* was induced in both ecotypes, GA and MP, in the presence of 10 μM (+Ni) and 100 μM (++Ni) $NiSO_4$; interestingly, *N. caerulescens* MP displayed higher constitutively levels of *NRAMP4* expression under all the conditions tested, suggesting a different regulation mechanism of the vacuolar *NRAMP4* gene expression in hyperaccumulator accessions of *Noccaea,* which may be associated with the Ni hypertolerance and hyperaccumulator phenotype (Mizuno *et al.,* 2005; Visioli *et al.,* 2014).

ZNT1 resulted to be highly expressed in both *N. caerulescens* accessions, in particular in GA ecotype, compared to the non-accumulator species *T. arvense*, which displayed very low transcript levels, confirming the high constitutive expression of *ZNT1* in hyperaccumulator plants (Pence *et al.*, 2000; Assunção *et al.*, 2001). The regulation of *NRAMP4* and *ZNT1* was also evaluated in *N. caerulescens* MP and *T. arvense* treated with excess Ni for several hours (6, 12, 18 and 24). Both genes appeared to be significantly upregulated in the shoots of the Ni hyperaccumulator plants (in particular after 18 and 12 hours of treatment, respectively) as compared to their controls, but no differences were found in *T. arvense* (**Fig. 4.3**), supporting the possible involvement of *NRAMP4* and *ZNT1* in Ni tolerance and accumulation in *N. caerulescens* MP.

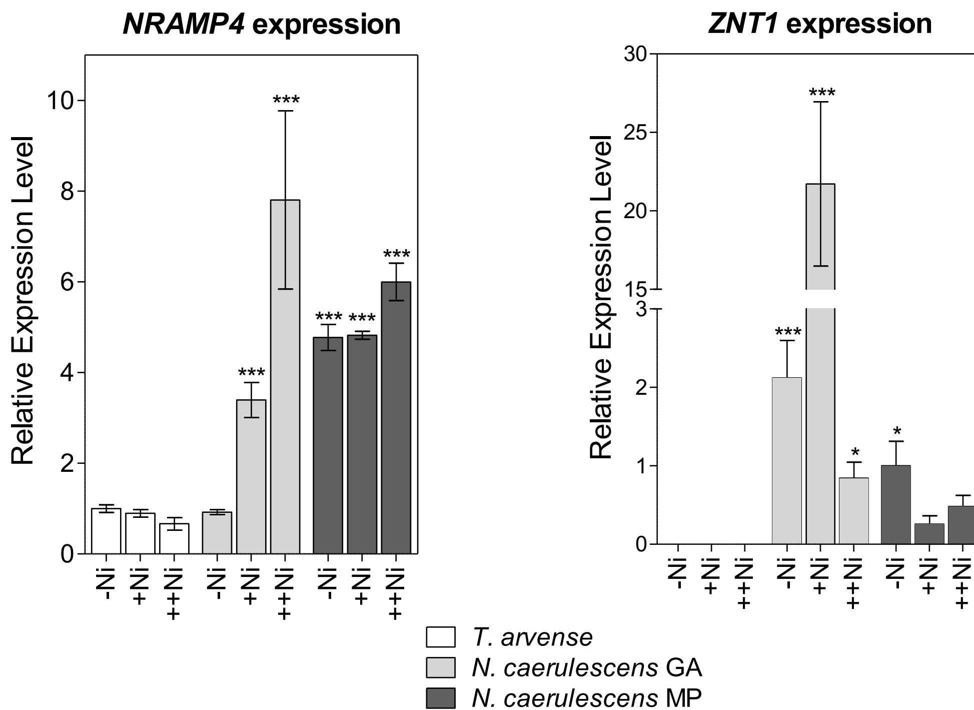

Figure 4.2: Real Time RT-PCR experiment testing the expression of *NRAMP4* and *ZNT1* relatively to the expression of the housekeeping gene *Actin2* in shoots of *T. arvense, N. caerulescens* GA and MP treated and not treated with Ni excess. -Ni, +Ni and ++Ni refer to 0, 10 and 100 μM of $NiSO_4$. Statistically significant variations, as resulting from the ANOVA test (n = 3), are marked by asterisks. Bars correspond to standard error, calculated as in Muller *et al.* (2002).

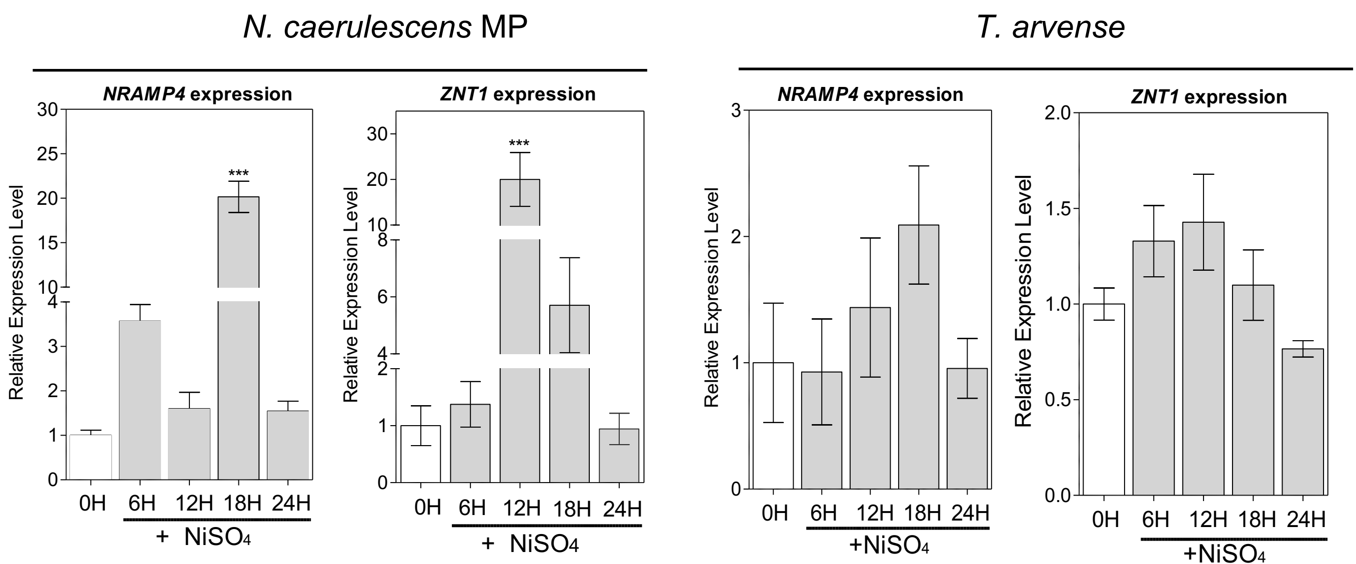

Figure 4.3: Expression analysis of *NRAMP4* and *ZNT1* (relatively to the expression of the housekeeping gene *Actin2* in shoots of *N. caerulescens* MP and *T. arvense* treated with Ni excess (10 μM of $NiSO_4$). Statistically significant variations, as resulting from the ANOVA test (n = 3), are marked by asterisks. Bars correspond to standard error, calculated as in Muller *et al.* (2002).

4.3 Yeast complementation assay with NcNRAMP4 and NcZNT1

The Ni-transport ability of both NRAMP4 and ZNT1 was investigated by yeast complementation assays, using a WT strain of *S. cerevisiae*. The complete CDS of both *NRAMP4* and *ZNT1* was amplified with specific primers from the cDNA of *N. caerulescens* MP and then transferred into a pADSL vector. The pre-culture of recombinant yeasts was diluted 1:100 and transferred into a selective SD-trp medium containing excess Ni (400 μM); OD_{600} nm was measured several times to determine the survival rate under these stressing conditions. The growth of recombinant yeasts was considered after 24 hours of exposure to Ni. The pADSL empty vector was used as control. As shown in **Fig. 4.4**, NcNRAMP4 significantly reduced the survival of recombinant yeast compared to the empty vector, whereas the opposite was observed for NcZNT1. Since there is no information on the Ni-transporting ability of these two genes in *N. caerulescens* MP and other Ni hyperaccumulator/hypertolerant plants, it is only possible to speculate that both proteins take part in the transport and homeostasis regulation of Ni *in planta*. Mizuno and colleagues (2005) found that NjNRAMP4 and NjZNT1 can transport Ni in the Ni-hyperaccumulator species *N. japonica*, a result in keeping with ours. In comparison with *AtNRAMP4* and *NcNRAMP4* from LC ecotype, *NjNRAMP4* cannot increase the survival rate of mutant yeast defective for Zn, Fe and Mn transport (Thomine *et al.,* 2000; Lanquar *et al.,* 2004; Oomen *et al.,* 2009), indicating that this gene may possess different metal binding properties in Ni-hyperaccumulating species. We cannot exclude that the effect caused by the heterologous expression of these two proteins in yeast may be linked to the competition of Ni with other micronutrients, as confirmed by the literature (Richau *et al.,* 2009; van der Pas *et al.,* 2019). *NRAMP4* and *ZNT1* are generally involved in regulating Fe, Zn and Mn homeostasis in plants (Curie *et al.,* 2000; Thomine *et al.,* 2000; Lanquar *et al.,* 2004). NRAMP4 in particular is known to mobilize metals from the vacuolar compartment into the cytoplasm (Lanquar *et al.,* 2005) and ZNT1 is required for metal uptake from the soil (Assunção *et al.,* 2010; Castro *et al.,* 2017). Therefore, it is possible that the expression of NRAMP4 in yeast may induce an over-accumulation of Ni in the cytoplasm and consequently cause the premature death of the cell, or the presence of Ni may perturb the homeostasis of other metals, which are required for optimal growth. Mizuno and colleagues (2005) have also observed that in *N. japonica* certain amino acid residues present in both NRAMP4 and ZNT1 seem to be correlated to the ability to transport Ni, although further investigations are required to clarify the metal-binding properties of these two genes.

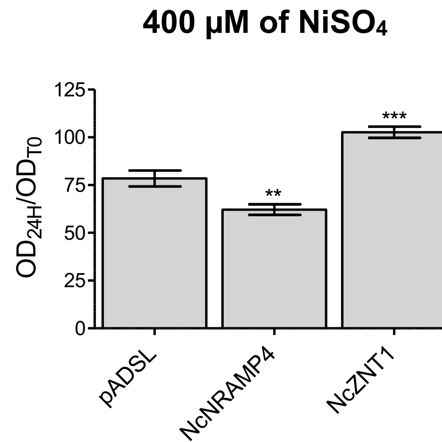

Figure 4.4: Liquid culture assay performed on recombinant *S. cerevisiae* WT (DY1457) expressing *NcNRAMP4* and *NcZNT1,* adding 400 µM $NiSO_4$ in the growth medium. The empty vector (pADSL) was used as control. Bars correspond to standard deviation (n = 3).

4.4 Generation of *A. thaliana* overexpressing *NcNRAMP4* and *NcZNT1* lines

In order to investigate whether an enhanced expression of both *NRAMP4* and *ZNT1* could increase Ni tolerance and accumulation in plants, *NcNRAMP4* and *NcZNT1* genes were expressed at high levels in *A. thaliana* under the control of the CaMV35S promoter. The CDS of *NcNRAMP4* was cloned in the final expression vector pH2GW7 (Hyg^R) using the Gateway recombination method, while *ZNT1* was fused into pMD1 (Kan^R) by direct cloning. Single transformed plants were tested for *NcNRAMP4* and *NcZNT1* expression by Real Time RT-PCR (Section 3.5; **Fig. 4.5 A-B**). Double 35S::*NRAMP4*/35S::*ZNT1* transgenic lines were also obtained by crossing plants carrying single 35S::*NRAMP4* and 35S::*ZNT1*. PCR was performed on their genomic DNA to confirm the presence of both genes, and the transgenic lines were propagated for further analyses.

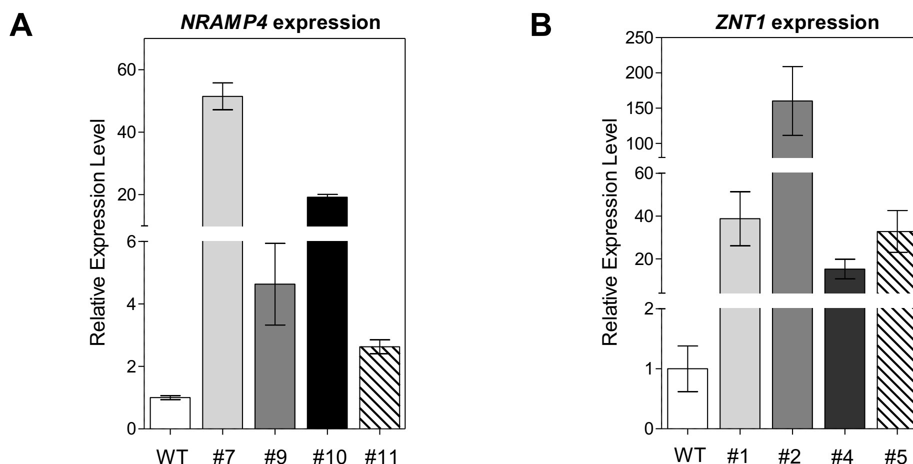

Figure 4.5: Expression analysis on *NcNRAMP4* (A) and *NcZNT1* (B) in *A. thaliana* transgenic lines. WT was used as calibrator sample.

4.5 Phenotypic analysis of *A. thaliana* transgenic lines

In order to analyze the *in vivo* effect of overexpressing these transporters *in planta,* a phenotypic characterization was performed on the 35S::*NRAMP4,* 35S::*ZNT1* and 35S::*NRAMP4*/35S::*ZNT1* transgenic lines of *A. thaliana* measuring the shoot biomass of plants grown in soil under controlled conditions. Transgenic lines overexpressing *NcNRAMP4* displayed significantly greater shoot biomass and total leaf area than the WT (**Fig. 4.6**). As for *ZNT1*, only two overexpressing lines (#2, #3) displayed greater shoot biomass than the WT (**Fig. 4.7**), although the differences with 35S::*NcNRAMP4* plants were smaller and there was a greater variability between the three transgenic lines. The phenotype of the double 35S::*NRAMP4*/35S::*ZNT1* lines was similar to that of 35S::*NcNRAMP4* (**Fig. 4.8**). These effects may either depend directly on the high levels of *NcNRAMP4* and *NcZNT1* expression in the transgenic lines or be indirectly linked to a possible alteration in the expression of other metal transporters, as speculated by Lin and colleagues (2016). In fact, differences were observed in the modulation of genes involved in regulation of nutrient homeostasis, such as *IRT1*, *IRT2*, *FRO2* and *MTP1* (Lin *et al.,* 2016) in *A. thaliana* transgenic lines overexpressing *ZNT1* of *N. caerulescens* LC, suggesting that metal homeostasis is a very complex mechanism under the control of several genes. From our data, it can be assumed that both NcNRAMP4 and NcZNT1 transporters could have induced some changes in the homeostasis of some micronutrients, such as Zn, Fe and Mn, required for plant development, with a consequent improvement of vegetative growth under normal conditions. In particular, the enhanced metal remobilization induced by the higher levels of *NcNRAMP4* expression seem to have a stronger effect on the growth of transgenic *A. thaliana* plants in comparison to *NcZNT1*, which is known to be particularly involved in Zn transport in *N. caerulescens* (Lin *et al.,* 2016).

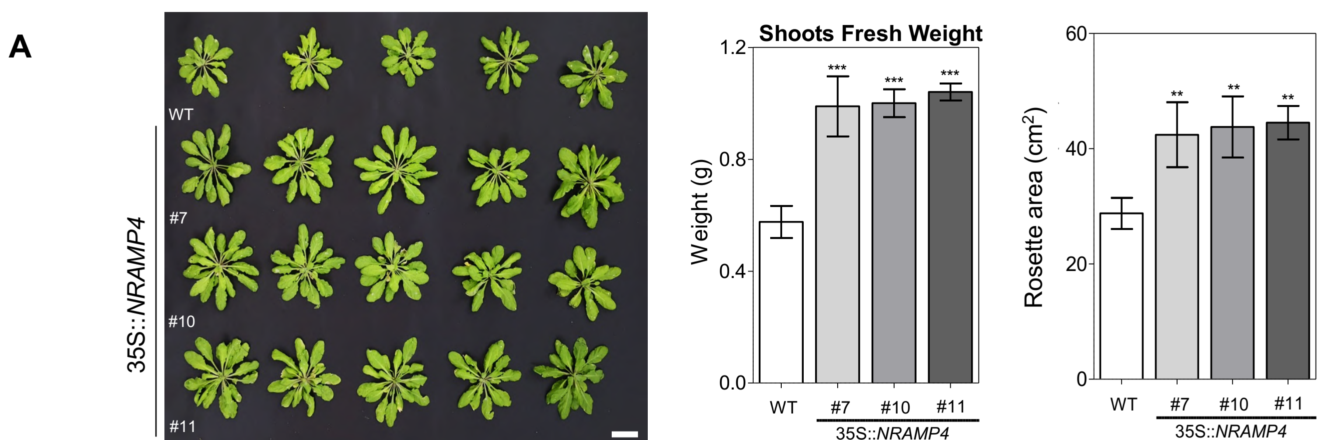

Figure 4.6: Shoot fresh weight and total leaf area in 4-week old *A. thaliana* WT and 35S::*NRAMP4* transgenic lines grown on soil. Values are means ± SD (n=6). Scale bars = 5 cm

B

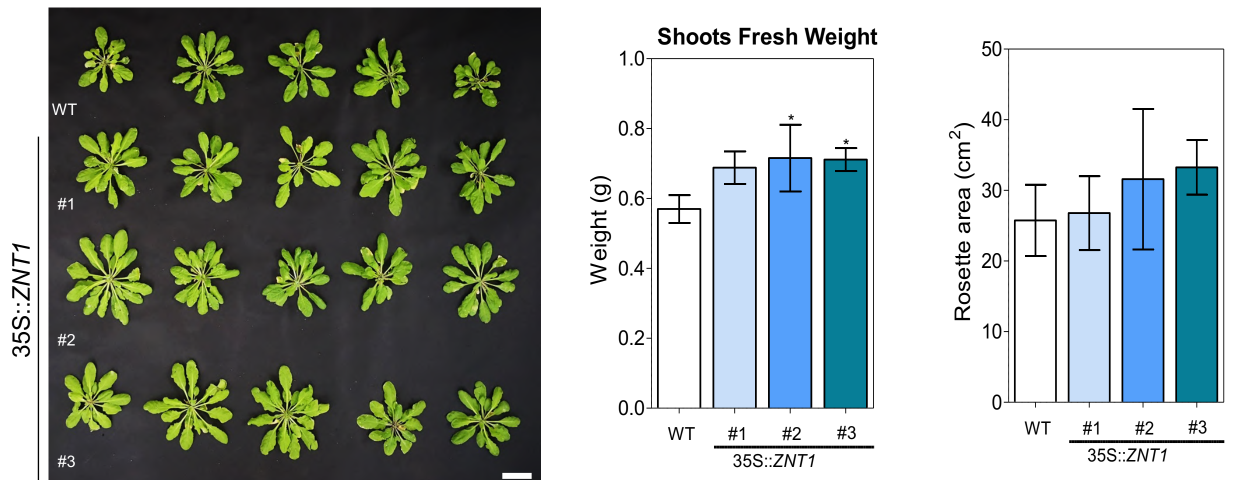

Figure 4.7: Shoot fresh weight and total leaf area in 4-week old *A. thaliana* WT and 35S::*ZNT1* transgenic lines grown on soil. Values are means ± SD (n=6). Scale bars = 5 cm

C

Figure 4.8: Shoot fresh weight and total leaf area in 4-week old *A. thaliana* WT and 35S::*NRAMP4*/35S::*ZNT1* transgenic lines grown on soil. Values are means ± SD (n=6). Scale bars = 5 cm.

4.6 Ni tolerance in *A. thaliana* on soil

To determine the possible impact of the overexpression of *NcNRAMP4* and *NcZNT1* in Ni tolerance and accumulation, plants of *A. thaliana* WT, single 35S::*NRAMP4,* 35S::*ZNT1* and double 35S::*NRAMP4*/35S::*ZNT1* transformed lines were cultivated in soil for 4 weeks under controlled conditions and then supplemented with 350 ppm of $NiSO_4$ (350 ppm) for 3 weeks. During the experiment, the dimension of rosette leaves and their phenotype were compared once a week for 21 days. In the first 2-weeks, no particular differences were observed considering all *A. thaliana* plants, which displayed similar phenotype; however, at the end of the Ni treatment, only *A. thaliana* WT showed visible chlorotic effect on the shoots, with a reduction of leaves dimension compared to transgenic lines (**Fig. 4.9**). This data confirms that Ni is toxic to plants when present at high concentration in the medium (see Fig.4.1; Freeman *et al.,* 2004). The amount of Ni accumulated into the shoots was also measured for all plants by ICP/MS analysis, considering also the micronutrients Zn and Fe, which have been demonstrated to possibly compete with Ni homeostasis *in planta* (Richau *et al.,* 2009; van der Pas *et al.,* 2019). Compared to the WT, all transgenic lines displayed lower accumulation of Ni, whereas no differences were found regarding shoot Zn content. Therefore, it is possible to directly correlate the chlorotic phenotype observed in *A. thaliana* WT upon metal treatment with higher Ni accumulation compared to the other lines. The lower accumulation of Fe found for 35S::*NRAMP4* and 35S::*ZNT1* transgenic lines could be associated with a possible competition between Ni and Fe for the same transporters, which may include members of *ZIP* and *NRAMP* gene families (see van der Pas *et al.,* 2019). In particular, high concentration of Ni into the soil could change metal homeostasis regulation mediated by *NcNRAMP4* and *NcZNT1*, reducing the accumulation of this micronutrient or its transport into the areal part (Lanquar *et al.,* 2005; Bastow *et al.,* 2018). It is interesting to notice that Fe accumulation in the shoots was found to be at least 10 times higher than Ni, indicating that probably these two proteins can strongly translocate more Fe than Ni. The same consideration could be valid for Zn as well, which seem to compete with Ni for the same proteins (Richau and Schat, 2009). In this experiment, the concentration of both Ni and Zn resulted to be similar in order of magnitude, suggesting that higher concentration of Ni could be required to induce a stronger competition between these two metals.

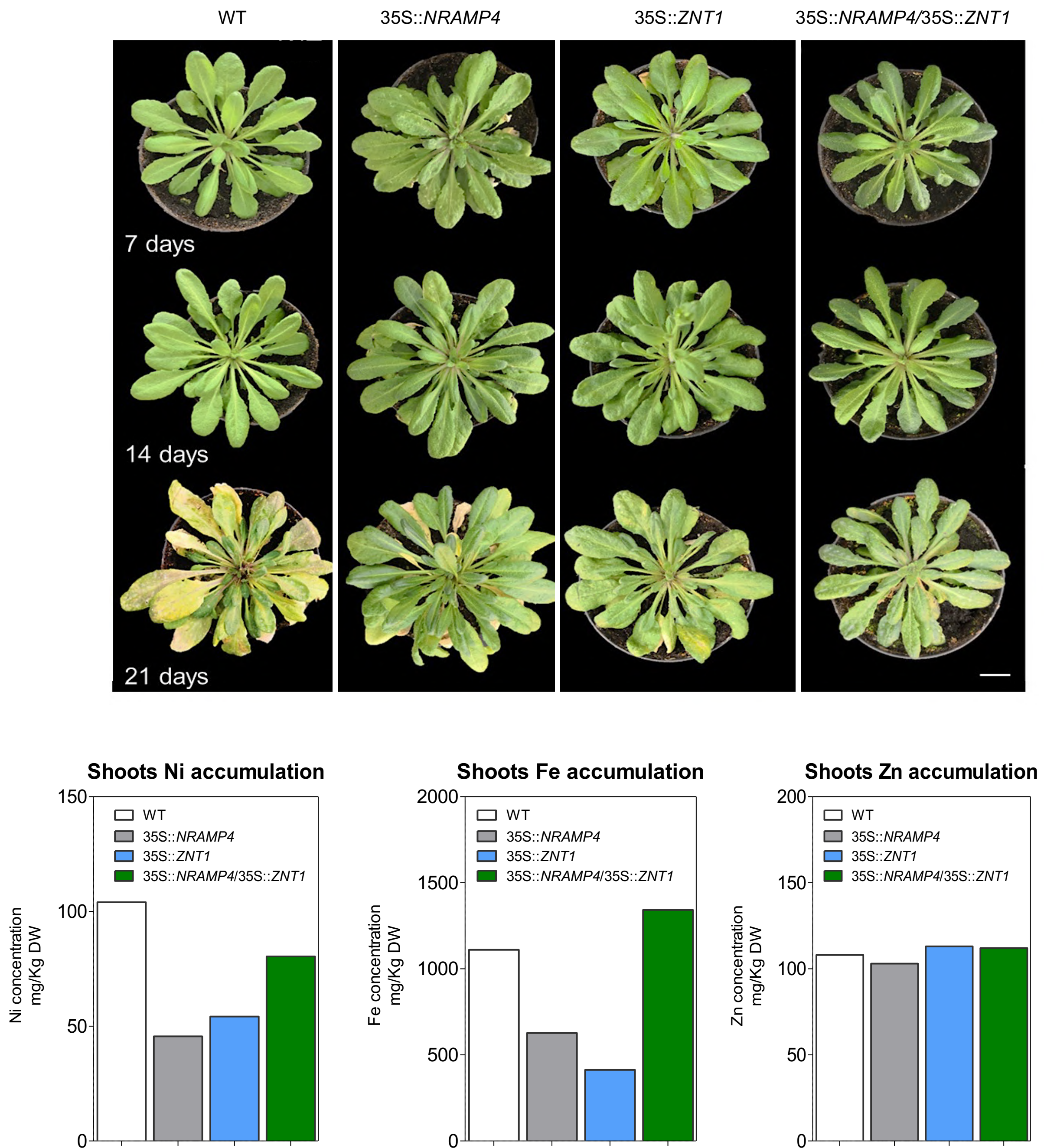

Figure 4.9: A) Growth of *A. thaliana* WT and transgenic lines in the presence of Ni excess for 3 weeks. Scale bars=3 cm. B) ICP-MS analysis on shoots of *A. thaliana* WT transgenic lines for 35S::*NRAMP4,* 35S::*ZNT1* and 35S::*NRAMP4*/35S::*ZNT1* to evaluate the accumulation of Ni, Fe and Zn levels respectively.

4.7 *In vitro* analysis

To examine the effect of *NcNRAMP4* or *NcZNT1* on Ni tolerance, WT, single 35S::*NRAMP4*, 35S::*ZNT1* and double 35S::*NRAMP4*/35S::*ZNT1* *A. thaliana* plants were vertically grown *in vitro* on Petri dishes on a MS medium with or without the addition of excess Ni (50 μM). A phenotypic characterization was then performed, measuring the length of their primary root.

After 10 days of treatment, single 35S::*NRAMP4* and double 35S::*NRAMP4*/35S::*ZNT1* lines displayed longer roots than the WT under all the conditions tested (**Fig. 4.10 A**), whereas transgenic lines overexpressing single *NcZNT1* did not differ from the WT (**Fig. 4.10 B**). The transgenic lines overexpressing *NcNRAMP4* and *NcNRAMP4*/*NcZNT1* also displayed significantly greater shoot fresh weight under control and excess Ni conditions (**Fig. 4.10 C; Fig. 4.11**), while 35S::*ZNT1* lines did not differ significantly from its WT. These data can be explained by taking the biological role of these transporters into account. In *A. thaliana* and *N. caerulescens*, NRAMP4 is normally required for the regulation of Zn, Fe and Mn homeostasis by vacuolar remobilization (Thomine *et al.,* 2000; Lanquar *et al.,* 2004; Oomen *et al.,* 2009). NRAMP4 in particular has been shown to be the primary source of Fe in germinating seeds (Lanquar *et al.,* 2005; Bastow *et al.,* 2018); this element is essential for plant growth and development, particularly for the functioning of chloroplasts and mitochondria, which require large amounts of this micronutrient (Lanquar *et al.,* 2010). Therefore, the overexpression of *NcNRAMP4* in *A. thaliana* presumably induced a greater Zn, Fe and Mn remobilization from the vacuolar compartment into the cytoplasm in comparison with the WT, which also displayed a significantly shorter primary root and more stunted growth than the 35S::*NRAMP4* lines. Since the double 35S::*NRAMP4*/35S::*ZNT1* transgenic lines displayed a similar phenotype to single 35S::*NRAMP4* transgenic lines, it can be presumed that this phenomenon mainly depends on the increased expression of *NcNRAMP4* rather than that of *NcZNT1*.

Therefore, it is possible that the phenotype observed *in vitro* for all plants could be principally linked to alterations in their metal homeostasis brought about by overexpression of *NcNRAMP4* and *NcZNT1* rather than the high levels of Ni, although a general reduction of root length was observed in all the lines upon Ni treatment.

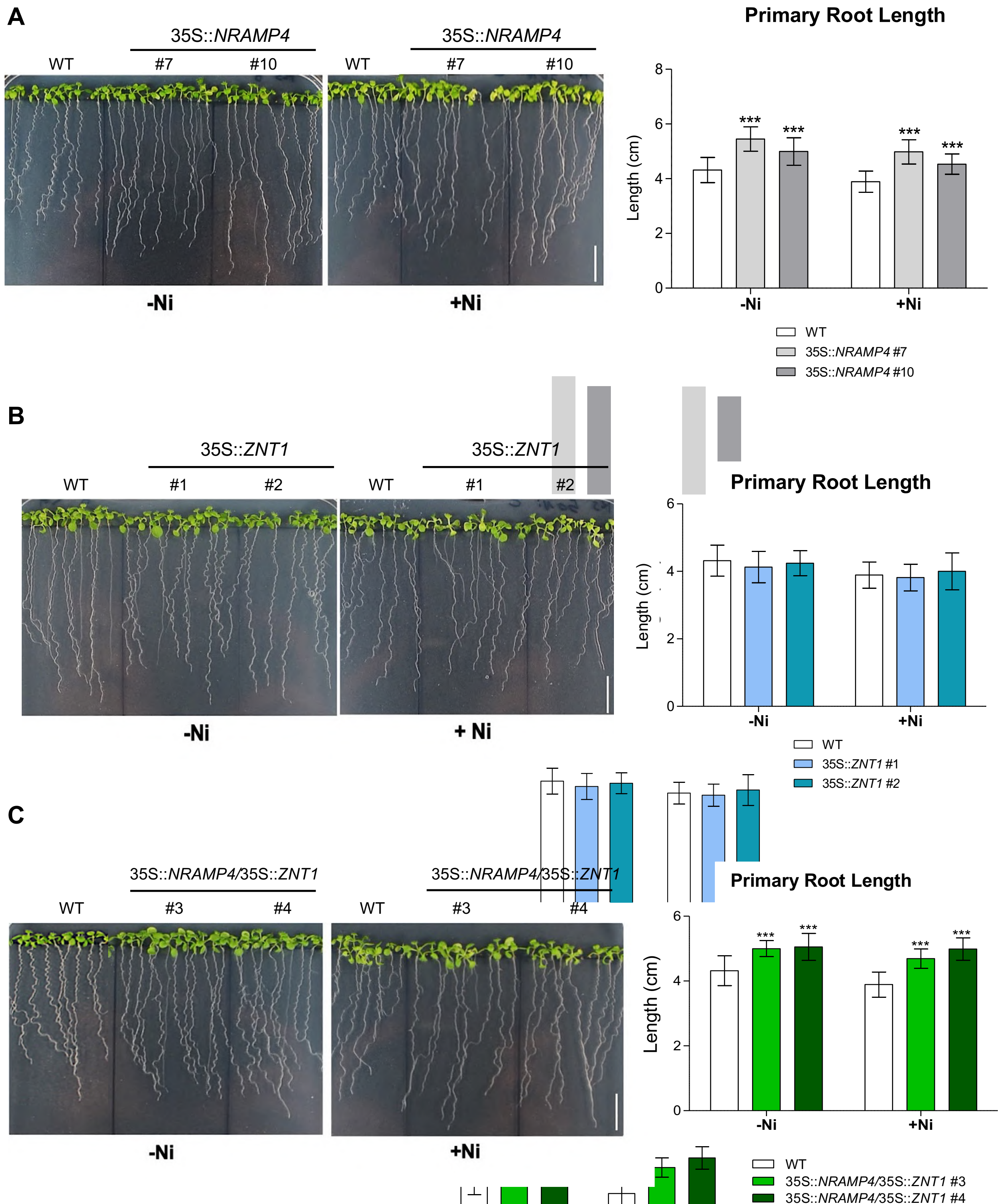

Figure 4.10: Primary root length in two lines overexpressing A) *NcNRAMP4,* B) *NcZNT1* and C) *NcNRAMP4/NcZNT1* and the wild-type at different Ni concentrations. -Ni and +Ni refer to 0 and 50 µM of $NiSO_4$. Values are means ± SD (n≥15). Scale bars = 1 cm.

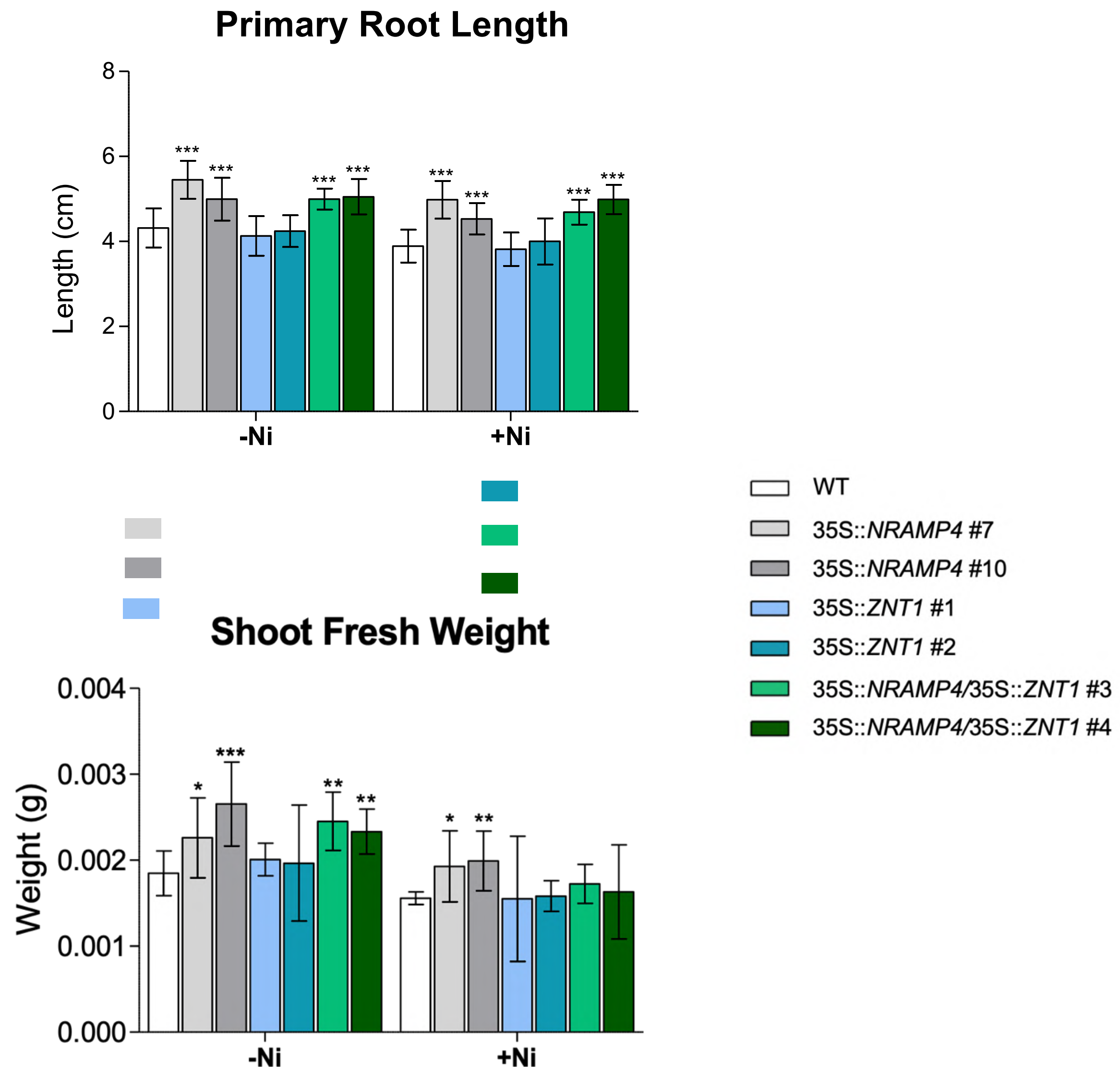

Figure 4.11: Summarized results relative to primary root length and the weight of shoots for plants grown in the absence (-) or presence of excess (+) Ni. Values are means ± SD (n≥15).

4.8 Ni tolerance and accumulation in hydroponic solution

WT and transgenic lines of *A. thaliana* plants overexpressing single 35S::*NRAMP4,* 35S::*ZNT1* and double 35S::*NRAMP4*/35S::*ZNT1* were used to assess the effect of these genes on Ni accumulation and tolerance. The plants were grown in a hydroponic solution containing excess Ni (20 µM) for 2 weeks. Five plants for each genotype were used for this experiment. Their phenotypes and Ni content in shoots were then analyzed.

As shown in **Fig. 4.12,** transgenic lines overexpressing *NcNRAMP4* and double *NcNRAMP4*/*NcZNT1* (line # 3) displayed significantly longer roots and greater shoot biomass than the WT, whereas no differences were observed between WT and the transgenic lines overexpressing *ZNT1*, according to *in vitro* analyses (Section 4.7).

It is possible that the overexpression of *NcZNT1* from *N. caerulescens* MP plants may not affect the growth of transgenic plants in the presence of excess Ni because the concentration of this element in the medium may partially limit Zn availability (Richau and Schat, 2009). Lin and colleagues (2016) found that the overexpression of *ZNT1* from the Zn hyperaccumulator plant *N. caerulescens* LC reduced the shoot biomass of transgenic lines in comparison with the WT under Zn-sufficient conditions, although the opposite result was found under Cd and Zn treatment.

The amount of Ni accumulated into the shoots was also measured by ICP/MS analysis; transgenic lines showed a significant reduction of Ni content in shoots compared to WT plants, except for a single line expressing 35S::*NRAMP4* (#7). These results confirmed that both *NcNRAMP4* and *NcZNT1* can modify Ni accumulation and homeostasis regulation *in planta.*

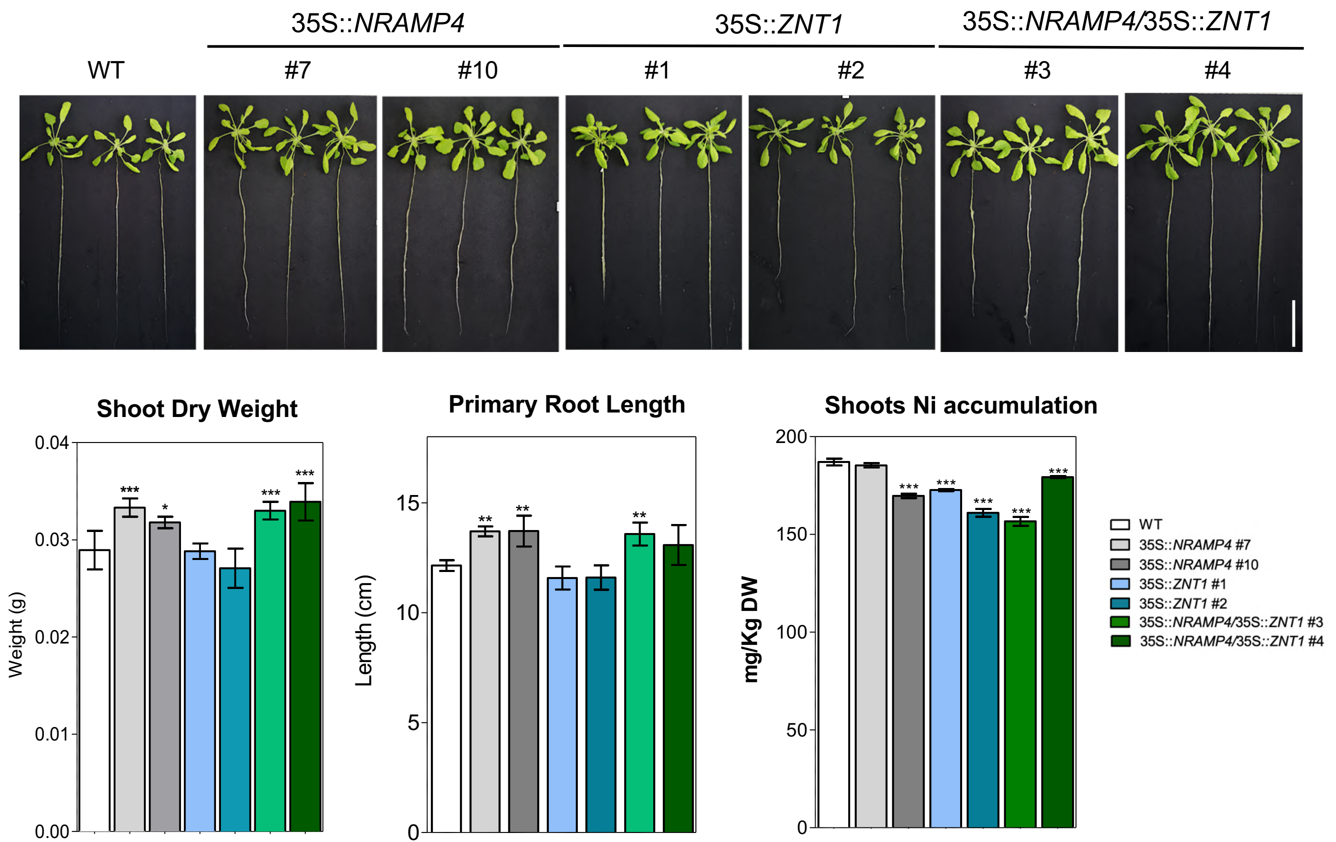

Figure 4.12: *A. thaliana* WT and transgenic lines overexpressing *NcNRAMP4, NcZNT1* and *NcNRAMP4/NcZNT1* in combination grown in the presence of Ni excess for 2 weeks. Primary root length, shoot dry weight and Ni accumulation in shoots were measured for all plants. Values are means ± SD (n=5) and ± SD (n=3) respectively. Scale bars = 3.5 cm.

4.9 Gene expression analysis of *MTP1* in the presence of Ni excess

The vacuolar transporter MTP1, belonging to the cation diffusion facilitator (CDF) family of metal transporters, has been fully characterized in both hyperaccumulator and non-accumulator plants (Ricachenevsky *et al.,* 2013). Although this gene is mainly associated with *in planta* Zn and Cd detoxification (Assunção *et al.*, 2001; van de Mortel *et al.*, 2006; Shahzad *et al.,* 2010), several experiments with mutant yeasts seem to indicate a broader affinity and the possibility to transport Mn, Cd, Co and Ni as well (Montanini *et al.*, 2007). *MTP1* was found to be constitutively highly expressed in the Ni hyperaccumulator plant *N. goesingense* (Gustin *et al.*, 2009), which possesses two spliced versions of the MTP1 gene (Persans *et al.,* 2001) characterized by different metal-binding properties.
It is therefore plausible that MTP1 may be involved in the transport and accumulation of Ni in several accessions of *N. caerulescens,* including ecotype MP. For this reason, we decided to investigate MTP1 in *N. caerulescens* MP, and more specifically, its role in Ni tolerance and accumulation.
The modulation of *MTP1* in the presence of excess Ni was analysed by Real Time RT-PCR on samples isolated from the shoots of *N. caerulescens* (GA and MP) and *T. arvense* plants treated with excess levels of this metal (**Fig. 4.13**). After 1 day of exposure to Ni, the *MTP1* gene was observed to be more abundantly expressed in the two ecotypes of *N. caerulescens,* MP and GA, than *T. arvense* both in the presence and absence of excess Ni (10 μM and 100 μM $NiSO_4$), according to the data available in the literature (Assunção *et al.*, 2001; van de Mortel *et al.*, 2006). In particular, MTP1 was strongly expressed in the Zn/Cd hyperaccumulator *N. caerulescens* GA under all the conditions tested, confirming its role in Zn/Cd detoxification by vacuolar storage (Becher *et al.,* 2004; Dräger *et al.*, 2004). The transcription levels of *MTP1* in both *T. arvense* and *N. caerulescens* GA were higher at 10 μM Ni than at 100 μM, whereas in *N. caerulescens* MP the relative expression of this gene was similar at both concentrations; in fact, *MTP1* expression did not appear to be induced by an excess of Ni.
These data could indicate that *MTP1*, together with the other genes previously found to be overexpressed in the presence of Ni excess (Visioli *et al.*, 2016), could be required in determining the Ni tolerance trait of *N. caerulescens* plants, as also suggested for other accessions of this genus (Persans *et al.,* 2001; Gustin *et al.,* 2009).

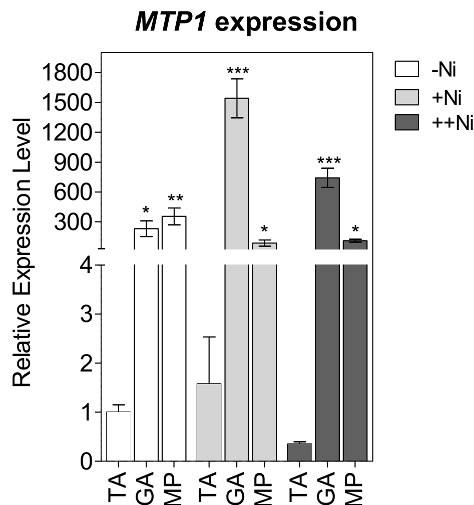

Figure 4.13: Real Time RT-PCR experiment testing the shoots-expression of *MTP1* relatively to the expression of the housekeeping gene *Actin2*. -Ni, +Ni and ++Ni refer to 0, 10 and 100 µM of $NiSO_4$. Statistically significant variations, as resulting from the ANOVA test (n = 3), are marked by asterisks. Bars correspond to standard error, calculated as in Muller *et al.* (2002).

4.10 CDS amplification of *MTP1*

The full-length CDS of *MTP1* from *N. caerulescens* MP and GA was amplified from its cDNA using a pair of specific primers designed on conserved regions of other *N. caerulescens* ecotypes present in databases (Section 3.6). In both ecotypes, two different CDSs of MTP1 of different length, as shown on agarose gel (**Fig. 4.14-4.15**), were identified. These two sequences, termed *MTP1-long* and *MTP1-short,* were also obtained by amplifying the complete CDS from the genomic DNA of both plant species, indicating that they are probably two different alleles. Previously, two spliced versions of the same *MTP1* gene were found in the Ni hyperaccumulator species *N. goesingense* (Persans *et al.,* 2001), which differed for the presence of absence of the His-rich loop and thus also displayed a different metal-binding specificity. Moreover, multiple allelic variants were also identified for NgMTP1 by Kim *et al.* (2004), which were characterized by the insertion or deletion of amino acid residues in the internal part of the gene. In order to compare these two sequences, the amplicons obtained by PCR from the cDNA and gDNA were cloned in a pGEM®-T easy vector and sequenced. The sequences were then aligned using ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2; Larkin *et al.*, 2007) and converted into protein sequences using the Translate tool of the ExPASy database (http://web.expasy.org/translate).
NcMTP1-long and *NcMTP1-short* differ for the presence/absence of the conserved His-loop region (**Fig. S1-S2**)**,** which act as a Zn sensor *in planta,* although it may also affect the metal substrate affinity of MTP1 (Kawachi *et al.,* 2008; Tanaka *et al.,* 2015).

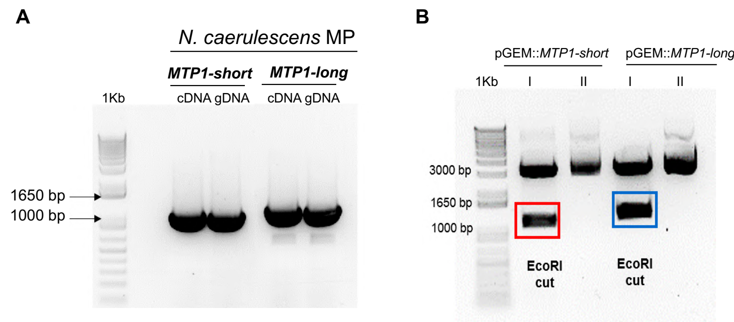

Figure 4.14: A) Amplification of the complete CDS of *NcMTP1-long* and *NcMTP1-short* from cDNA and gDNA of *N. caerulescens* MP. B) Agarose gel electrophoresis of the pGEM::*NcMTP1-short* and pGEM::*NcMTP1-long* cut with EcoRI enzyme.

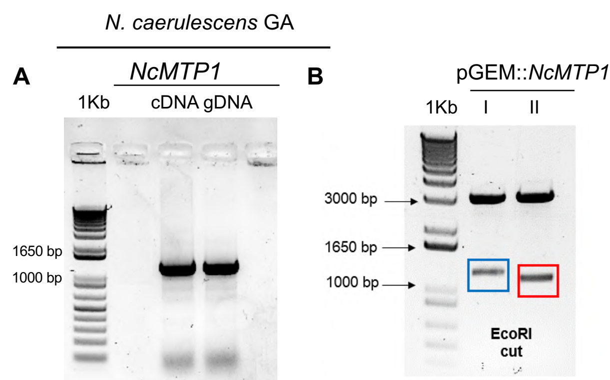

Figure 4.15: A) Amplification of the complete CDS of *NcMTP1-long* and *NcMTP1-short* from cDNA and gDNA of *N. caerulescens* GA. B) Agarose gel electrophoresis of the pGEM::*NcMTP1-long* (I) and pGEM::*NcMTP1-short* (II) cut with EcoRI enzyme.

4.11 NcMTP1-long and NcMTP1-short protein localization in plants

MTP1 is generally recognized as a gene encoding for a tonoplast protein required for the detoxification of metals in both non-accumulating and hyperaccumulator plants (Persans *et al.,* 2001; Gustin *et al.,* 2009), although a plasma membrane localization has also been proposed for *NgMTP1b* (Kim *et al.,* 2004). Since at least two different MTP1 forms were found both in the cDNA and gDNA of *N. caerulescens* ecotypes GA and MP, subcellular localization experiments were performed to evaluate the tissue expression of *MTP1-long* and *MTP1-short.*

The analysis of the subcellular protein localization of NcMTP1-long and NcMTP1-short was performed by producing constructs fusing the complete CDS of *NcMTP1-long* and *NcMTP1-short,* lacking the stop codon, to the *eGFP* reporter gene lacking the start codon. The fusions were then transferred into pMD1, and the final constructs were used for plant transformation.

The subcellular localization of these proteins was evaluated by transient expression in transfected *N. tabacum* protoplasts isolated and purified from leaves. A strong signal for *NcMTP1-long*::eGFP was

detected at the tonoplast, confirming previous data (**Fig. S3**)**,** whereas no clear result emerged for *NcMTP1-short*::eGFP. Co-localization experiments were therefore performed on *N. tabacum* leaves using the same constructs. Various sub-cellular markers were used in combination with the pMD1-*MTP1-long::*eGFP and pMD1-*MTP1-short::*eGFP constructs for the transient expression.

As shown in **Fig. 4.16,** both *NcMTP1-long* and *NcMTP1-short* co-localized clearly only with the tonoplast marker RFP::*SYP51* and no other sub-cellular marker (data not shown), confirming that both *NcMTP1-long* and *NcMTP1-short* are vacuolar transporters.

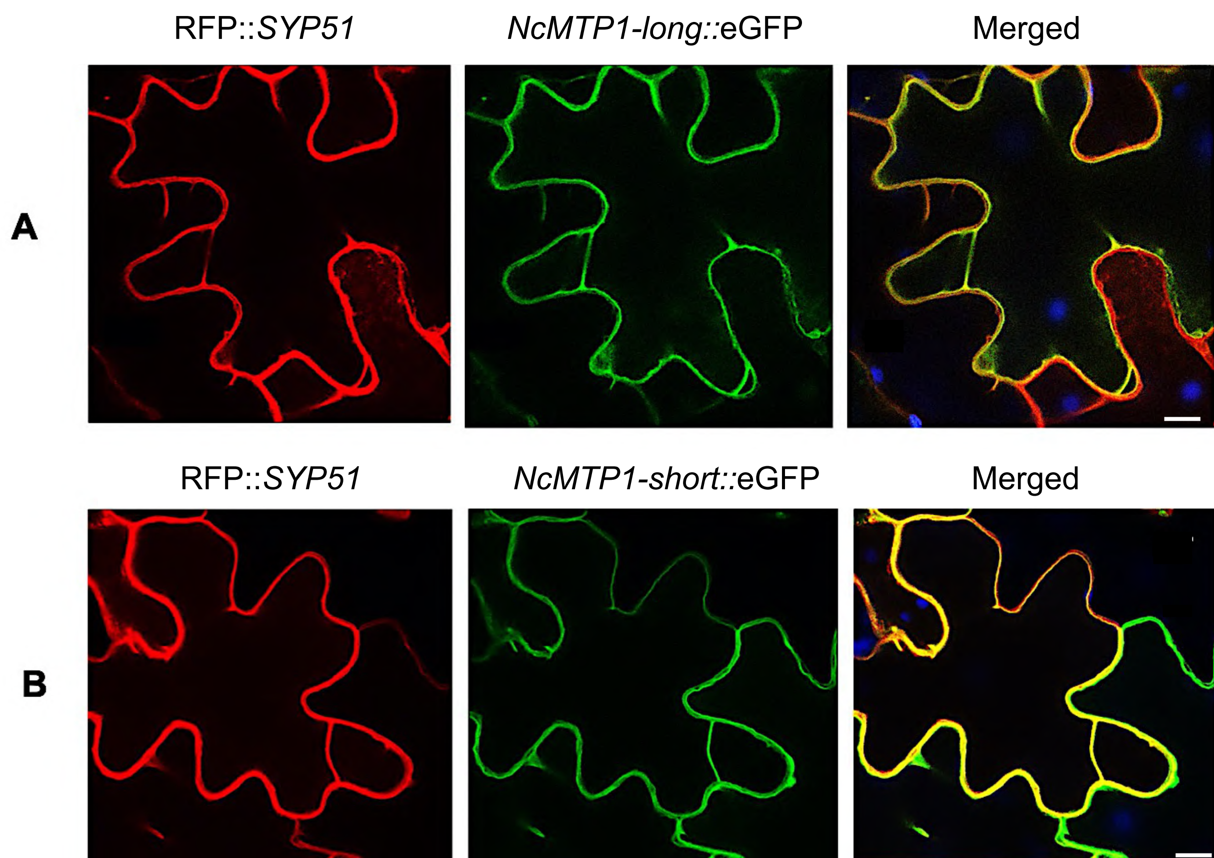

Figure 4.16: Co-localization experiments in *N. tabacum* leaves with *MTP1-long* and *MTP1-short* fused to eGFP reporter gene. A) Tonoplast marker RFP-SYP51 fluorescence signal; B) MTP1-eGFP fluorescence signal; C) merge of RFP-SYP51 and MTP1-eGFP fluorescence signals. Scale bar = 10 μm

4.12 Metal transport analysis of *MTP1* proteins by yeast complementation

In *A. thaliana*, MTP1 is required for the vacuolar detoxification of excess Zn under stressful conditions; no other metal seems to be transported by this protein (Desbrosses-Fonrouge *et al.,* 2005; Ricachenevsky *et al.,* 2013), although a broader metal-binding specificity has been proposed for this gene in other species. In the Ni hyperaccumulator plant *N. goesingense*, for instance, MTP1 could complement Cd and Co-sensitivity in yeast, as well as Zn (Persans *et al.,* 2001; Kim *et al.,* 2004).

Moreover, the short NgMTP1t2, lacking the highly conserved His-loop region, seems to differ from NgMTP1t1 in its affinity for metals, Ni in particular (Persans *et al.,* 2001).

The metal-binding properties of NcMTP1-long and NcMTP1-short of the two *N. caerulescens* ecotypes, MP and GA, was investigated by heterologous expression in mutant yeast. The CDS of AtMTP1, known as a Zn transporter (Desbrosses-Fonrouge *et al.,* 2005), was also considered for this set of experiments. Two strains of *S. cerevisiae* were used for the tests: the double mutant *zrc1cot1*, hypersensitive to Zn and Co (Kawachi *et al.,* 2012), and the WT parental strain DY1457.

The strategy used to generate pADSL-MTP1 constructs of all the plant species is showed in **Fig. 4.17.** The amplification of the *NcMTP1-long* and *NcMTP1-short* CDS of *N. caerulescens* MP and GA, as well as that of *AtMTP1*, was performed by high fidelity PCR on the cDNA of the plants. The amplicons were cloned in the pGEM®-T easy vector and sequenced. All the sequences were then digested with *SpeI* and *SalI* restriction enzymes and ligated under the control of the ADH promoter in the final expression vector pADSL prepared previously. pADSL::*MTP1* constructs were used to transform *S. cerevisiae* WT and *zcr1cot1* mutant strains using the lithium acetate/single-stranded carrier DNA/polyethylene glycol method (Agatep *et al.*, 1998). Selective recombinant yeasts selected in SD-trp solid medium were used in all the experiments.

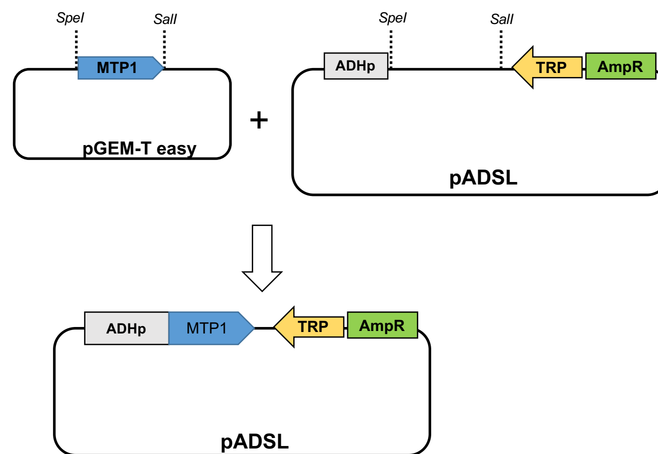

Figure 4.17: Preparation of pADSL::*MTP1* constructs for yeast complementation assay. Trp is the selective marker necessary to select recombinant yeasts in selective SD-trp medium.

4.12.1 Zinc resistance assays

To confirm that the His-loop region is not essential for Zn-transporting activity (Tanaka *et al.,* 2015), recombinant *zrc1cot1* mutant yeasts expressing *NcMTP1-long, NcMTP1-short* and *AtMTP1* were grown for 3 days in a selective medium (SD-trp) in the presence of excess (500 μM) Zn, after which spot assays and liquid culture assays were performed. AtMTP1 was used as a control, since its role in Zn transport (Desbrosses-Fonrouge *et al.*, 2005).

Both NcMTP1-long and NcMTP1-short proteins were able to complement the *zrc1cot1* defective phenotype, displaying no significant difference with AtMTP1 (**Fig. 4.18**). Interestingly, *NcMTP1-short* from *N. caerulescens* MP displayed a lower affinity for Zn than *NcMTP1-long,* probably due to the absence of specific amino acid residues which have been recently proposed to be required for high-affinity Zn transport (Wang *et al.,* 2018). In general, these data confirm that the Zn-transporting activity mediated by MTP1 does not depend on the presence of the His-loop region, which is principally required for Zn homeostasis in the cell (Tanaka *et al.,* 2015), and that both MTP1 proteins are functional transporters.

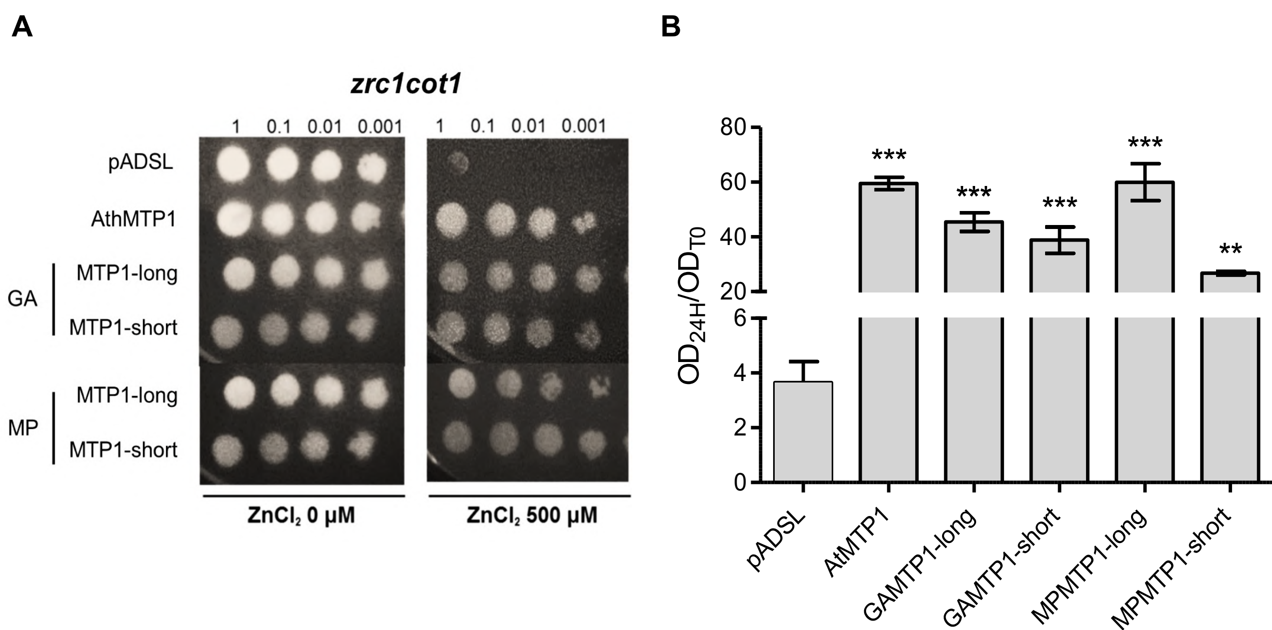

Figure 4.18: A) Double mutants *zrc1cot1* transformed with pADSL and pADSL harbouring *MTP1-long* and *MTP1-short* of *N. caerulescens* grown for 3 days at 28°C in the presence of Zn excess. Serial dilutions were spotted on modified SD-trp selective medium adding 500 µM of $ZnCl_2$. Spots were made with 10 µl of yeast culture diluted at OD_{600} nm indicated in the figure. B) Liquid culture assay performed on *S. cerevisiae zrc1cot1* adding 500 µM $ZnCl_2$. Values are means ± SD (n=3).

4.12.2 Nickel resistance assays

In addition to Zn, the putative Ni-transporting ability of *MTP1-long* and *MTP1-short* was investigated by liquid culture assays. For this set of experiments, *S. cerevisiae* WT and *zrc1cot1* mutants expressing *NcMTP1-long* and *NcMTP1-short* were used. A pre-culture of recombinant yeasts was diluted 1:100 and then transferred into a selective SD-trp medium modified by adding excess Ni (400 µM). OD_{600} nm was measured several times to determine the survival rate under these stressful conditions.

The expression of AtMTP1 and NcMTP1-long of *N. caerulescens* ecotype MP and GA reduced the survival of recombinant DY1457 yeast in comparison to the pADSL vector, although no significant differences were detected considering MTP1-short of both ecotypes (**Fig. 4.19 A**). This result can be explained

considering that Ni is known to compete with other micronutrients, Zn in particular (Assunção *et al.,* 2008; Richau and Schat, 2009), and that NcMTP1-long and NcMTP1-short may differ in their ability to transport Ni, as also suggested by other authors (Persans *et al.,* 2001). High concentrations of Ni in the growth medium may therefore perturb Zn homeostasis in WT yeast, although this organism possesses all the transporters needed to modulate metal homeostasis, including *zrc1* (Kawachi *et al.*, 2012). The same analysis was also performed with the double mutant *zrc1cot1*. As shown in **Fig. 4.19 B**, no significant differences were obtained considering all the MTP1-long proteins of *N. caerulescens* and *A. thaliana* in comparison with the empty vector, whereas the NcMTP1-short of MP limited significatively the growth of recombinant yeast, probably due to an altered metal-binding specificity brought about by the absence of the His-loop region in this protein (Persans *et al.,* 2001; Kawachi *et al.,* 2008).
These results would suggest that in *N. caerulescens* MP MTP1 could not be directly involved in the transport and overaccumulation of Ni, which probably depends on the activity of other low-affinity transporter proteins (see van der Pas *et al.,* 2019).

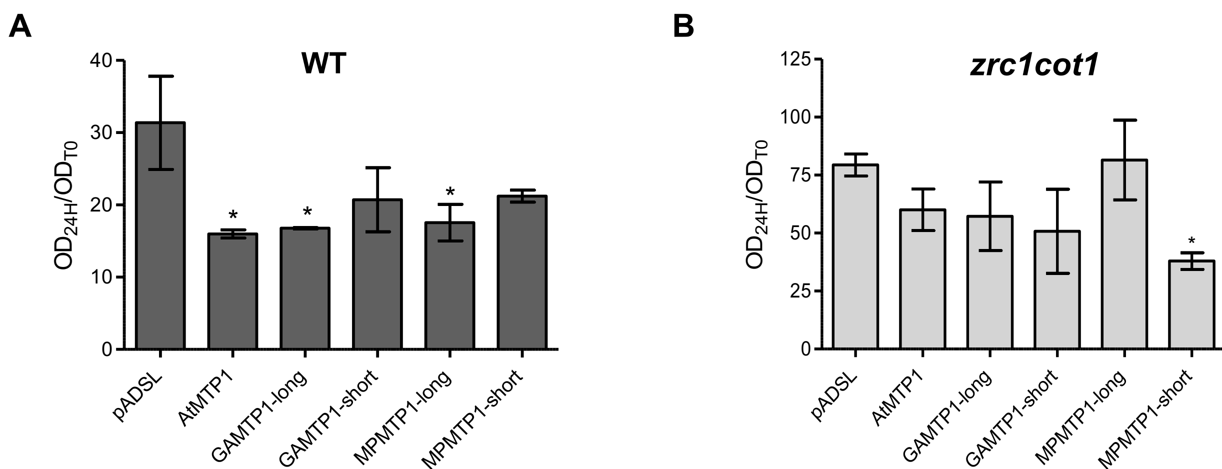

Figure 4.19: Liquid culture assay performed on A) *S. cerevisiae* WT (DY1457) and B) *zrc1cot1* double mutant adding 400 μM $NiSO_4$ in the medium. Empty vector was used for control, and AtMTP1 was included as negative control. Values are means ± SD (n=3).

4.12.3 Cobalt resistance assay

Since *N. caerulescens* MP has adapted to Ni-rich and Co-rich soils (Visioli *et al.,* 2014), it is plausible that *MTP1-long* and *MTP1-short* possess a different Co-binding specificity. Previous research works have revealed that unlike *NgMTP1t2-short*, which seems to prefer Ni*, NgMTP1t1-long* is able to transport Zn, Co and also Cd (Persans *et al.,* 2001); moreover, the removal of the His-loop from AtMTP1 has been observed to partially change its affinity, and increase Zn and Co transport in recombinant yeast (Kawachi *et al.,* 2008), as also found with a modified version of *Triticum urartu TuMTP1* (Wang *et al.,* 2018).

For this reason, the possible involvement of NcMTP1 in Co transport was investigated by liquid culture assays using the double mutant *zrc1cot1* defective strains of *S. cerevisiae.*

As shown in **Fig. 4.20**, all the MTP1 proteins were able to complement the defective phenotype of the *zrc1cot1* double mutant, although *MTP1-short* of *N. caerulescens* MP failed to display significant differences with the empty vector. This may be due to i) the presence/absence of amino acid residues affecting its metal-binding specificity (Wang *et al.*, 2018) and/or ii) increasing Co transport into the cytosol can reduced the binding selectivity for other essential nutrients, leading to a condition of nutrient deficiency, as also suggested by literature (Assunção *et al.,* 2001; Nishida *et al.,* 2012; Deng *et al.,* 2019).

From these data it is possible to assume that MTP1-long and MTP1-short of *N. caerulescens* MP and GA can regulate Co transport in a different manner, also considering the habitat to which these two ecotypes are native.

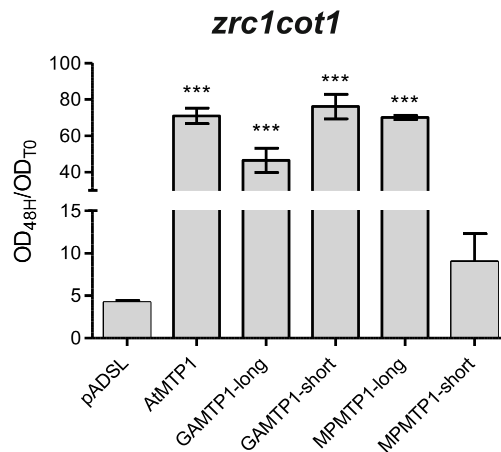

Figure 4.20: Liquid culture assay performed on *S. cerevisiae zrc1cot1* double mutant adding 600 µM $Co(NO_3)_2$ in the medium. The empty vector was used for control, and AtMTP1 was included as negative control. Values are means ± SD (n=3). Scale bars = 1 cm.

4.13 Transformation of *A. thaliana mtp1+/+* mutants with 35S::*MTP1* constructs

Yeast complementation assays confirmed the different metal-binding properties of *NcMTP1-long* and *NcMTP1-short* of *N. caerulescens* MP (Section 4.12). Therefore, to clarify the role of both transporters *in planta,* the complete CDS of these genes was amplified from the cDNA of *N. caerulescens* MP using specific primers (Section 3.6) and cloned under the control of the strong promoter CaMV35S. We also included AtMTP1 as a control, given its role in Zn transport in *Arabidopsis* (Desbrosses-Fonrouge *et al.,* 2005). The pH2GW7*::NcMTP1-long,* pH2GW7::*NcMTP1-short* and pH2GW7*::AtMTP1* constructs were separately introduced into *A. thaliana mtp1*+/+ homozygous mutants by floral-dip transformation.

Analysis of metal tolerance and accumulation in transgenic lines is hence scheduled to better clarify the role of both *NcMTP1-long* and *NcMTP1-short* in *N. caerulescens* plants.

5. CONCLUSIONS

This research work focuses on *N. caerulescens* ecotype Monte Prinzera (MP, Italy), native to a serpentine soil in Italy, characterized by its ability to accumulate and tolerate high concentrations of Ni, as well as Zn (Assunção *et al.,* 2003; Richau and Schat, 2009; Visioli *et al.,* 2012). We decided to investigate the role of the vacuolar transporters *NRAMP4* and *MTP1* and the plasma membrane transporter *ZNT1*, given their putative role in the regulation of Ni homeostasis in various Ni hyperaccumulator species of the *Noccaea* genus (Persans *et al.,* 2001; Kim *et al.,* 2004; Mizuno *et al.,* 2005; Taylor and Macnair, 2006).
Under Ni excess, the expression of *NRAMP4* was significantly greater in the shoots of *N. caerulescens* GA and MP than in *T. arvense,* i.e. the control, which displayed no significant modulation in gene expression, consistently with previous data (Visioli *et al.,* 2014). Interestingly, constitutive high levels of *NRAMP4* expression were found in *N. caerulescens* MP, under both standard and excess Ni conditions, consistently with the data observed by Weber *et al.* (2004), van de Mortel *et al.* (2006) and Visioli *et al.* (2014); regarding *ZNT1, N. caerulescens* GA and MP displayed a positive modulation of its expression when Ni was present at excess concentrations, confirming the previous observations regarding the major role of ZNT1 in Zn and Cd transport *in planta* (Pence *et al.,* 2000; Assunção *et al.,* 2001; van de Mortel *et al.,* 2006). These data therefore suggest the possible involvement of these two proteins in response to excess Ni *in planta,* as indicated (Visioli *et al.,* 2014). The Ni-binding properties of *NcNRAMP4* and *NcZNT1* were investigated by complementation assays using recombinant WT strain of *S. cerevisiae* expressing both transporters. In comparison with the empty vector, *NcNRAMP4* significantly reduced the survival of recombinant yeast after Ni treatment and the opposite result was found for *NcZNT1*. These data are in keeping with what observed by Mizuno *et al.* (2005), strongly suggesting a direct role in Ni transport of both NRAMP4 and ZNT1 in Ni hyperaccumulator and hypertolerant plants. However, the effect caused by the expression of these genes in yeast could also be correlate to a competition between Ni and other micronutrients, as previously demonstrated (Richau *et al.,* 2009; see van der Pas *et al.,* 2019). In order to determine their effect on Ni tolerance and accumulation *in planta,* single 35S::*NRAMP4* and 35S::*ZNT1* and double 35S::*NRAMP4*/35S::*ZNT1 A. thaliana* transgenic lines were obtained and employed for a variety of assays. A phenotypic characterization of these plants showed that the lines overexpressing single 35S::*NRAMP4,* 35S::*ZNT1* and double 35S::*NRAMP4*/35S::*ZNT1* are characterized by a significant increase in their shoot biomass as compared to WT plants, although the greatest impact on growth was brought about by *NRAMP4*. *In vitro* analyses were thus performed to better elucidate the role of these genes in Ni tolerance. Single 35S::*NRAMP4* and double 35S::*NRAMP4*/35S::*ZNT1* lines displayed longer roots than *A. thaliana* WT under all the conditions tested, but no significant differences in the phenotype were observed in the lines expressing *NcZNT1*.

It is possible that the differences in phenotype can be associated to an alteration of the general metal homeostasis regulation linked to the overexpression of *NcNRAMP4* and *NcZNT1* genes rather than a specific response to Ni in the medium.

On soil, all transgenic lines displayed a clearly greater tolerance to Ni excess and much lower levels of this metal in their shoots than the WT, which also displayed evident signs of chlorosis. Fe and Zn accumulation in shoots was also measured, to clarify whether their homeostasis was altered, as previously reported in *Noccaea* species (Richau *et al.,* 2009; see van der Pas *et al.,* 2019). Single 35S::*NRAMP4* and 35S::*ZNT1* lines displayed lower levels of Fe than the WT, although the opposite result has been established in the double 35S::*NRAMP4*/35S::*ZNT1* lines, suggesting that high concentrations of Ni in the soil may affect Fe homeostasis *in planta* (see van der Pas *et al.,* 2019). Finally, the expression of *NcNRAMP4* and *NcZNT1* significatively reduced the Ni-shoot content in transgenic lines when grown in hydroponic solution, strongly suggesting an involvement of these genes in Ni tolerance and accumulation in *N. caerulescens* MP.

In addition to *NcNRAMP4* and *NcZNT1,* this work also focused on *MTP1*, a well-described vacuolar transporter belonging to the cation diffusion facilitator (CDF) family of metal transporters.

At first, an expression analysis on *MTP1* in response to excess Ni was performed on the shoots of *N. caerulescens* (GA and MP) and *T. arvense* plants exposed for 1 day to high levels of this metal. Both hyperaccumulator ecotypes of *Noccaea* constitutively expressed this gene*,* consistently with previous investigations (Assunção *et al.*, 2001; van de Mortel *et al.*, 2006). In particular, *N. caerulescens* GA displayed the highest levels of *MTP1* expression, confirming the major role of this protein in the detoxification of Zn and Cd (Becher *et al.,* 2004; Dräger *et al.,* 2004; Talke *et al.,* 2006; Shahzad *et al.,* 2010). Interestingly, MTP1 seems to have a different response in these two ecotypes of *Noccaea*: *N. caerulescens* MP displayed high levels of MTP1 expression under all the conditions tested, whereas in *N. caerulescens* GA MTP1 was strongly induced by the presence of excess Ni in the medium. These data probably reflect differences in the mechanism regulating the expression of MTP1 in these two ecotypes, which are characterized by a different affinity for metals. Moreover, MTP1 may also contribute to the Ni tolerance trait in *N. caerulescens*, as proposed for other accession of this genus (Persans *et al.,* 2001; Gustin *et al.,* 2009). Two different coding sequences of MTP1, characterized by different length, were found in the cDNA and gDNA of both ecotypes of *N. caerulescens*, MP and GA. Subcellular localization experiments have shown that both MTP1-short and MTP1-long are localized in the vacuolar compartment, confirming previous studies (Persans *et al.,* 2001; Kim *et al.,* 2004) and suggesting that MTP1-short is also involved in metal detoxification by vacuolar compartmentalization. The metal transport properties of NcMTP1-long and NcMTP1-short were also investigated by heterologous expression in mutant yeast (WT and *zrc1cot1* double mutants). Zn, Ni and Co were considered because they may compete for the same transporters *in planta* (Kawachi *et al.,* 2008; Richau and Schat, 2009; Tanaka *et al.,* 2015).

All the MTP1 proteins rescued the defective phenotype of the double mutant *zrc1cot1* under excess Zn conditions, with no significant differences between MTP1-long and MTP1-short of both ecotypes, confirming that the His-loop is not essential for Zn transport activity (Kawachi *et al.,* 2012; Tanaka *et al.,* 2015). As regards Ni tolerance, a significant reduction in yeast growth was found in the recombinant *S. cerevisiae* WT yeast expressing the AtMTP1 and NcMTP1-long of both MP and GA ecotypes, and only MTP1-short of *N. caerulescens* MP reduced the growth of recombinant *zrc1cot1*. These data seem to indicate that MTP1 is probably not directly involved in Ni transport, and that the defective phenotype could be the result of an altered nutrient homeostasis regulation in recombinant yeast (van der Pas *et al.,* 2019), although MTP1-long and MTP1-short displayed differences in their ability to transport Ni.
Finally, Co was considered as metal of interest for the reason that this micronutrient is present at high concentrations in the native soil of the MP ecotype of *N. caerulescens* and it is known to strongly compete with Ni (Nishida *et al.,* 2012; Wang *et al.,* 2018; Deng *et al.,* 2019). Interestingly, only the MTP1-short of *N. caerulescens* MP failed to rescue the defective phenotype of the double *zrc1cot1* upon exposure to Co, while all the other MTP1 proteins significantly increased the survival of yeast. This result suggests that some residues that are absent in MTP1-short may be linked to the ability of the protein to transport Co, as also proposed by Wang *et al.* (2018). In general, MTP1-short and MTP1-long displayed many differences regarding Zn, Ni and Co transport in MP and GA ecotypes of *N. caerulescens,* although their precise role *in planta* remains to be clarified.

In summary, all these data strongly suggest the possible involvement of the vacuolar transporter NRAMP4 in Ni tolerance and accumulation in *N. caerulescens* MP, whereas ZNT1 and MTP1 seem to have a minor role in this complex biological process, thus requiring further analyses in order to confirm these assumptions.

6. SUPPLEMENTARY MATERIAL

Figure S1: Protein alignment of AtMTP1, NcMTP1-long and NcMTP1-short of *N. caerulescens* MP and GA ecotype. Black highlighting indicates nucleotide identity in all the sequences; gray highlighting indicates identity in most of the sequences considered; red box indicates the conserved His-loop region.

```
AtMTP1              1 MESSSPHHSHIVEVNVGKSDEERIIVASKVCGEAPCGFSDSKNASGDAHERSASMRKLCI
NcMTP1-long_MP      1 MESSS----HIIEVNGGRSDEERRVVASKVCGEAPCGFSDAKNVSGDAKERNASMRKLCI
NcMTP1-short_MP     1 MESSS----HIIEVNGGRSDEERRVVASKVCGEAPCGFSDAKNVSGDAKERNASMRKLCI
NcMTP1-long_GA      1 MESSS----HIIEVNGGRSDEERRVVASKVCGEAPCGFSDAKNVSGDAKERNASMRKLCI
NcMTP1-short_GA     1 MESSS----HIIEVNGGRSDEERRVVASKVCGEAPCGFSDAKNVSGDAKERNASMRKLCI
                      *****    ** *** * *****  *************** ** **** ** ********

AtMTP1             61 AVVLCLVFMSVEVVGGIKANSLAILTDAAHLLSDVAAFAISLFSLWAAGWEATPRQTYGF
NcMTP1-long_MP     57 AVVLCLVFMSVEIVGGIKANSLAIMTDAAHLLSDVAAFAISLFALWAAGWEATPRQTYGF
NcMTP1-short_MP    57 AVVLCLVFMSVEIVGGIKANSLAIMTDAAHLLSDVAAFAISLFALWAAGWEATPRQTYGF
NcMTP1-long_GA     57 AVVLCLVFMSVEIVGGIKANSLAIMTDAAHLLSDVAAFAISLFALWAAGWEATPRQTYGF
NcMTP1-short_GA    57 AVVLCLVFMSVEIVGGIKANSLAIMTDAAHLLSDVAAFAISLFALWAAGWEATPRQTYGF
                      ************ *********** ****************** ****************

AtMTP1            121 FRIEILGALVSIQLIWLLTGILVYEAIIRIVTETSEVNGFLMFLVAAFGLVVNIIMAVLL
NcMTP1-long_MP    117 FRIEILGALVSIQLIWLLTGILVYEAILRLLTETSEVNGFLMFAVATFGLLVNIIMAVML
NcMTP1-short_MP   117 FRIEILGALVSIQLIWLLTGILVYEAILRLLTETSEVNGFLMFAVATFGLLVNIIMAVML
NcMTP1-long_GA    117 FRIEILGALVSIQLIWLLTGILVYEAILRLLTETSEVNGFLMFAVATFGLLVNIIMAVML
NcMTP1-short_GA   117 FRIEILGALVSIQLIWLLTGILVYEAILRLLTETSEVNGFLMFAVATFGLLVNIIMAVML
                      *************************** *  ************ ** *** ******* *

AtMTP1            181 GHDHGHSHGHGHGHGHDHHNHSHGVTVTTH----HHHHDHEHGHSHGHGEDK-HHAHGDV
NcMTP1-long_MP    177 GHDHGHSHGHG----HDHGNHSHGVTVTTHDHDPTHDHDHDDGHGHSHGEDNQDEAHGDV
NcMTP1-short_MP   177 GHDH------------------------------------DDGHGHSHGEDNQDEAHGDV
NcMTP1-long_GA    177 GHDHGHSHGHG----HDHGNHSHG-----EDHDPTHDHDHDHDHDDGHGEDNHDEAHGDV
NcMTP1-short_GA   177 GHDH----------------------------------------DDGHGEDNHDEAHGDV
                      ****                                           ****    *****

AtMTP1            236 TEQLLDKSKTQVAAKEKRKRNINLQGAYLHVLGDSIQSVGVMIGGAIIWYNPEWKIVDLI
NcMTP1-long_MP    233 TEQLLEKPKQ---EKEKKKRNINLQGAYLHVLGDSIQSVGVMIGGAAIWYNPKWKIIDLI
NcMTP1-short_MP   201 TEQLLEKPKQ---EKEKKKRNINSQGAYLHVLGDSIQSVGVMIGGAAIWYNPKWKIIDLI
NcMTP1-long_GA    228 TEQLLEKPKQ---EKEKKKRNINLQGAYLHVLGDSIQSVGVMIGGAAIWYNPKWKIIDLI
NcMTP1-short_GA   197 TEQLLEKPKQ---EKEKKKRNINLQGAYLHVLGDSIQSVGVMIGGAAIWYNPKWKIIDLI
                      ***** * *     *** ***** ********************** ***** *** ***

AtMTP1            296 CTLAFSVIVLGTTINMIRNILEVLMESTPREIDATKLEKGLLEMEEVVAVHELHIWAITV
NcMTP1-long_MP    290 CTLAFSVIVLGTTINMIRSILEVLMESTPREIDATKLEKGLLEMEEVVAVHELHIWAITV
NcMTP1-short_MP   258 CTLAFSVIVLGTTINMIRNILEVLMESTPREIDATKLEKGLLEMEEVVAVHELHIWAITV
NcMTP1-long_GA    285 CTLAFSVIVLGTTINMIRNILEVLMESTPREIDATKLEKGLLEMEEVVAVHELHIWAITV
NcMTP1-short_GA   254 CTLAFSVIVLGTTINMIRNILEVLMESTPREIDATKLEKGLLEMEEVVAVHELHIWAITV
                      ****************** *****************************************

AtMTP1            356 GKVLLACHVNIRPEADADMVLNKVIDYIRREYNISHVTIQIER
NcMTP1-long_MP    350 GKVLLACHVNVTPQADADMVLNKVVDYIRREYKISHVTVQIER
NcMTP1-short_MP   318 GKVLLACHVNVTPQADADMVLNKVVDYIRREYNIVHVTVQIER
NcMTP1-long_GA    345 GKVLLACHVNVTPQADADMVLNKVVDYIRREYNIVHVTVQIER
NcMTP1-short_GA   314 GKVLLACHVNVTPQADADMVLNKVVDYIRREYNIVHVTVQIER
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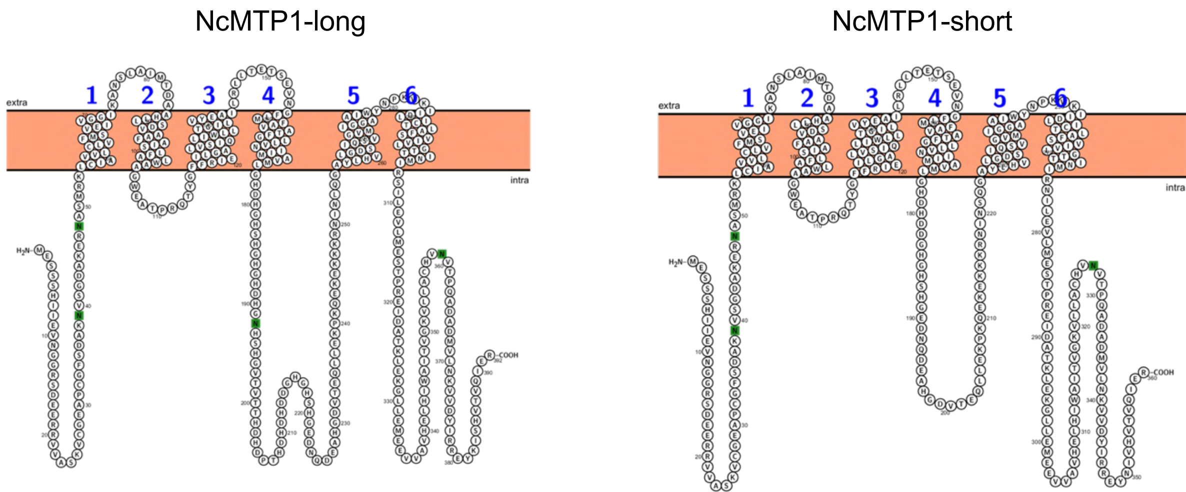

Figure S2: Secondary structure of NcMTP1-long and NcMTP1-short obtained using the Protter tool: an interactive protein feature visualization and integration with experimental proteomic data (Omasits *et al.*, 2014).

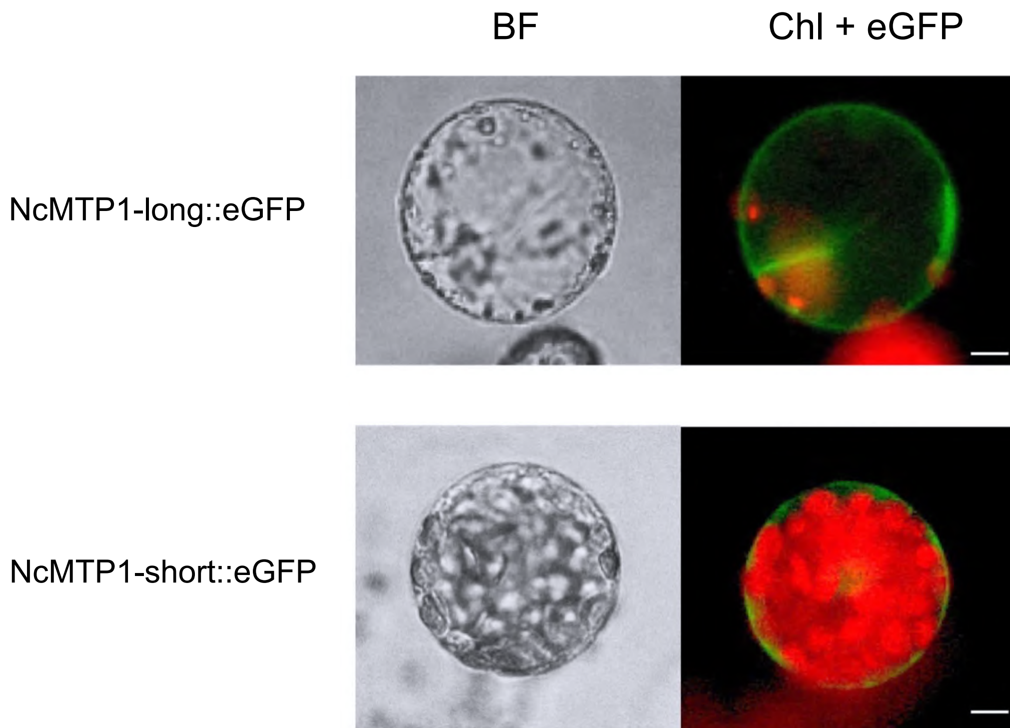

Figure S3: Preliminary data on the subcellular localization of NcMTP1-long::eGFP and NcMTP1-short::eGFP proteins transiently expressed in *N. tabacum* protoplasts. From left to right: bright-field image and a combined image of eGFP and chlorophyll fluorescence. Legend: BF (Bright Field), Chl + GFP (merged signal of chlorophyll and eGFP). Scale bars = 10 µm.

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Identification of miRNAs involved in response to Zn excess in *Arabidopsis* species

1. INTRODUCTION

1. Non-coding RNAs in plants

Since the middle of the last century, the role of non-coding RNAs (ncRNAs) in various organisms has been studied (see Managadze *et al.,* 2011; Palazzo *et al.,* 2015). Different classes of ncRNAs have been defined based on their biogenesis pathways and length of the mature sequence (Axtell, 2013): in general, the most important categories of ncRNAs are small ncRNAs (sRNAs), which include small interfering RNAs (siRNAs) and microRNAs (miRNAs), Piwi-interacting RNAs (piRNAs) (Borges and Martiensenn, 2015; Brant *et al.,* 2018), ribosomal RNAs (rRNAs) and transfer RNAs (tRNAs), although new emerging classes of secondary sRNAs have been recently identified (Axtell, 2013; Brant and Budak, 2018). In plants, sRNAs and other ncRNAs are responsible for the modulation of gene expression by RNAi mechanism, acting at the transcriptional and post-transcriptional level, thus representing essential components of gene regulatory networks (Majumdar *et al.,* 2017; Brant and Budak, 2018; Yu *et al.,* 2019). The class of siRNAs can also be divided into 3 different subcategories: heterochromatic siRNAs (hc-siRNA), gradual secondary siRNAs (phasiRNA) and epigenetically activated siRNAs (easiRNA; Wang *et al.,* 2019). A more extended classification of these molecules was described by Axtell (2013). Some recent works in plants and animals have also focused the attention on the important roles of long non-coding RNAs (lncRNAs; >200 nt in length) in the development and the adaptation to environmental conditions (Wang *et al.,* 2017; Singh *et al.,* 2018; Calixto *et al.,* 2019), although much of information is still missing. Interestingly, siRNAs and lncRNAs are both involved in the RNA-directed DNA methylation (RdDM) process and gene silencing, a very complex epigenetic pathway in plants, implicated in many biological processes, including response to stresses (Matzke and Mosher, 2014).

The emerging regulatory features of ncRNAs in plant development and response to many abiotic and biotic stressful conditions have been widely studied in animals and plants in last years, due to advancement in the field of biotechnology and bioinformatics (Adams *et al.,* 2018; Bant *et al.,* 2018; Wu *et al.,* 2019). It is important to note that miRNAs and siRNAs both represent fundamental small RNA regulatory molecules which share common properties in plants, although many differences between these two categories must be taken into consideration (**Table 1**; Kamthan *et al.,* 2015; Zhu *et al.,* 2016; Morgado and Johannes, 2017). In particular, these RNAs are similar in size (20–24 nt), although the pathway of biogenesis, the precursor structures and modes of action are different (Axtell, 2013; Kamthan *et al.,* 2015). Many enzymes take part in these processes, including the class of Dicer-like nucleases (DCLs) enzymes, which are fundamental for the biogenesis of small RNAs in plants (Henderson *et al.,* 2006; Yu *et al.,* 2017). The roles of these proteins have been well described in *Arabidopsis*: miRNAs are produced by the activity of DCL1, whereas DCL3 is involved in the biogenesis of 24-nt heterochromatic siRNAs (hcsiRNAs; Xie

et al., 2005) and DCL4 and DCL2 are required for the processing of dsRNAs to generate 20- to 22-nt long short interfering RNAs (see Kamthan *et al.,* 2015). To perform their functions, both miRNAs and siRNAs are loaded into the RNA-induced silencing complex (RISC) by the activity of Argonaute (AGO) proteins (Brant and Budak, 2018). sRNAs can regulate the expression of their target/s at transcriptional and post-transcriptional level principally by mRNA cleavage, inhibiting translation (Brodersen *et al.,* 2008) and other mechanisms which have been described in more detail in recent research works (Rogers and Chen, 2013; Tamiru *et al.,* 2018).

Properties	miRNA	siRNA
Origin	Distinct genomic loci. Encoded by their own genes	Encoded by transposons, viruses, heterochromatin
Biogenesis (nature of precursor)	Single RNA molecules that include an imperfect stem–loop secondary structure	Long bimolecular RNA duplexes or extended hairpins
Evolutionary conservation	Nearly always conserved in related organisms	Rarely conserved in related organisms
Nature of regulatory target	Regulate different genes	Mediate the silencing of the same (or very similar) genes from which they originate

Table 1: Comparison of miRNAs and siRNAs in plants (Kamthan *et al.,* 2015)

2. MicroRNAs

MicroRNAs (miRNAs) are small non-coding RNAs, 20-24 nucleotides in length (Jones-Rhoades *et al.,* 2006; Axtell *et al.,* 2011), estimated to represent 1% of the expressed genome in complex organisms (Sunkar *et al.,* 2004). A hundred to several hundreds of *MIRNA* (*MIR*) genes have been identified in plant genomes, belonging to different families (Budak and Akpinar, 2015). In particular, miRNAs can be classified as "intergenic" or "intronic", depending on their location in the genome. miRNAs are evolutionarily ancient and many miRNA:target–gene interactions are broadly conserved (Floyd and Bowman, 2004). Present in both plants and animals, these regulatory molecules are also found in prokaryotes, such as *Escherichia coli* (Altuvia *et al.,* 2004), which contains more than 50 sRNAs, and metazoan (Bartel, 2018), and are believed to have played an essential role in the evolution and the appearance of multicellular organisms. The genome of *Arabidopsis* contains over 100 miRNA-encoding loci (Bonnet *et al*., 2004; Jones-Rhoades and Bartel, 2004; Sunkar *et al.,* 2004), with an estimated 250-300 miRNA-encoding genes. Twenty miRNAs have been reported to be present only in *A. thaliana* (Bartel and Bartel, 2003) representing more than 40 genes.

2.1 Genome organization of *MIR* genes in plants

Although the role and biogenesis of miRNAs resulted to be similar between plants and animals, many aspects regarding the regulation pattern and genome organization of miRNAs are substantially different

(Bologna *et al.,* 2013; Li *et al.,* 2018). Genes encoding miRNAs in plants (called *MIR* genes) are generally found in intergenic areas of the genome, but many of these are also found in sense or antisense orientation (Brown *et al.,* 2008; Tchatchou *et al.*, 2017). Unlike metazoan, most plant *MIR* genes are intergenic and rarely tend to be arranged in tandem (Voinnet, 2009). *MIR402* is the only recognized gene within an intron in *Arabidopsis*, although 25% of human miRNA genes are encoded within introns, suggesting a different genome organization between plants and animals (Li *et al.,* 2018; Zhao *et al.,* 2018).

2.2 Biogenesis of plant miRNAs

The biogenesis and maturation of plant miRNAs is a complex mechanism involving several steps and many enzymes (Bartel *et al.,* 2004; Rogers and Chen, 2013; Ackhar *et al.,* 2016; **Fig. 1**). Plant *MIR* genes are transcribed by RNA polymerase II (RNA Pol II) into long primary microRNAs, called pri-miRNAs, ranging from hundreds to thousands of nucleotides in length (Bartel *et al.,* 2004). One of the main differences in the biogenesis of miRNAs between animal and plant kingdoms is that in animals pri-miRNAs are smaller and more similar to each other than in plants (Waterhouse *et al.,* 2005; Li *et al.,* 2018). RNA pol II is responsible for the transcription of *MIR* genes, although plants have evolved other RNA polymerases with a role in siRNA biogenesis (Axtell *et al.,* 2013; Kamthan *et al.,* 2015). Once formed in the nucleus, primary miRNAs (pri-miRNAs) are encoded in capped and polyadenylated RNAs (Voinnet, 2009; Xie *et al.,* 2015) and then converted by endonuclease RNase III DICER-LIKE 1 (DCL1) into stem-loop structures. *A. thaliana* contains 4 DCL (Dicer-like) proteins with distinct functions in small RNA biogenesis (Yu *et al.,* 2017). In animals, this step of pri-miRNA maturation consists of i) cleavage by Drosha and ii) processing by the joint action of the Dicer, Hyponastic leaves 1 (HYL1) and Serrate (SE) enzymes (Chen, 2009; Voinett, 2009), which also appears to be involved in the regulation of the transcription of intronless genes in *Arabidopsis* (Speth *et al.,* 2018). In contrast, since plants lack a Drosha homologue, in this kingdom both processes are performed by the RNase-III-like protein, Dicer-Like 1 (DCL1), which is also involved in the maturation of pri-miRNAs in the nucleus. This process produces a miRNA/miRNA∗ duplex (Voinnet, 2009; Ackhar *et al.,* 2016). Many research works have also described an interaction of the Dawdle (DDL) RNA-binding proteins with DCL1, required for the accumulation and biogenesis of sRNAs (Zhang *et al.,* 2018). The nuclear heterodimeric cap-binding complex (CBP) has been observed to promote the accumulation of some, but not all, miRNAs (Rogers and Chen, 2013). Before incorporating of mature miRNA into an Argonaut (AGO)-containing RNA-induced silencing complex (RISC), the mature miRNA/miRNA∗ duplex is methylated on the 2' OH of the 3' terminus by Hua Enhancer 1 (HEN1) to prevent any degradation by Small RNA degrading nuclease (SDN), a class of exonuclease enzymes (Axtell *et al.,* 2011; Zhao *et al.,* 2014). miRNA∗, also known as "passenger strand", was presumed to be a by-product of the miRNA biogenesis pathway (Khvorova *et al.,* 2003; Schwarz *et al.*, 2003), with no biological function, although more recent reports have proved that it also

has significant functionality in *Arabidopsis* (Liu *et al.,* 2017). The RNA strands are now better defined as "miRNA-3p" and "miRNA-5p", according to whether they originate from the 5' arm or the 3' arm of their hairpin precursor (Kozomara and Griffiths Jones, 2014; Yashin *et al.,* 2015). Both these RNA molecules can then be loaded into an AGO1-containing miRISC (Fang *et al.,* 2016), forming the miRNA-RISC complex responsible for the interaction with the target/s. This functional complex can be exported from the cytoplasm before or after its assembly by HASTY (HST) (Achkar *et al.,* 2016), although recent studies seem to indicate a greater possibility for the second hypothesis (Bologna *et al.,* 2018). Most of plant miRNAs have been reported to recognize their target/s through perfect or almost perfect complementarity, therefore inducing gene silencing by cleavage, inhibition of translation (Wang *et al.,* 2019), transcriptional regulation (Liu *et al.,* 2018), DNA methylation or Histone-modification (Khraiwesh *et al.,* 2012). Moreover, miRNA-RISC complex can also be responsible for the generation of a double stranded RNA (dsRNA), which can act as a substrate for the activity of the DCL4 protein in order to produce secondary siRNAs (Carbonell, 2019; de Felippes, 2019). The turnover and the degradation of miRNAs in cell is a very complex regulated mechanism, which required several proteins with many functions, in order to maintain a correct balance of intracellular miRNA levels (Wang *et al.,* 2019).

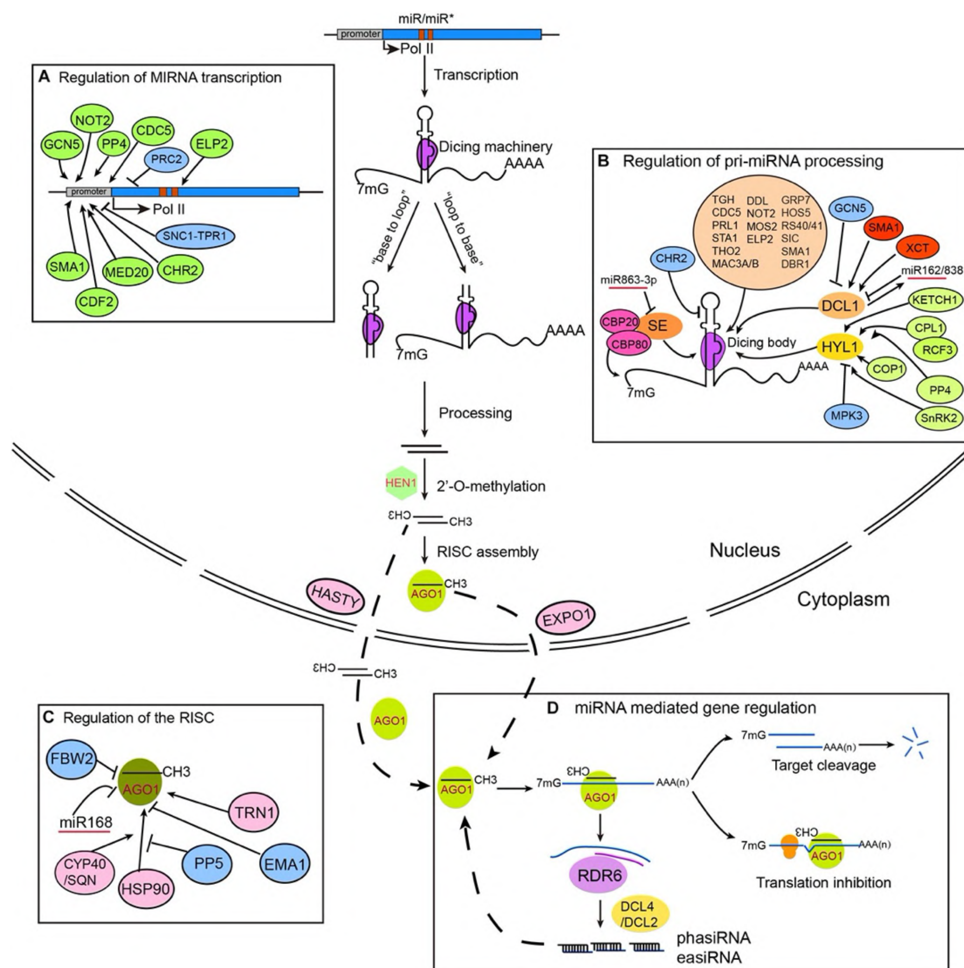

Figure 1: miRNA biogenesis in plants (Wang *et al.,* 2019).

3. Next-generation sequencing of plant small RNA (sRNA-Seq): general overview

Over the past years, the advent of Next-Generation Sequencing of small RNAs (sRNA-Seq) has permitted the exploration of the biological roles of these molecules in many organisms (Morgado and Johannes, 2017; Zhou *et al.,* 2020), including plants. However, since thousands or millions of sRNAs can be produced and captured all together in one single experiment of sequencing, the identification and the classification of the different sRNAs is a very tedious process, which requires a lot of information concerning their i) precursors, ii) the properties of the mature molecules, iii) their putative target sites and many others (Morgado and Johannes, 2017). Numerous computational tools have been developed in past years in order to detect sRNAs in newly sequencing libraries and, possibly, identified new candidates (Rueda *et al.,* 2015), although the prediction of sRNAs based on sequencing data remains mainly inaccurate. A detailed description of the best *in silico* tools currently available is described in the review of Morgado and Johannes (2017).

Among the various categories of sRNA, the miRNA class has gained great interest in the past years (Borges and Martienssen, 2015; Ma *et al.,* 2015). The first approach used to identify miRNAs was based on direct cloning and subsequent sequencing, although different biases, including the size of sRNAs, their numerous functions in organisms and others, made this system too limited to obtain a large amount of high-quality data (Prabu and Mandal, 2010). The Sanger sequencing method has represented the first system applied for the massive analysis of miRNAs from different samples, although it was very expensive and required a large amount of resources (see Hu *et al.,* 2017). Progresses were obtained over the years, leading to improvements in the entire process. Actually, the standard pipeline normally applied for the identification of miRNAs requires a strong correlation between biology and bioinformatics (Akhtar *et al*., 2016). In particular, a standard investigation on miRNAs is composed by many steps, including i) preparation of small RNA libraries, ii) high performance sequencing through Next-Generation Sequencing (NGS) technologies (Simon *et al.,* 2011; Ma *et al.,* 2015; Peng *et al.,* 2018) and iii) a robust computational analysis using the *in silico* tools which are actually available (Hannoufa *et al.,* 2018; Xu *et al.,* 2019). Modern sequencing technologies represent a very powerful system to obtain huge amounts of data at genomic or transcriptomic level of many tissues, cells and developmental stages of organisms, including plants (Turner *et al.,* 2012; Slatko *et al.,* 2018). Among the different strategies and the maximum performance of the recent years, the sequencing provided by Illumina represents the most promising, thanks to high-resolution data and cost-effectivness (Slatko *et al.,* 2018). The NGS platforms have allowed to study the expression pattern of miRNAs under different conditions, get more information on the secondary structures of miRNA precursors and identify new sRNA variants (Conesa *et al.,* 2016; Backofen *et al.,* 2017; Zhou *et al.,* 2020).

One of the most important steps to obtain a good sequencing performance is the preparation of the libraries (Dart-Dascot *et al.,* 2018).
The majority of the protocols actually available require the ligation of a couple of oligonucleotide adapters to the ends of the sRNA sequence (5' and 3'), containing primers for sequencing, the generation of complementary DNA (cDNA) by reverse transcription and subsequent amplification by PCR (see Liu *et al.,* 2007). To avoid possible contaminations and a reduction of the quality of the data, several steps during these processes are of fundamental importance, including the separation of sRNAs with other small RNA molecules, the formation of primer dimers due to the bond between the adapter sequences and many other limitations related to the PCR reaction (Lu *et al.,* 2007). Therefore, several strategies have consequently been developed in order to decrease these biases during library preparation and increase the final yield of the entire process; these include gel separation with PAGE or solid-phase reversible immobilization (SPRI) on magnetic beads (Lunding *et al.,* 2010), although some other adjustments must still be undertaken in order to obtain reduced biases (Dabney *et al.,* 2012).
The tipical workflow for sRNA-Seq analysis comprise several steps: generation of raw sequences from libraries and their alignment with data sets available, identification of miRNAs and their secondary structures, and analysis of the predicted target/s (Liao *et al.,* 2018; Xu *et al.,* 2019; Zhou *et al.,* 2020). Many computational tools have been recently developed (Ferdous *et al.,* 2017; Chen *et al.*, 2018; Xu *et al.,* 2019), which were extensively applied in many research works in order to identify miRNAs in different plant species, including *Arabidopsis*, maize, *B. napus* and others (Banerjee *et al.,* 2016; Zhou *et al.,* 2020).

4. miRNAs are involved in response to different stresses in plants

Over the past few decades, different factors have contributed to reducing crop yield with negative implications on food security, including human activities, biotic and abiotic stresses, climate change and others (Djami-Tchatchou *et al.,* 2017; Hatfield *et al.,* 2018). To support population growth in the years to come and to improve productivity without consuming further arable lands, a possible solution to these problems is represented by the generation of GMO crops tolerant to adverse environmental cues (Datta *et al.,* 2013; Gupta, 2015; Djami-Tchatchou *et al.,* 2017). In addition, different genomic tools have been developed over the past few years to induce gene silencing in plants, including the application of artificial miRNA technology (amiRNA), which represents a very powerful technique (Zhang *et al.,* 2019). However, GMO-RNAi free approaches have been recently taken in consideration as valid alternative to the use of genetic engineering (Worrall *et al.,* 2019; Dalakouras *et al.,* 2020), to overcome the problems related to genetic modified organisms in several states.
The plant's responses to different environmental stresses requires complex and coordinated regulation mechanisms involving different biochemical, metabolic and physiological aspects of plant development and nutrition, which must be finely modulated (Jeandroz *et al.,* 2017; Woodrow *et al.,* 2017).

Several genes ad molecules, including ncRNAs (Sunkar *et al.,* 2012), are sensitive to different types of stress and seem to participate in these complex responses principally acting at transcriptional and post-transcriptional levels (Shriram *et al.,* 2016). In particular, high-throughput and deep/degradome sequencing technologies have proved the extended activity and the strong impact of sRNAs in regulating gene expression in many organisms (Barciszewska-Pacak *et al.,* 2015; Alptekin and Budak, 2016; Zhou *et al.,* 2020).

Most plant miRNAs are involved in post-transcriptional gene regulation, which mainly occurs through mRNA cleavage (Rogers and Chen, 2013; Lin *et al.,* 2016), although they can also modulate their target levels by chromatin remodeling or epigenetic modification, as recently described (Kumar *et al.,* 2018).

It is remarkable that most targets of miRNA are transcription factors (TFs), which enhance and regulate gene expression in plants and are essential for plant signaling (Samad *et al.,* 2017; Jalmi *et al.,* 2018). The coordinate responses and regulatory network of miRNA-TFs are fundamental for plant development, growth and response to stress (Sunkar *et al.,* 2012; Samad *et al.,* 2017). After several attempts during the years, a set of conserved miRNAs in plants has been demonstrated to play key roles in physiological, metabolical and morphological adaptation to many environmental factors (see Xu *et al*., 2019; **Fig. 2**), confirming their importance during the evolution. Many of these miRNAs are responsive to diversified stresses in *A. thaliana*, *B. juncea*, tobacco, maize and other crops, including saline stress, heavy metals, heat, drought, cold and others (Yin *et al.,* 2015; Singh *et al.,* 2017; Liu *et al*., 2019; Pegler *et al.,* 2019). Moreover, many plant sRNAs, including miRNAs, are also involved in the response against various biotic stresses, which comprise bacteria, viruses, fungi and nematode parasites (Chauhan *et al.,* 2017; Soto-Suárez *et al.,* 2017; Brant *et al.,* 2018). It is notable that the recent progress made regarding the study of the role of miRNA-target/s in different food and industrial crops could also represent a relevant way to find a new solution to solve most of the environmental problems (Tang *et al.,* 2017; Xu *et al.,* 2019; Wani *et al.,* 2020). However, the majority of miRNAs that have been identified in different plants are able to respond only to a single stress, which seems not to be sufficient to achieve this goal (Xu *et al.,* 2019). Many research works are currently focused on multi-stress responsive miRNAs, with a big impact on plant growth and development, thus representing a potential target for the genetic improvement of plants (Ebrahimi-Khaksefidi *et al.,* 2015; Jalmi *et al.,* 2018; Wani *et al*., 2020). For example, the highly conserved miR156 and miR169 can modulate the expression levels of members belonging to the family of Squamosa Promoter binding protein-Like (SBP) and Nuclear Factor Y (NFY) transcription factors (Chen *et al.,* 2010; Zhang *et al.,* 2017), which are involved in many biological processes. These two miRNAs were proved to be responsive to different abiotic stresses, which comprise salinity stress in *Triticum aestivum* (Pandey *et al*., 2014) and *Glycine max* (Li *et al*., 2011), oxidative stress in *A. thaliana* (Zhou *et al.,* 2008) and *Populus tremula* (Jia *et al.,* 2009), heavy metal stress in *Oryza sativa* (Ding *et al.,* 2011) and others. A list of

miRNAs explored for crop engineering against environmental cues is better described in the works of Basso *et al.* (2020) and Xu *et al.* (2019).

Furthermore, recent progress in the study of epigenetics have also led the attention to the emerging functions of miRNAs as fundamental epigenetic regulators (Rajewsky *et al.,* 2017; Xu *et al.,* 2019). In particular, small ncRNAs seem to be directly involved in the activation of DNA damage responses (DDR) in cell, a complex mechanism which defines the survival or the death of a cell under specific stressful conditions (Michelini *et al.,* 2018; Bellato *et al.,* 2019), thus confirming the involvement of these molecules in the regulation of genome stability and genome editing in organisms.

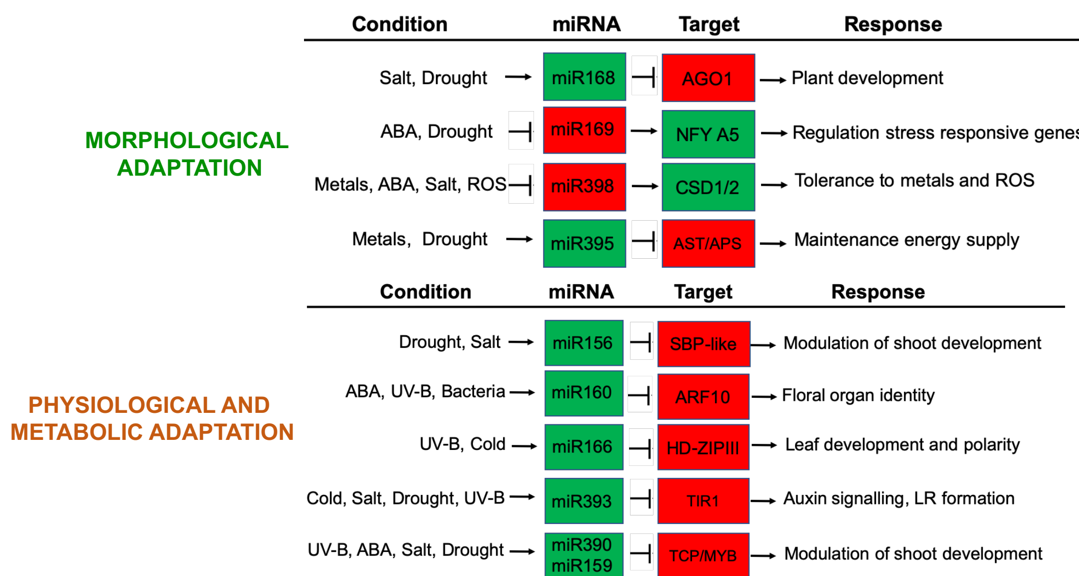

Figure 2: Most conserved and well-studied miRNAs in *Arabidopsis* are involved in the plant's response to different stresses (adapted from Khraiwesh *et al.,* 2012). The colour green corresponds to a positive modulation whereas the red colour corresponds to a negative modulation.

5. Roles of miRNAs in response to heavy metal stress in plants

Metal pollution caused by anthropogenic activities is a serious problem all over the world (Fang *et al.,* 2013; Singh *et al.,* 2016). When present at high concentrations, metals disturb many fundamental processes in plants, such as water balance, mineral nutrition, nutrient homeostasis and photosynthesis (Dal Corso *et al.,* 2008; Yruela, 2009). In general, plants tend to avoid metal toxicity by employing a variety of strategies, such as i) reducing the root uptake of metals, ii) increasing chelation by secreting an abundance of ligands (Hall, 2002; Seth *et al.,* 2011), iii) enhancing signal transduction to control biological responses and iv) producing antioxidant enzymes to prevent ROS toxicity (Sharma *et al.,* 2012; Huang *et al.,* 2019). Microarray analyses and high-throughput deep/degradome sequencing have been carried out in order to better elucidate the possible implications of miRNAs in response to heavy metal stress in

plants (Jian *et al.*, 2018; Gao *et al.,* 2019; Ding *et al.,* 2020). Several conserved and non-conserved miRNAs, with different biological roles, have been thus identified in rice (Ding *et al.,* 2013), *B. napus* (Xie *et al.,* 2007; Jian *et al.,* 2018), *M. truncatula* (Zhou *et al.,* 2012), tobacco (He *et al.,* 2016), maize (Gao *et al.,* 2019) and *A. thaliana* (Ding and Zhu, 2009; Barciszewska-Pacak *et al.,* 2015).

It is interesting to notice that plant miRNAs take part in the most important actions required for heavy metal tolerance, which include i) complexation of metals, ii) oxidative stress response and iii) signal transduction (Nikalje, 2018). In particular, genome-wide analysis have shown that the majority of miRNA targets are involved in plant tolerance to heavy metals (Yang and Chen, 2013), with a role in antioxidation, phytohormone signaling, detoxification and miRNA biogenesis.

All these data not only indicate the importance of miRNAs during evolution (see Jalmi *et al.,* 2018), but they can also underline the possible use of miRNAs as targets to increase the tolerance to heavy metal stress in plants (Noman and Aquell, 2017).

5.1 miRNAs involved in regulation of plant development

Different strategies are known to be involved in heavy metal stress response in plants. The first line of defence is represented by physical barrier (Emamverdian *et al.,* 2015): the development of cuticule, cell walls and trichomes is characterized by high variability and plasticity that depends on environmental conditions (Sarret *et al.,* 2002; Harada *et al.*, 2010; Curie and Mari, 2017). For example, metal storage in trichomes is known as a defense mechanism against negative effects in many plant species (Sarret *et al.,* 2002; Hauser *et al.,* 2014), but several studies have also proved the plasticity of root and leaf development under metal stress (Kochian *et al.,* 2005; Farid *et al.,* 2013). The first general symptom of heavy metal exposure in plants is the inhibition of root length, induced by hormones, essentially because it is the first organ exposed to metals with a fundamental role in absorption of micro-macro nutrients (Kochian *et al.,* 2005; Rellán-Álvarez *et al.,* 2006; Lequeux *et al.,* 2010).

The conserved roles of two miRNAs, miR390 and miR393, regarding the modulation of root architecture under several stressful conditions have been reported in many plant species, thanks to computational and experimental procedures (Soto *et al.,* 2012; Jalmi *et al.,* 2018; Lu *et al.,* 2018).

miR390 is conserved in many plant species, including *A. thaliana*, rice and maize, and it is normally involved in the biogenesis of tasiRNAs from *TRANS-ACTING SIRNA3 (TAS3)* mRNAs in order to modulate *AUXIN RESPONSIVE FACTOR (ARF)* genes, which are fundamental for auxin signaling (Xia *et al.,* 2017). High levels of miR390 induce the cleavage of TAS3 under stressful conditions in many plant species, leading to a degradation of Auxin responsive factors (ARFs) (Marin *et al.,* 2010; Iglesias *et al.,* 2014). Different metals have been shown to change miR390 regulation in plants, including Cd, Al, and Hg (Zhou *et al.,* 2012; Ding *et al.*, 2016; Lu *et al.,* 2018); in addition, this molecule is also regulated by

drought stress in cowpea (Barrera-Figueroa *et al.,* 2011), hence confirming that the plasticity of root development is a strategy adopted to cope with several stressful conditions.

miR393 is also required for the regulation of root development in plants, targeting the auxin receptor *TIR1*, which has a role in auxin signaling (Windels *et al.,* 2011). A modulation of miR393 expression was found in several plant species exposed to metal stress including *M. truncatula*, maize, *B. napus*, barley and others (Fang *et al.,* 2013; Bai *et al.,* 2017; Jian *et al.,* 2018; Gao *et al.,* 2019). In addition, more recent works have determined the correlation between the expression levels of *TIR1*, targeted by miR393, and the response to abiotic stress *in planta*; in fact, overexpression of miR393 increased the tolerance of *Agrostis stolonifera* transgenic plants to drought, cold and salinity stress, thus confirming the importance of miR393-target interaction in organisms (Li and Zhang, 2015; Zhao *et al.,* 2018).

Changes in leaves, necrosis, decrease in leaf number and size and decreased photosynthetic activity are negative effects caused by excess of metals (Emamverdian *et al.,* 2015). Many studies on plants have demonstrated an important role of many miRNAs in leaf development (see Yang *et al.,* 2018). For example, the highly conserved miR319 is responsible for shoot and floral organs development by the modulation of TEOSINTE-BRANCHED/CYCLOIDEA/PCF (TCP) TFs levels (Efroni *et al.,* 2008; Koyama *et al.,* 2017). This RNA molecule is involved in the regulation of leaf senescence via JA biosynthesis, which is known to be involved in plant responses to Cd (see Lei *et al.,* 2020), targeting positive regulators like WRKY53 (Schommer *et al.,* 2008). In particular, these proteins, which belong to the class of WKRY TFs, play different roles in response to biotic and abiotic stresses including excess Zn (Broadley *et al.,* 2007; Phukan *et al.,* 2016).

All these data confirm the importance of miRNAs in hormone signaling *in planta*, with a big impact on plant growth and development (Li and Zhang, 2015).

5.2 Oxidative stress response

In response to various types of environmental stress, such as drought, salinity and high concentration of metals, plants and other organisms produce and accumulate reactive oxygen species (ROS) and free radicals, which represents one of the most serious problems for crop yield (You and Chan, 2015). The production of ROS, such as hydrogen peroxide and superoxide radical anions, is considered an important parameter to understand the general conditions of plants. The effects of ROS are very serious for plants growth, since they can cause lipid peroxidation, damage to nucleic acids and protein degradation (see Huang *et al.,* 2019). The antioxidant systems of plants include several enzymatic scavengers, such as superoxide dismutases (SODs), peroxidases and catalases, but ascorbate, tocopherol, carotenoids and glutathione are equally important, since they are involved in the defence processes (Mittler *et al.,* 2004; Choudhury *et al.,* 2016). SODs are the first line of defence against ROS in plants (Asada, 2006; Jajic *et al.,* 2015); the most important are iron SOD (FeSOD), manganese SOD (MnSOD), and copper-zinc SOD

(Cu/Zn-SOD), which are localized in different cellular compartments. Several plant miRNAs participate to the response against oxidative stress (Panda and Sunkar, 2015), including miR398 and miR408 which are the most conserved multi-stress responsive miRNAs among plants (Pilon, 2017). In particular, ROS production by heavy metals and other types of stresses is responsible for inducing MAPK signaling *in plants,* a process which seems to be also regulated by the activity of miR398b/c (Smeets *et al.,* 2013).

5.2.1 miR398 modulates Cu-SODs levels under several biotic/abiotic stress factors in plants

miR398 is an important RNA molecule with a conserved role in the response to many biotic and abiotic stress factors, by the modulation of SODs expression. This miRNA is highly conserved in *A. thaliana* and other plant species (Wang *et al.,* 2014; Leng *et al.,* 2017; Li *et al.,* 2019); in particular, MIR398 is encoded by three loci in *Arabidopsis* (*MIR398a, MIR398b,* and *MIR398c*), two of which, miR398b and miR398c, have the same identical sequence, while the other one (miR398a) differs only for the last nucleotide (Sunkar *et al.,* 2006). The mature miR398b has four targets in *Arabidopsis*: the two Cu/Zn superoxide dismutase (CSD) enzymes *CSD1* and *CSD2*, the Cu chaperone for superoxide dismutase (CCS1) *CCS*, which delivers copper to CSD1 and CSD2, and *COX5b–1*, a zinc-binding subunit of cytochrome *c* oxidase (Sunkar and Zhu, 2004; Guan *et al.,* 2013; Shahbaz and Pilon, 2019). Various abiotic stresses have been observed to modulate the expression of miR398 in plants. In *Arabidopsis*, miR398a and miR398b/c are down-regulated by intense light, methyl viologen (MV) and high concentrations of metals (Cu and Fe), with a subsequent increase in *CSD1-CSD2* transcripts levels (Sunkar *et al.,* 2006) and resistance to oxidative stress. The modulation of miR398 expression under a variety of abiotic stresses, including excess levels of metals, has also been recently investigated in many plant species (Candar-Cakir *et al.,* 2016; Kang *et al.,* 2017; Gao *et al.,* 2019; Li *et al.,* 2019), confirming its conservative role as general regulator for abiotic stress response *in planta.* Interestingly, manipulation of miR398 expression levels in rice has recently demonstrated the fundamental role of this molecule in the growth and development of this plant (Zhang *et al.,* 2017), thus representing a promising target for crop improvement.

5.3 Metal complexation

Many conserved miRNAs have been observed to take part in metal complexation processes in plants. For example, the small RNA library isolated from *B. napus* subjected to Sulphur deficiency and Cd stress revealed a set of miRNAs that respond significantly to these treatments (Huang *et al.,* 2010; Yuan *et al.,* 2016; Jian *et al.,* 2018), including miR395. This RNA molecule is known to play a key role in regulating S homeostasis in plants, but it is also significantly modulated in the presence of high levels of metals (Zhang *et al.,* 2013; Li *et al.,* 2017; Jian *et al.,* 2018).

Sulphur is an essential element for plant nutrition: it is taken up by the roots from the soil and incorporated into many molecules involved in primary and secondary metabolism (Raush *et al.,* 2005; Matthewman *et al.,* 2012). In addition, different studies have proved a direct link between S metabolism and response to heavy metals, confirming the importance of this nutrient *in planta* (Ernst *et al.,* 2008). The expression of miR395 has a fundamental role in sulphate accumulation and allocation (Liang *et al.,* 2010; Matthewman *et al.,* 2012); in particular, the *ATP sulphurylase* (encoded by *APS* genes) and sulphate transporter 2;1 (*SULTR2;1*) genes, both involved in sulphur absorption and homeostasis, have been found to be targeted by miR395 in response to S contents in the plant cell (Matthewman *et al.,* 2012).
A positive modulation of miR395 expression was also found in *A. thaliana* and *B. napus* plants exposed to Cd and Cu (Zhang *et al.,* 2013; Jagadeeswaran *et al.,* 2014), confirming its involvement in response to heavy metal stress, with the reallocation of sulphate for the production of GSH and phytochelatins (Gielen *et al.,* 2016). It is important to note that other miRNAs (miR159, miR167) are required for the detoxification of metals by regulating the expression of genes coding for ABC and NRAMP metal transporter proteins (Yang and Chen, 2013).

6. Nutrient deficiency response and homeostasis

The general pathway for the uptake, transport and allocation of a nutrient in plants is a very complex mechanism, characterized by the activity of many proteins, including metal transporters and TFs. These processes depend on many aspects, such as i) type of nutrient, ii) redox balance in the cell, iii) mineral availability, iv) organs or tissues considered and v) developmental stage of the plant (Sperotto *et al.,* 2014). Nutrient deficiency can be brought about by several factors, including drought, high or low temperatures, salt, soil composition, pH and other parameters that may affect nutrient bioavailability.
Moreover, excessive levels of heavy metals in the soil can cause symptoms of nutrient deficiency, because they can compete with several micronutrients (Dal Corso *et al.,* 2013; Zeng *et al.,* 2014; Lešková *et al.,* 2017; Loix *et al.,* 2017). Plants have evolved a variety of strategies to avoid nutrient deficiency and enhance metal uptake from the soil, including regulation of the expression of metal transporter proteins and variation of the root architecture (Jung and McCouch, 2013). Interestingly, deep/degradome sequencing and genome wide analysis on *Arabidopsis*, *B. napus,* poplar and other plant species have revealed that several miRNAs, belonging to different families, play a key role in regulating mineral nutrition, therefore increasing nutrient uptake and correct balance (Paul *et al.,* 2015; Samad *et al.,* 2017; Noman *et al.,* 2019). As shown in **Fig. 3**, under conditions of limited nutrient availability a specific miRNA can be translocated from shoots to roots via the phloem (1), reducing the activity of the target/s (2) and enhancing the uptake of nutrients from the soil and their translocation to the shoots (3-4). These data strongly suggest the role of miRNAs in mineral plant nutrition, also representing an interesting point for further analysis (see Paul

et al., 2015). It is notable that numerous scientific studies have also shown that exogenous plant sRNAs can be assimilated from different sources by many organisms, including mice and humans, with several biological effects, hence supporting a possible cross-kingdom communication by diet/plant-derived miRNAs and their possible application for the treatment of human diseases (Li *et al.,* 2018; Fu *et al.,* 2019).

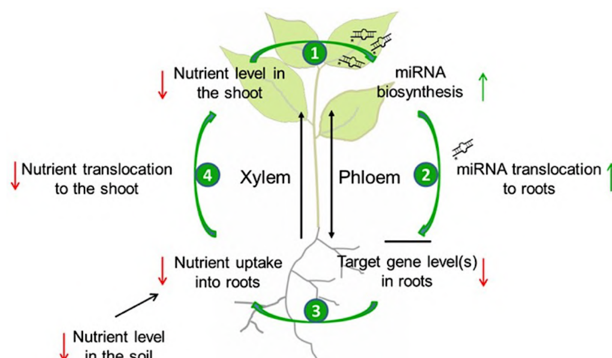

Figure 3: Diagram of the systemic regulation of nutrient-responsive miRNA in plants under conditions of limited nutrient availability (Kehr, 2013).

6.1 Phosphate and Sulphate homeostasis

Inorganic phosphate (Pi), necessary for plant growth, is usually scarcely available in the soil solution. Hence, the regulation of Pi transport into the plants, mediated by miRNAs and their specific targets, is one of the most studied and interesting regulation pathways in plants (Paul *et al.,* 2015). The miR399 family of miRNAs seems to be involved in multiple nutrient starvation responses in plants (Chiou *et al.,* 2006; Hu *et al.,* 2015). In *Arabidopsis*, six members (a–f) of the miR399 family have been identified to date. In general, the expression levels of *PHO2*, a key transporter involved in phosphate mobilization, are regulated by miR399, which is responsible for the mRNA cleavage mediated by the interaction with its 5' UTR region (Chiou *et al.,* 2006). Under conditions of Pi starvation, the enhanced expression of miR399 leads to a reduction in the *PHO2* levels in the roots, improving the flow of Pi towards the shoots. Interestingly, miR399 was observed to be a phloem-mobile long-distance molecule in *Arabidopsis*, therefore suggesting that this molecule is an essential factor for Pi homeostasis (Hu *et al.,* 2015). Other miRNAs regulate phosphate transport in *Arabidopsis*, such as miR778, miR827, and miR2111. A similar regulation pattern was observed for sulphur (S): in *Arabidopsis*, S starvation affected the expression of miR395 (Matthewman *et al.,* 2012). S is essentially assimilated into cysteine, methionine, various Fe–S proteins and other important molecules involved in both primary and secondary metabolism. Under conditions of low S availability, SLIM1 induces the expression of miR395, which modulates *APS* and *SULTR* genes, improving S transport towards the shoots (Matthewman *et al.,* 2012).

6.2 Copper homeostasis

Copper is one of the most important micronutrients for plant growth and development, therefore required in many fundamental biological processes, including i) photosynthesis, ii) respiration, iii) protection against oxidative stress, iv) biosynthesis of lignin and other compounds (Peñarrubia *et al.,* 2015). Many enzymes and proteins require Cu as cofactors or as a constituent, and this element is therefore one of the most abundant micronutrients in plants. One-third of the total copper (Cu) in the shoots of *A. thaliana* has been estimated to be located in its chloroplasts (Shikanai *et al.,* 2003; Aguirre *et al.,* 2016; Printz *et al.,* 2016). The soluble carrier Plastocyanin (PC), which is localized in the thylakoid lumen of chloroplasts (Joliot and Joliot, 2006), represents one of the most abundant and important Cu-protein in *Arabidopsis*. PC is essential for plants, since it is required for the electron transport between the cytochrome *b6f* complex and PSI (see Katoh, 2005). Superoxide dismutases (SODs) form the second largest class of copper enzymes in the cell; as previously described, they are involved in the scavenging of reactive oxygen species (ROS), produced by several stress factors, including metals (Mittler *et al.,* 2004; Aguirre *et al.,* 2016). Two Cu/Zn-SOD isoforms are present in *Arabidopsis*; one is localized in the cytoplasm (CSD1) and the other in the chloroplast stroma (CSD2). The modulation of CSDs levels under stress is regulated by several factors, including the activity of miRNAs (Yamasaki *et al.,* 2007). Because Cu is required for a variety of biological processes, the balance and uptake of copper in plants are very delicate and finely tuned mechanisms involving many molecules, including miRNAs (Schulten and Krämer, 2018; Shahbaz and Pilon, 2019). Cu deprivation is responsible for changes in root and leaf architecture, and stunted growth (Pilon *et al.,* 2006; Printz *et al.,* 2016); on the other hand, excess levels of Cu are also toxic to plants, because they can induce the production of ROS by Fenton reactions (Yamasaki *et al.,* 2007). Copper homeostasis in the plant cell is therefore regulated by the modulation of both PC and Cu/Zn-SOD levels, being the most important Cu sinks.

6.2.1 Role of the Cu-miRNAs in copper balance

Since photosynthesis is the most important process for photoautotrophic organisms, plants tend to maintain an adequate flow of copper to PC for the electron transport chain reaction, by reducing when necessary other copper proteins that are less indispensable (Abdel-Ghani *et al.,* 2006; Peñarrubia *et al.,* 2015). Various miRNAs, termed Cu-miRNAs, have been proved to efficiently regulate Cu balance and homeostasis in cells (Pilon *et al.,* 2017; Shahbaz and Pilon, 2019), including miR398. In *Arabidopsis*, the higher levels of miR398 brought about by Cu starvation down-regulate CSD1, CSD2, thus increasing copper supply to PC (Sunkar *et al.,* 2006; Beauclair *et al.,* 2010). Promoter analyses on miR398 and other Cu-related genes have revealed the presence of several GTAC motifs, corresponding to copper

responsive elements (CURE), suggesting a conserved, complex mechanism regulating Cu availability in plants. The transcription factor SPL7, which has an important role in modulating copper homeostasis in plants (Araki *et al.,* 2018), can recognize the GTAC motifs present on the promoter regions of many copper-responsive genes, including various SODs and copper proteins, but also different miRNAs (Pilon, 2017; Shahbaz and Pilon, 2019). In particular, miR397, miR398, miR408 are the most highly conserved miRNAs in plants, since their importance in the regulation of Cu homeostasis *in planta*, as also demonstrated by tandem target mimicry analysis (Cuperus *et al.,* 2011; Shahbaz and Pilon, 2019). Interestingly, the overexpression of miR397 in rice significatively increased the overall yield in comparison to the WT, representing the first application of a miRNA-based biotechnology in crops (Zhang *et al.,* 2013). miR408 regulates a variety of Cu-related genes encoding copper-binding proteins belonging to the phytocyanin family, which are involved in electron-transfer between proteins (Choi and Davidson, 2011), as well as Laccase-like multicopper oxidases (LMCO; Carriò-Segui *et al.,* 2019). These genes encoding proteins which are responsible for the oxidative polymerization of lignin, fundamental for the formation of the cell-wall under normal and abiotic stressful conditions, a process which depends on the concentration of Fe in the growth medium (Schuetz *et al.,* 2014; Loix *et al.,* 2017; Carriò-Segui *et al.,* 2019). Many experiments on several plant species have proved the characteristic of miR408 as a conserved multi-stress responsive miRNA, thus representing a potential target for the generation of GMO crops more resistant to environmental stresses (Ma *et al.,* 2015; Kuo *et al.,* 2018; Guo *et al.,* 2018; Xu *et al.,* 2019). Moreover, gene coding for an ascorbate oxidase, which is essential for the biogenesis of the anti-oxidant Ascorbate (Bielen *et al.,* 2013), was identified as the target of miR408 (see Carrió-Seguí *et al.,* 2019), suggesting the involvement of this RNA molecule in regulating the response to oxidative stress *in planta.* miR397 and miR857, are mainly expressed in the vasculature, and play a key role in regulating the expression of non-essential Cu-proteins (Wang *et al.,* 2014; Zhao *et al.,* 2015). Modulation of miR397, miR398, miR408 and miR857 expression levels mediated by SPL7 TF in *Arabidopsis* depends on the Cu levels in the plant cell (see Araki *et al.,* 2018; **Fig. 4**)**.** In particular, SPL7 induces the expression of Cu-miRNAs and various genes encoding the Cu transporter in conditions of Cu deficiency (Pilon *et al.,* 2017), thus reducing the expression of non-essential Cu proteins and increasing the distribution of Cu among the various groups of PC proteins (Gifford *et al.,* 2008; Schulten and Krämer, 2018). Interestingly, expression levels of SPL7 *in planta* seem to be also modulated by miR156 and miR157 (Valdes-Lopez *et al.,* 2010; Yu *et al.,* 2012), suggesting the possible involvement of the two miRNAs in the Cu homeostasis regulation and the coordinate roles between miRNAs in this process.

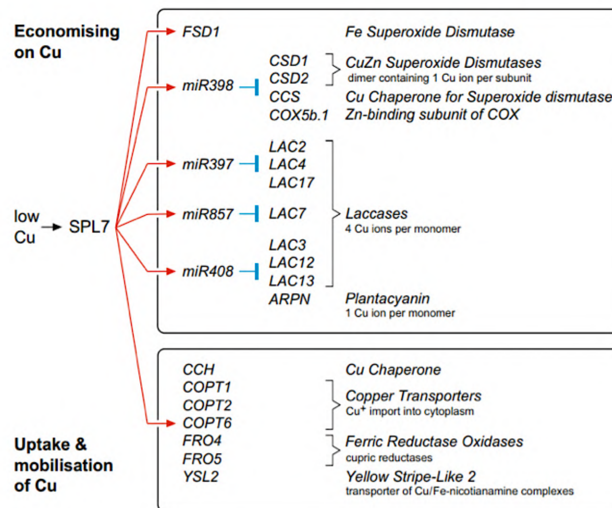

Figure 4: Cu-deficiency response in *Arabidopsis* mediated by the transcription factor Squamosa Promoter binding protein-Like 7 (SPL7) under conditions of low Cu availability (Schulten and Krämer, 2018).

6.2.2 Cu-miRNAs are modulated by high levels of metals

The roles of Cu-miRNAs in response to metal stress and mineral nutrition have been studied in last years. An altered expression of miR398 was found in many plant species exposed to metals (Cuypers *et al.,* 2011; Burklew *et al.,* 2012; Remans *et al.,* 2012). High concentrations of Zn and Cd in the medium were observed to induce the expression of miR398 in *A. thaliana* and *B. napus*, with a reduction in Cu-SOD expression levels (Remans *et al.,* 2012), but excess Cu and Fe induced the opposite response (Sunkar *et al.,* 2006). The presence of Cd seems to stimulate Cu-deficiency responses in *Arabidopsis*, probably due to a competition between Cu and Zn/Cd in the cell (Gielen *et al.,* 2016). Cd in particular induced the SPL7-dependent Cu-deficiency response, down-regulating miR397, miR398, miR408 and miR857 in *A. thaliana* and other plants (Gayomba *et al.,* 2013; Gielen *et al.,* 2016; Gao *et al.,* 2019). Given the presence of GTAC DNA motifs in their promoter regions, it can be presumed that these proteins, together with the other Cu-related genes, are under the control of SPL7 TF. Interestingly, a similar regulation pattern was also found for Zn (Gielen *et al.,* 2016), an element that can affect the redox balance in plants. These Cu-miRNAs seem to possess a direct role in regulating Zn nutrition *in planta* (Shi *et al.*, 2013; Zheng *et al.,* 2019); furthermore, miR398b is also regulated by excess levels Al, Hg, Pb in many plant species (Jalmi *et al.,* 2018), confirming its possible involvement in heavy metal tolerance in plants (Shriram *et al.,* 2016; Xu *et al.,* 2019).

2. AIM OF THE WORK

The aim of this work was to identify miRNAs putatively involved in the response to excess Zn; this micronutrient is one of the most abundant in the earth crust and can be toxic when present at high concentrations (Broadly *et al.,* 2007). Different miRNAs have been found to be modulated by variable concentrations of Zn, as well as Cd, which is high similar to Zn, thus confirming the involvement of these small RNA molecules in response to nutrients (Shi *et al.,* 2013; Liang *et al.,* 2015; Paul *et al.,* 2015).
In order to obtain more information about the possible involvement of miRNAs in the adaptation to excess metal, in this experiment we have considered *A. thaliana* and the metallicolous ecotype I16 (Lombardy, Italy) of the Zn/Cd hyperaccumulator *A. halleri*, a model species for metal hyperaccumulation and hypertolerance studies (Becher *et al.,* 2004; Verbruggen, 2015).
At first, a miRNA-Seq analysis was performed on sRNAs isolated from the shoots of the following plants: *A. thaliana* subjected to excess Zn for 1 week, the untreated controls, and untreated *A. halleri*. Various miRNAs were found to be modulated differently in the two *Arabidopsis* species, including many metal-responsive miRNAs. According to data available in the literature, some miRNAs were selected as putative Zn-responsive miRNAs and experimentally validated by Northern Blot and Real Time RT-PCR.
Given their relevant role in response to abiotic stress (Pilon *et al.,* 2017), greater attention was paid to the Cu-responsive miRNAs that appeared to be differently modulated in *A. halleri*. This class of miRNAs is believed to be important for the plant's ability to adapt to environmental factors, and essential for the basal tolerance to Cd in *Arabidopsis* (Gielen *et al.,* 2016; Schulten and Krämer, 2018). Copper homeostasis in *A. thaliana* is fundamental for plant growth and development, because this micronutrient is a cofactor for several enzymes involved in key biological processes, such as photosynthesis and respiration (Paul *et al.,* 2015). Several research works have elucidated the role of Cu-miRNAs in response to abiotic stresses (Sunkar *et al.,* 2006; Gayomba *et al.,* 2013; Ma *et al.,* 2015), including high concentration of metals, indicating a possible competition between Cu and other micronutrients. Among these miRNAs, miR398b and miR408 were considered for further analyses. These two miRNAs are normally modulated under Cu-deficiency conditions and in the presence of abiotic stresses (Sunkar *et al.,* 2006; Zhao *et al.,* 2015; Zu *et al.,* 2018). To clarify the possible role of miR398b and miR408 in response to excess Zn, the modulation of *MIR398b* and *MIR408* genes under Zn treatment was initially analyzed by Real Time RT-PCR performed on plants of *A. thaliana* and *A. halleri*. Their promoter sequences were then amplified from the gDNA of *A. thaliana* and *A. halleri* and fused to the *GUS* reporter gene. Transgenic lines of *A. thaliana* expressing pMIR398b::*GUS* and pMIR408::*GUS* were generated and the response of both genes to Zn and Cu was tested to verify whether Zn and Cu compete with each other (Remans *et al.,* 2012). Finally, the effect of overexpressing the stem-loop precursor of *A. halleri* miR408 on Zn tolerance was assessed by producing *A. thaliana* transgenic lines and growing them in the presence of excess levels of this element.

3. MATERIALS AND METHODS

3.1 Plant material and growth conditions

In this study different species belonging to the Brassicaceae family were used:

- *Arabidopsis thaliana* ecotype Col-0 (non-accumulator);
- *Arabidopsis halleri* ecotype I16 (Zn/Cd hyperaccumulator; Lombardy, Italy).

3.1.1 Growth conditions and Zn treatment

Seeds of *A. thaliana* were sterilized in Petri dishes on water-soaked Whatman filter paper and incubated for 2 days at 4°C in the dark to break seed dormancy. Plant cultivation *in vitro* and *in vivo* was performed as previously described. For sRNA-Seq experiment, plants of *A. thaliana* and *A. halleri* were grown hydroponically in half-strenght Hoagland's nutrient solution for 3 weeks and transferred on soil under controlled conditions (16 h light/8 h dark, illumination 100–120 μmol $m^{-2}s^{-1}$, day/night temperature 22°C/18°C). One pool of *A. thaliana* plants was grown adding 500 μM of $ZnCl_2$ for 1 week and then leaves were collected for sRNA-Seq analysis. The other *A. thaliana* and *A. halleri* plants were supplemented with water and samples were collected as indicated.

3.2 sRNA-Seq analysis

3.2.1 Samples preparation, sRNAs extraction and library construction

Three sets of sRNAs were prepared from samples of *A. thaliana*, *A. thaliana* + Zn and *A. halleri*. Each sample was derived from the original pool (5 plants) prepared from Zn-free or Zn-treated leaves. Small RNAs were extracted with mirPremier® microRNA Isolation Kit and quantified with NanoDrop™ One^{C} Microvolume UV-Vis Spectrophotometer (Thermo Scientific™). The purity and integrity (RIN) of sRNAs was also verified by agarose gel (4%) and Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA). Each library underwent flow-cell cluster generation and bridge amplification using the Illumina HiSeq 2500 platform (Solexa/Illumina).

3.2.2 miRNAs identification

Reads were processed using the miRPlant tool (An *et al.,* 2014). First, reads were trimmed using the adaptor sequence (TGGAATTCTCGGGTGCCAAGG) and filtered to remove bases with a quality score lower than 17.

All the reads with a final length lower than 10 bases were removed and were not considered for further analysis. The retained remaining reads were aligned against the genome *A. thaliana* miRBase (http://www.mirbase.org/index.shtml) hairpin database (with bowtie not allowing any mismatch). The miRDeep-P (Yang and Li, 2011) pipeline was then applied to identify the differentially expressed miRNAs and categorize them into the known families. In miRNAs quantification, only reads which completely covered a mature miRNA, were considered. In addition, reads which were mapped on multiple gene loci, were evenly distributed among matching genes.

3.2.3 Differential miRNAs Expression Analysis

The differential miRNA expression analysis between the samples was performed using the edgeR package (Robinson *et al.,* 2010). The read counts were normalized using the TMM normalization method implemented in the EDASeq package. Differentially expressed miRNAs were identified based on a False Discovery Rate-corrected p-value lower than 0.05.

3.3 Experimental validation of miRNAs expression

To validate experimentally miRNAs expression levels obtained with sRNA-Seq analysis, two different approaches were used: Northern Blot analysis on mature miRNAs and Real time RT-PCR on pre-miRNAs.

3.3.1 Northern Blot analysis

For miRNA blotting, 1.5 µg of small RNAs was separated on 15% denaturing polyacrylamide gels containing 7 M urea and transferred to Hybond N^+ nylon in a Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad, USA) membrane. Blots were hybridized over-weekend with respective miRNA-complementary oligonucleotides (**Table 3.1**) that had been end-labeled with Ambion® mirVana™ Probe & Marker Kit using radioactive P^{32} at 42°C in moderate CG hybridization buffer (1% w/v BSA, 0.5 M Na_2HPO_4, 15% v/v formamide, 1 mM EDTA, 7% w/v SDS) after pre-hybridization for at least 2 hours. Blots were then washed three times at 42°C in 1× SSC and 0.1% SDS for 10 min each time and the signal of the radiolabeled probes was then detected on a phosphorimaging screen.

Table 3.1

Gene	Probe name	Sequence 5’-3’
RNA_U6	probe_U6RNA	TCATCCTTGCGCAGGGGCCA
miR157abc_5p	probe_miR157	GTGCTCTCTATCTTCTGTCAA
miR159c	probe_miR159c	AGGAGCTCCCTTCAATCCAAA
miR398bc_3p	probe_miR398bc	TGTGTTCTCAGGTCACCCCTG

3.3.2 Real Time RT-PCR

Real Time RT-PCR analysis was performed on the RNA samples purified from *A. thaliana, A. thaliana* + Zn and *A. halleri* used for sRNA-Seq analysis. RNA extraction and cDNA synthesis were performed as previously described. Real Time RT-PCR analysis was performed using the KAPA SYBR® FAST ABI Prism® 2X qPCR Master Mix (Kapa Biosystems, Wilmington, MA, USA). The primers used are listed in **Table 3.2.** The $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) was applied to organize the expression data.

Table 3.2

Gene	Primer name	Primer sequence (5’-3)
MIR157	miR157c_F	GGTTGTTGACAGAAGATAGAG
	miR157c_R	CACAAAAGAGTAGAGATGCAG
MIR159	miR159_F	GAGGACAAGATTAGAGGAACT
	miR159_R	CAATCCAAACGAAGAGAAGA
MIR390	miR390_F	GTAGAGAAGAATCTGTAAAGCT
	miR390_R	AGTAAGAAGAGCCAATGAAACT
MIR395	miR395_F	CTTTCAAATTCCCACATGTTCTT
	miR395_R	ACTTCAGTGTTATATACAAACCAA
MIR398b	miR398b_F	TGAAGGTAGTGGATCTCGAC
	miR398b_R	GGGTGACCTGAGAACACATG
MIR408	miR408_F	GGCCAATTTCAAAGGTTAGATT
	miR408_R	TTCAGCTCCACTTCATGAATG
ACTIN (2/8)	#F_ACT2	GAACTACGAGCTACCTGATG
	#R_ACT2	CTTCCATTCCGATGAGCGAT

3.4 Expression analysis of pre-miR398b and pre-miR408 under Zn treatment

To quantify the relative expression of *pre-miR398b* and *pre-miR408* in *A. thaliana* and *A. halleri* under Zn excess, Real Time RT-PCR was performed. For the experiment, *A. thaliana* and *A. halleri* were grown hydroponically for two weeks in standard half-strenght Hoagland's solution and then transferred for several hours in a modified solution containing Zn excess (20 μM of $ZnSO_4$ for *A. thaliana;* 100 μM of $ZnSO_4$ for *A. halleri*). The following steps (RNA extraction, cDNA synthesis, genomic DNA removal and Real Time RT-PCR) were performed as previously indicated.

The expression of the targets was evaluated by Real Time PCR with specific primers reported in **Table 3.3.** The $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) was applied to organize the data.

Table 3.3

Gene	ATG number	Primer name	Sequence 5'-3'
miR398B	AT5G14545	398b_F	TGAAGGTAGTGGATCTCGAC
		398b_R	GGGTGACCTGAGAACACATG
miR408	AT2G47015	408_F	GGCCAATTTCAAAGGTTAGATT
		408_R	TTCAGCTCCACTTCATGAATG
ACT2/8	AT3G18780	ACT2/8_F	AACATTGTGCTCAGTGGTGG
	AT1G49240	ACT2/8_R	GACCTTAATCTTCATGCTGCT

3.5 Promoter analysis of miR398b and miR408

3.5.1 Constructs preparation and *A. thaliana* transformation

The promoter regions of *MIR398b* and *MIR408* were amplified by means of High-Fidelity Platinum® *Pfx* DNA Polymerase (Thermo Fisher Scientific) from genomic DNA of *A. thaliana* and *A. halleri;* the amplified fragments were cloned into the pGEM®-T easy vector (Promega), sequenced and used as template for following cloning strategies. Obtained amplicons were cloned in the pGEM®-T easy vector (Promega). Both pGEM-pMIR398b and pGEM-pMIR408 constructs were cut using specific restriction enzymes to allow directional cloning of the pMIR398b and pMIR408 fragments into the pBI121*::GUS* previously prepared. The primers used are listed in **Table 3.4.**

The final vectors pBI121-pMIR398B*::GUS* and pBI121-pMIR408::*GUS* of both *A. thaliana* and *A. halleri* were introduced by electroporation into competent *A. tumefaciens* cells, strain EHA105. which were used for *A. thaliana* transformation by floral dip (Clough and Bent, 1998).

Transformed plants with pBI121-pMIR398B::*GUS* and pBI121-pMIR408::*GUS* were selected *in vitro* for their resistance to kanamycin (50 mg/L).

Table 3.4

Gene	Primer name	Primer sequence (5'-3)
pMIR398b	#F_HindIII_p398b	AAGCTTAACCGATTCTAATAAGCCAAATA
	#R_BamHI_p398b	GGATCCGTCGAGATCCACTACCTTCA
pMIR408	#F_HindIII_p408	AAGCTTAACAATCTCTAATCTACAAACAAA-
	#R_XbaI_p408	TCTAGATAACCTTTGAAATTGGCCAGTT

3.6 GUS histochemical assay

Plants were incubated overnight at 37°C in GUS reaction buffer (100 mM sodium phosphate buffer pH 7.0, 1 mM EDTA pH 8.0, 2 mM potassium ferrocyanide, 2 mM potassium ferricyanide, 1% Triton X-100, 500 mg/L 5-bromo-4-chloro-3-indolyl β-D-Glucuronide in dimethylformamide) to detect *GUS* expression (Basu *et al.*, 2003). Chlorophylls were then removed by incubating tissues in ethanol 70% at 70°C. A Leica MZ16 microscope (Leica Microsystem GmbH) was used to examine the plants.

3.6.1 Treatments on pMIR398::*GUS* and pMIR408::*GUS* transformed plants

Plants transformed with the pMIR398B::*GUS* and pMIR408::*GUS* cassette were grown *in vitro* in solid MS medium. Metal stress treatments were performed for 6 hours in Petri dishes with 1X MS modified medium. The following conditions were performed: MS (control condition); 250 μM $ZnSO_4$; 50 μM $CuSO_4$; 250 μM $ZnSO_4$ + 50 μM $CuSO_4$.

3.7. Generation of *A. thaliana* transgenic lines overexpressing *pre-AhMIR408*

3.7.1 Construct preparation and *A. thaliana* transformation

The complete sequence of *pre-MIR408* were amplified from genomic DNA of *A. halleri* (**Table 3.5**) and the amplified fragment were cloned into the pGEM®-T easy vector (Promega), sequenced and utilized as template for following cloning strategies. *pre-AhMIR408* was cloned in the Gateway pDONR201 vector (Thermo Fisher Scientific, Waltham, MA, USA) and then transferred to the expression vector pH2GW7 by LR recombination (The Gateway® LR Clonase TM enzyme mix kit, Thermo Fisher Scientific). The construct pH2GW7-*pre-AhMIR408* was then introduced by electroporation into competent *A. tumefaciens* cells, strain GV3101 and used for *A. thaliana* transformation by floral dip (Clough and Bent, 1998). Transformed plants with pH2GW7-*pre-AhMIR408* were selected *in vitro* for their resistance to hygromycin (15 mg/L) and the integration of the transgene in the genome of *A. thaliana* plants was confirmed by PCR using a combination of specific primers.

Transformed plants were tested for *MIR408* expression by RT-PCR (data not shown) and 3 indipendent lines were propagated until stable homozygous T3 lines were obtained, which were used for experimentation.

Table 3.5

Gene	Primer name	Sequence 5'-3'
pre-AhMIR408	#F_att_pre-MIR408	*GGGGACAAGTTTGTACAAAAAAGCAGGCT*CTGGCCAATTTCAAAGGTTAGA
	#R_att_pre-MIR408	*GGGGACCACTTTGTACAAGAAGCTGGGT*AAACCCATAGCTTGTCGTCAG

3.7.2 Zn tolerance assay: *In vitro* analysis

Seeds of *A. thaliana* WT and 35S::*pre-AhMIR408* were germinated and grown on standard MS and MS medium modified with 50 µM (+Zn) and 100 µM of $ZnSO_4$ (++Zn). The primary root length and shoot fresh weight was measured after 10 days.

3.8 Statistical analysis

Statistical analysis of data was analyzed using the one-way analysis of variance (ANOVA) followed by a post hoc Tukey's test performed with GraphPad Prism 7 (GraphPad Software) as previously described.

4. RESULTS AND DISCUSSION

4.1 sRNA-Seq analysis

In the past years, high-throughput sequencing technologies have been applied to study in greater detail the biological roles of several miRNAs *in planta* in different processes, including biotic and abiotic stressful conditions, nutrient homeostasis, hormone signaling and response to heavy metals (Mendoza-Soto *et al.,* 2012; Pegler *et al.,* 2019; Jiu *et al.,* 2019; Wani *et al.,* 2020).

In this work, a sRNA-Seq analysis was performed on Zn-treated and control *A. thaliana* and *A. halleri* plants with the aim of identifying Zn-responsive miRNAs. Zinc represents one of the essential micronutrients for plants, especially for its known role as a cofactor for the activity of numerous enzymes, response against herbivores and pathogens (Cabot *et al.,* 2019) and others, although high concentrations of this element are toxic to plants (Broadley *et al.,* 2007). Recent studies have been focused on the role of sRNAs in response to variable concentrations of Zn (see Zeng *et al.,* 2019). Several miRNAs have been confirmed to be involved in response to heavy metals (Jalmi *et al.,* 2018; Ding *et al.,* 2020), although no information is actually available regarding their possible involvement in hyperaccumulator and hypertolerant plants, which therefore remains an aspect of great interest.

For this reason, in addition to *A. thaliana*, *A. halleri* was also used in this work to possibly understand the involvement of RNA molecules in the mechanisms of Zn hypertolerance and hyperaccumulation.

In this experiment, three sets of sRNAs were prepared from samples of 3-week-old *A. thaliana* (control), *A. thaliana* treated with excess Zn (500 μM $ZnSO_4$) and *A. halleri*. Five plants per treatment were used for the extraction of sRNAs. The shoots were harvested, frozen in liquid nitrogen and small RNAs were extracted by means of mirPremier® microRNA Isolation Kit following the manufacturer's instructions. The quality of the RNA samples was checked with an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA) and subsequently the three RNA samples were sent to IGA Technology Services (Udine, Italy) for sRNA library construction and sequencing using the Illumina HiSeq 2500 platform. The raw reads have been filtered, trimmed and processed to remove adapter dimers, low quality reads, common RNA families (tRNA, rRNA, snRNA) and repeats as described in Section 3.2. The remaining sequences were then aligned (with no more than two mismatches in the three libraries) to known *Arabidopsis* miRNAs listed in miRBase database (http://www.mirbase.org/index.shtml; Kozomara and Griffiths Jones, 2014) and the miRDeep-P pipeline (Yang and Li, 2011) was applied to categorized miRNAs. The analysis of the differently modulated miRNAs in the samples was performed as described in Materials and methods (Section 3.2).

In total, 100 microRNAs were found to be expressed differently in *A. thaliana*, *A. thaliana* + Zn and *A. halleri*, which belonged to the 44 conserved families that have already been characterized and involved in many biological processes (see Pegler *et al.,* 2019; **Fig. 4.1 A-B**).

Although the majority of these small RNA molecules and their targets are well known in *Arabidopsis*, the psRNA Target program (http://plantgrn.noble.org/psRNATarget/; Dai *et al.,* 2011) was used to predict the targets of each miRNA. Both 5p and 3p mature sequences were identified and used for the statistical analysis, since the single RNA strands are known to have a biological role *in planta* (Liu *et al.,* 2017).

The results obtained by sRNA-Seq were compared with each other to obtain more information on the possible different regulation of miRNAs expression in *A. thaliana* and *A. halleri.*

The results obtained can be summarized as follows:

- *A. halleri* vs *A. thaliana*: 33 microRNAs are significatively up-regulated and 67 down-regulated (**Table S1-S2**). In particular, the majority of these miRNAs are involved in plant development (20 miRNAs up-regulated; 27 miRNAs down-regulated), abiotic stress responses (6 miRNAs up-regulated; 13 miRNAs down-regulated) and nutrient homeostasis (5 miRNAs up-regulated; 8 miRNAs down-regulated), although also miRNAs with a role in biotic stress responses (1 miRNA up-regulated; 1 miRNA down-regulated) were identified. For several miRNAs it was not possible to determine putative target and their biological function, and therefore they were considered as "unknown" (1 miRNA up-regulated; 18 miRNAs down-regulated);

- *A. halleri* vs *A. thaliana* + Zn: 33 microRNAs are significatively up-regulated and 63 down-regulated (**Table S3-S4**). Most of the differentially expressed miRNAs play a role in plant development (20 miRNAs up-regulated; 26 miRNAs down-regulated), abiotic stress responses (6 miRNAs up-regulated; 11 miRNAs down-regulated) and nutrient homeostasis regulation (5 miRNAs up-regulated; 8 miRNAs down-regulated). miRNAs with a role in biotic stress responses (1 miRNA up-regulated; 1 miRNA down-regulated) were also identified, and many others for which it was not possible to determine putative target and biological function ("unknown"; 1 miRNA up-regulated and 17 miRNAs down-regulated);

- *A. thaliana* + Zn vs *A. thaliana:* 4 microRNAs are significatively up-regulated, with a role in plant development (1 miRNA), nutrient homeostasis (1 miRNA), response to abiotic stress (1 miRNA) and 4 down-regulated (**Table S5-S6**), with a role in plant development (1 miRNA), although for the other three it was not possible determine the target (considered "unknown"; 1 miRNA up-regulated and 3 miRNAs down-regulated).

It is noteworthy that certain miRNAs expressed in *A. halleri* in a constitutive manner can be related to heavy metal tolerance in plant species (Jalmi *et al.,* 2018; Noman *et al.,* 2019; Ding *et al.,* 2020).
Most of them are involved in response to abiotic and biotic stresses and regulation of plant development, and many others take part in oxidative stress responses and nutrient homeostasis regulation (Khraiwesh *et al.,* 2012; Kehr *et al.,* 2013; Jalmi *et al.,* 2018). Furthermore, many of these sRNAs targeted various transcription factors which are fundamental for many biological processes, including mineral nutrition and hormone signaling (Samad *et al.,* 2017; Jalmi *et al.,* 2018; Pegler *et al.,* 2019). It is notable that certain miRNAs were also previously identified as involved in the response to variable Zn concentrations in many plants (Shi *et al.,* 2013; Zeng *et al.,* 2019), hence confirming their involvement in mineral nutrition. These data seem to suggest that the activity of certain miRNAs could be important in the hyperaccumulator plants' ability to adapt to excess Zn. Nevertheless, we must also consider that some metal-responsive miRNAs, which were observed to be differentially modulated, are also implicated in other biological processes, including plant development (Li and Zhang, 2016). Because *A. halleri* and *A. thaliana* are two different plant species, we cannot therefore exclude that the expression of several miRNAs may in part depend on their different developmental profile.

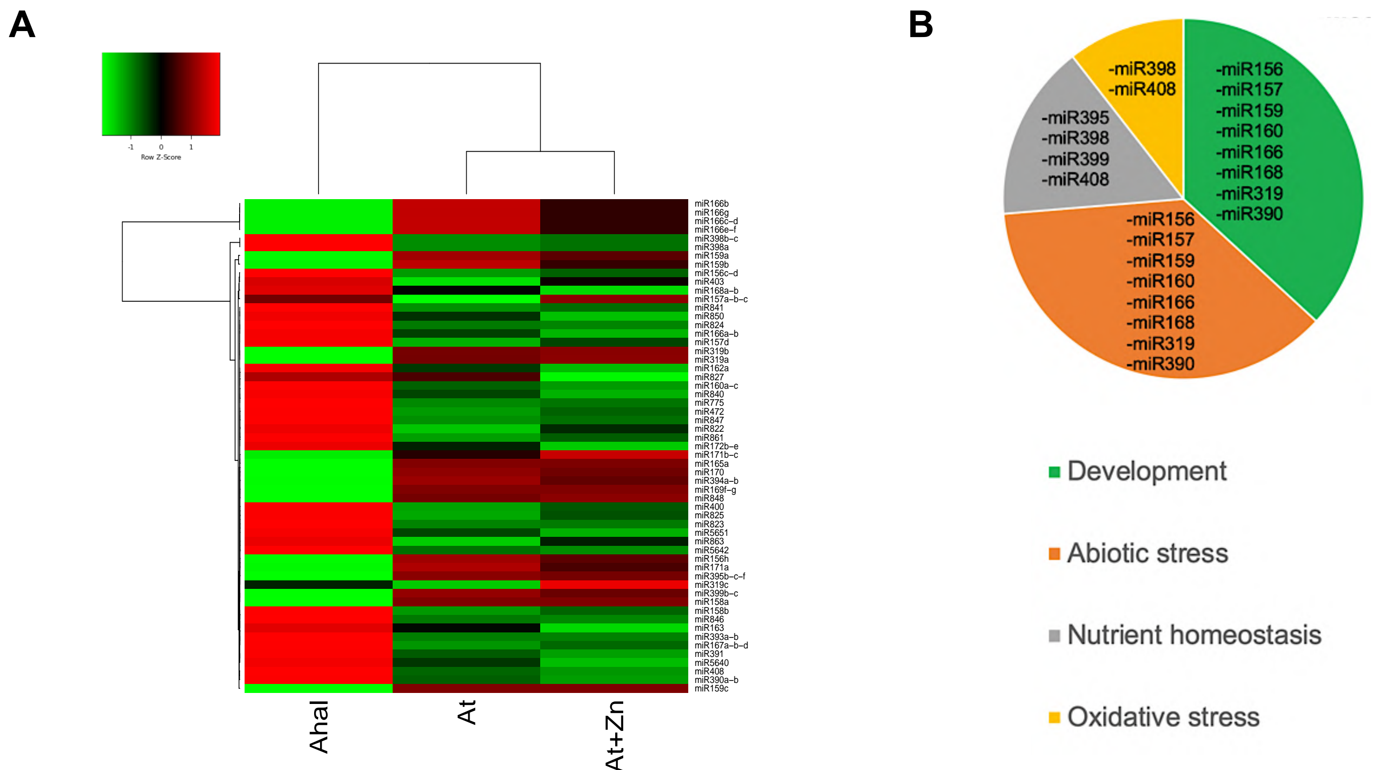

Figure 4.1: A) Heatmap of the normalized read count representing the hierarchical clustering of miRNA expression in *A. thaliana, A. thaliana* + Zn and *A. halleri.* The heatmap was made by R software. B) Circle chart of the functional classes into which the *A. halleri* miRNAs expressed differently in control and + Zn *A. thaliana* are grouped.

4.2 Validation of miRNA-Seq analysis

To validate the miRNA-Seq analysis data, two groups of conserved metal-responsive miRNAs were taken into account, since their potential importance in response to excess Zn (Mendoza-Soto *et al.,* 2012; Shi *et al.,* 2013; Zeng *et al.,* 2019): miRNAs involved in plant development and those playing a role in nutrient homeostasis.

4.2.1 miRNAs involved in plant development

Among the various roles of plant miRNAs, in recent years their ability to regulate development, growth and senescence under normal or stressful conditions has been studied (Xie *et al.,* 2010; Shriram *et al.,* 2016). Several miRNA targets are transcription factors (TFs), that modulate different genes involved in a variety of processes, including adaptation to the environmental conditions (Shriram *et al.,* 2016; Shu *et al.,* 2016; Samad *et al.,* 2017). The most relevant miRNAs that were observed to be differently expressed in *A. halleri* and *A. thaliana* were considered for the experimental validation and are described in greater detail below.

4.2.1.1 miR157

miR157, like miR156, is one of the most conserved small regulatory molecules in plants (Axtell and Bowman, 2008). This miRNA plays several roles in plant development, modulating the expression of some members of SQUAMOSA PROMOTER-LIKE (SBP or SPL) TFs (He *et al.,* 2018). In particular, the synergic action of both miR156 and miR157 has a strong impact on shoot development in *Arabidopsis,* inducing changes in leaf development (Wang *et al.,* 2015; Xu *et al.,* 2016; He *et al.,* 2018). Many researches have proved a modulation of miR156-miR157 by metal exposure (Jalmi *et al.,* 2018; Sanz-Carbonell *et al.,* 2019) and the response to Zn deficiency condition (Shi *et al.,* 2013), confirming that excess metal can modify plant growth and development (Dal Corso *et al.,* 2013). Interestingly, a possible direct correlation between SBP transcription factors and the regulation of Zn homeostasis in plants has been recently reported (Shulten *et al.,* 2019), suggesting a possible involvement of miR157 in mineral nutrition. In addition, the highly similar miR156 seems to take part in the regulation of the cellular levels of phytochelatins, which are important for the responses to Al, Cd and other metals (Yang and Chen, 2013). In this work, only a slight difference was observed in the expression of miR157 between untreated an Zn-treated *A. thaliana* plants, and constitutive lower levels of gene expression were found in *A. halleri* in comparison with (treated and control) *A. thaliana*. Northern Blot analysis and Real Time RT-PCR were therefore performed on the RNA extracted from the shoots of control and Zn-treated *A. thaliana* and *A. halleri* plants in order to confirm these data. As shown in **Fig. 4.2**, Real Time RT-PCR on the precursor

sequence displayed no significant differences in miR157 expression between the two plant species; however, miR157 was down-regulated in *A. thaliana* upon Zn exposure and constitutively low expressed in *A. halleri* (B), thus confirming the sRNA-Seq analysis data and indicating possible differences in the miR157 expression pattern in these two plant species. It is noteworthy that the same down-regulation of miR157 has been described in maize subjected to Cd exposure (Gao *et al.,* 2019), as well as for the very similar miR156 (Bukhari *et al.,* 2015; Jalmi *et al.,* 2018). This result may indicate that this type of modulation of miR156/157-target genes expression levels could be a conservative response mechanism to metal stress in plants (Yang and Chen, 2013; He *et al.,* 2016; Jalmi *et al.,* 2018), which probably implies a morphological adaptation to these conditions and the regulation of the activity of phytochelatins.

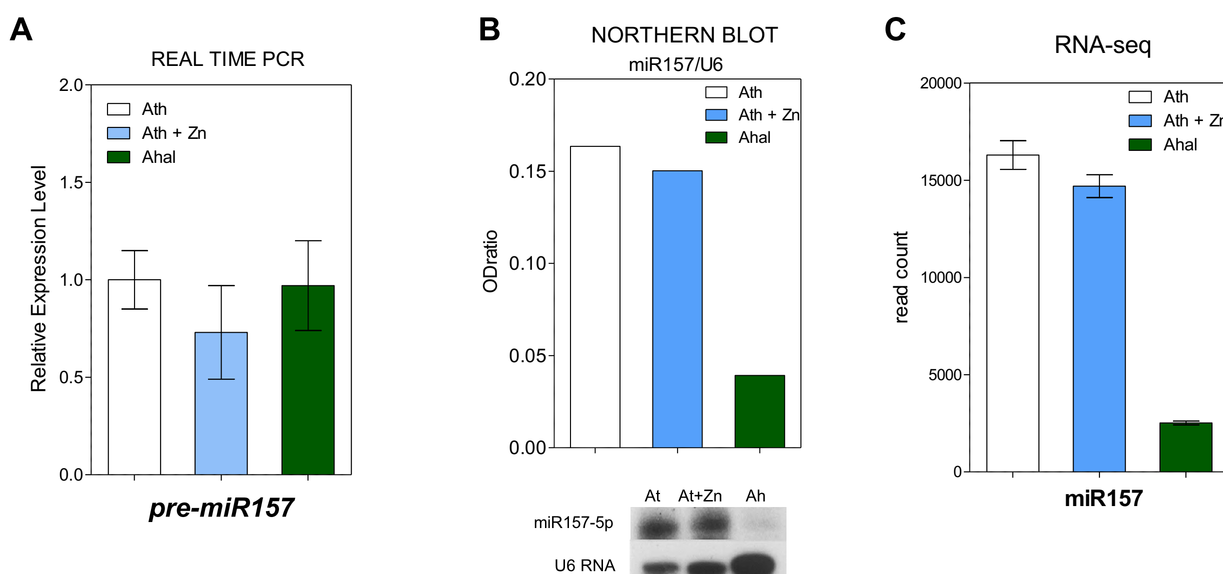

Figure 4.2: Relative expression of *pre-miR157* in *A. thaliana, A. thaliana* + Zn and *A. halleri* resulting from Real Time RT-PCR (A) and Northern Blot Analysis (B) data validation of the sRNA-Seq experiment (C).

4.2.1.2 miR159

miR159 family is one of the most ancient in plants (Axtell *et al.,* 2005). These small regulatory RNAs have been confirmed to target members of the gibberellic acid MYB (GAMYB) or GAMYB-like genes in monocots, dicots and gimnosperms (Li *et al.,* 2016), which are not only involved in the regulation of seed germination and anther formation, but also many other aspects of plant growth and development (Samad *et al.,* 2017; Zhao *et al.,* 2017; Millar *et al.,* 2019). miR159 in particular modulates the expression of two members of the MYB family of TFs, MYB33 and MYB65, regulating cell proliferation, reproduction and programmed cell death in *Arabidopsis* (Li *et al.,* 2016). Moreover, it seems to strongly interact with another conserved miRNA, miR319, which modulates TEOSINTE BRANCHED (TCP) TFs fundamental for plant growth, as demonstrated by Short Tandem Target Mimic Technology (Danisman, 2016; Koyama

et al., 2017; Peng *et al.,* 2018). Research works have recently reported that miR159 is modulated by various abiotic stresses, including metal excess and N starvation (Paul *et al.,* 2015; Gao *et al.,* 2019; Zuluaga and Sonnante, 2019). Since this miRNA is conserved among plant species, it is possible that the miR159-MYB interaction could be important for metal tolerance, probably due to a remodulation of plant development under these conditions (Mendoza-Soto, 2012; Zhou *et al.,* 2012; He *et al.,* 2016). In particular, MYB59 is involved in the response to Cd stress in *A. thaliana* (Fasani *et al.,* 2019) and OsARM1 is responsive to Arsenic in rice (Wang *et al.,* 2017), hence confirming the importance of these TFs in many biological processes *in planta*. In our experiments, miRNA-Seq analyses revealed an up-regulation of miR159 expression in *A. thaliana* upon Zn exposure, and constitutively higher levels of gene expression in *A. halleri*. Real Time RT-PCR and Northern Blot analysis were performed, confirming the modulation pattern of miR159, in agreement with the data available in the literature relative to other plant species treated with metal excess (Jalmi *et al.,* 2018; **Fig. 4.3**), although the opposite result was found in *B. napus* exposed to Cd (Zhou *et al.,* 2012). Interestingly, miR159 appears to be also involved in the regulation of the expression levels of ABC transporters, which play a role against metal stress in plants (Zhou *et al.,* 2012; Yang and Chen, 2013; Song *et al.,* 2014). Therefore, it is possible that high levels of Zn in the medium could modulate the expression of miR159 and of some members of ABC transporter genes, in order to avoid metal toxicity, as well as MYB TFs levels, which could participate to heavy metal stress responses in plants (Fasani *et al.,* 2019). Nevertheless, it is also important to note that miR159 is involved in the regulation of plant development (Li *et al*., 2016; Zhao *et al.,* 2017), suggesting that the different expression observed in *A. thaliana* and *A. halleri* plants could be ascribed to differences in their growth rather than a putative constitutive mechanism allowing *A. halleri* to respond to excess metal, although it remains only an assumption.

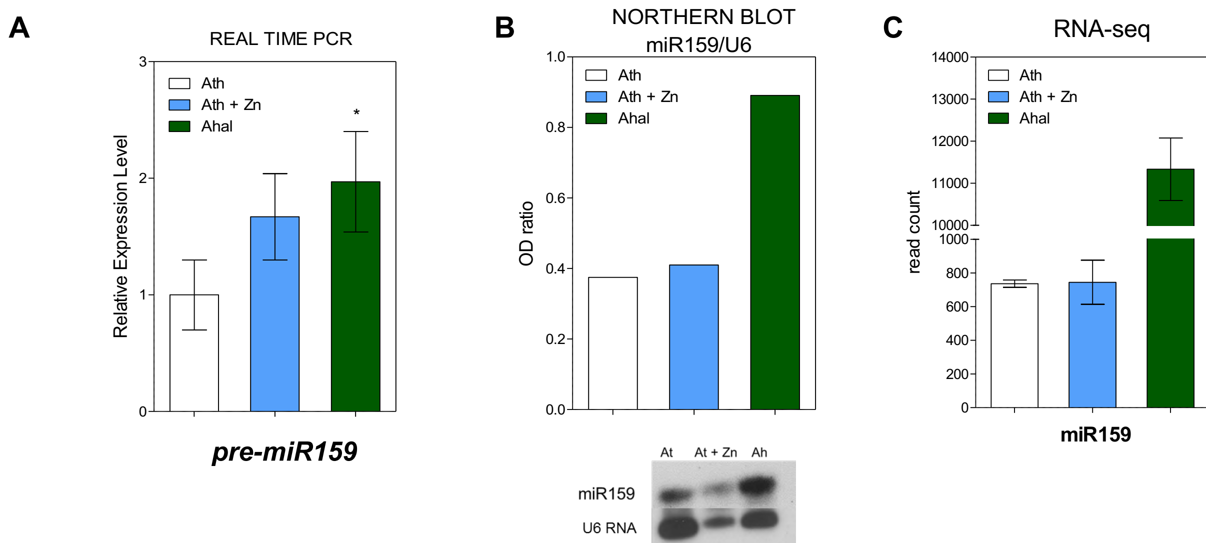

Figure 4.3: Relative expression of *pre-mi*R*159* in *A. thaliana, A. thaliana* + Zn and *A. halleri* resulting from Real Time RT-PCR (A) and Northern Blot Analysis (B) data validation of the sRNA-Seq experiment (C).

4.2.1.3 miR390

Changes in growth and development upon metal exposure are an important strategy to avoid problems of toxicity representing a typical response to stress displayed by *Arabidopsis* and other plant species. The plasticity of root development determines the architecture of the entire root system, regulating water and nutrient uptake but also responding to heavy metal stress (Kochian *et al.,* 2005; Marin *et al.,* 2010). This complex mechanism is mainly regulated by hormones, such as auxin and hormone-responsive genes, and must be finely tuned to maximize the uptake of essential elements and to avoid toxicity in the presence of heavy metals (Marin *et al.,* 2010). miR390 and miR393 in particular play key roles in the root development of various plants, under normal and stressful conditions (Mendoza-Soto *et al.,* 2012; Ding *et al.,* 2016, Lu *et al.,* 2018). Particularly, in *Arabidopsis*, rice, and other plants, miR390 is modulated by non-essential elements such as Cd and Hg (Zhou *et al.*, 2012; Ding *et al.,* 2016, Lu *et al.,* 2018) inducing the formation of lateral roots avoiding cellular damages. As shown in **Fig. 4.4,** miR390 was up-regulated in *A. thaliana* upon exposure to Zn and lower levels of miR390 expression were found in *A. halleri*, although the differences were not significant. Down-regulation of miR390 expression was previously proved in *M. truncatula* and rice exposed to metal stress (Chen *et al.,* 2012) and overexpression of miR390 reduced the tolerance to Cd in transgenic plants (Ding *et al.,* 2016), confirming the direct link between miR390-target genes and response to this type of stress. Real Time RT-PCR analysis has confirmed the pattern of miR390 expression found by sRNA-Seq analysis, also suggesting possible differences in the modulation of plant development in response to metal stress in *A. thaliana* and *A. halleri*, which grow in diverse soils (Staňová *et al.,* 2012). The low levels of miR390 expression observed in *A. halleri*, and the consequent impact on the root architecture by the regulation of the expression of ARF TFs, may reflect a different adaptability to the presence of Zn excess between these species (Jalmi *et al.,* 2018) which are native to different type of soils.

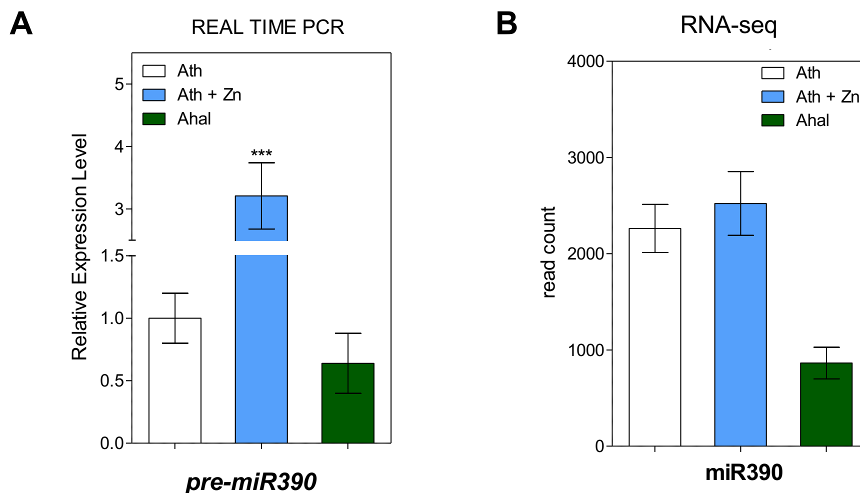

Figure 4.4: Relative expression of *pre-miR390* (A) in *A. thaliana, A. thaliana* + Zn and *A. halleri* resulting from the Real Time RT-PCR data validation of the sRNA-Seq experiment (B).

4.2.2 miRNAs involved in nutrient homeostasis

The acquisition of essential nutrients from the soil is fundamental for plant growth and development (Sperotto *et al.,* 2014); under conditions of nutrient starvation, plants activate various responses to enhance mineral uptake and maintain a balanced nutrient homeostasis (Mendoza-Soto *et al.,* 2012; Jung and McCouch, 2013). Given that heavy metals are chemically or physically similar to essential micronutrients, the presence of high concentrations of toxic elements in the soil has been observed to provoke nutrient deficiency symptoms in the plant (Dal Corso *et al.,* 2013), therefore inducing a variety of responses to counteract these stressful conditions. In *Arabidopsis* and other plant species, several miRNAs have been observed to play key roles in the regulation of nutrient homeostasis by modulating their target and enhancing metal uptake and homeostasis (Liang *et al.,* 2015; Paul *et al.,* 2015; Noman *et al.,* 2019). In this work, certain nutrient-responsive miRNAs displayed a different modulation pattern in the two plant species, *A. thaliana* and *A. halleri*, thus suggesting that the Zn/Cd hyperaccumulation/hypertolerance trait of the latter species could also be determined by miRNAs. Some metal-responsive miRNAs were therefore considered for the experimental validation.

4.2.2.1 miR395

miR395 was one of the first miRNAs to be described as involved in the plant's response to nutrient deficiency and metal stress (Jones-Rhoades and Bartel, 2004). miR395 normally regulates the assimilation and distribution of sulphur (S) in plant tissues by targeting *SULTR* and *APS* genes (Matthewman *et al.,* 2012). The fundamental role of miR395 in response to Cd has been largely demonstrated in several plant species, indicating the involvement of this small RNA molecule in alleviation of oxidative stress by modulation of S homeostasis (Yang and Chen, 2013; Zhang *et al.,* 2013; Chmielowska-Bąk *et al.,* 2014). In this work, sRNA-Seq analysis showed that miR395 seems to be constitutively up-regulated in *A. halleri* compared to *A. thaliana*, which also displayed a positive modulation of miR395 expression under Zn treatment, consistently with the pattern observed under Cd treatment (Zhang *et al.,* 2013; Jagadeeswaran *et al.,* 2014; Gao *et al.,* 2019). As shown in **Fig. 4.5**, *pre-miR395* was up-regulated in *A. thaliana* after exposure to Zn, confirming the Cd and Zn may induce similar regulation pattern of miR395 expression (Gielen *et al.,* 2016). Significantly higher levels of *pre-miR395* were also found in *A. halleri* as compared to *A. thaliana*, indicating a possible constitutive expression of this RNA in this Zn/Cd hyperaccumulator plant. The fact that miR395 is up-regulated by excess Zn may indicate that high S levels for the synthesis of phytochelatins could be a mechanism required by plants to avoid metal toxicity, including Cd (Ernst *et al.,* 2008), and that can be constitutively adopted by *A. halleri* to respond to excess levels of metals (Yang and Chen, 2013; Zhang *et al.,* 2013; Fan *et al.,* 2018).

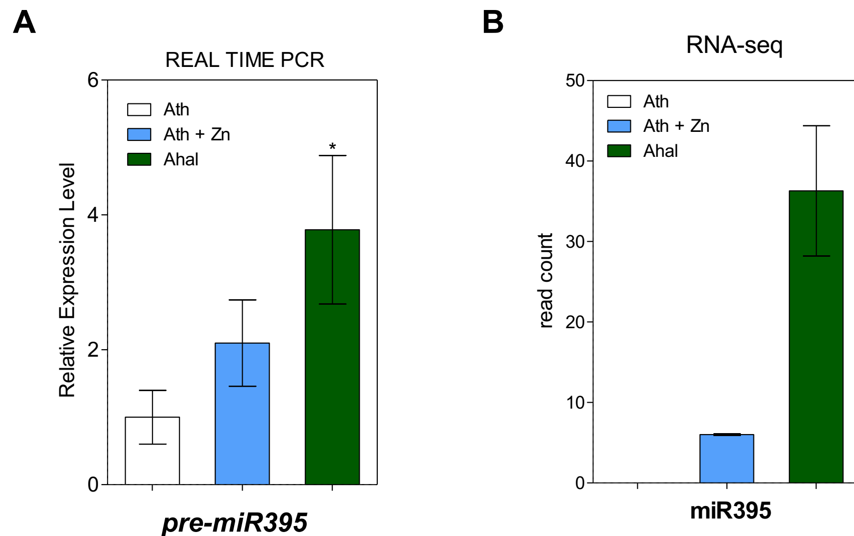

Figure 4.5: Relative expression of *pre-miR395* (A) in *A. thaliana, A. thaliana* + Zn and *A. halleri* resulting from Real Time RT-PCR and Northern Blot (B) data validation of the sRNA-Seq experiment.

4.2.2.2 miR398 and miR408

Copper (Cu) is one of the most important micronutrients for plants (Paul *et al.*, 2015), since it is necessary for several biological processes, including photosynthesis and respiration; Cu starvation, brought about by certain types of stress, is therefore potentially very dangerous (Abdel-Ghani *et al.,* 2006). Much research in recent years has clarified the role of several Cu-miRNAs in response to Cu, Cd and Zn (Pilon *et al.,* 2017; Schulten and Krämer, 2018; Zheng *et al.,* 2019). The two conserved Cu-responsive miRNAs, miR398b and miR408, were used for the experimental validation of the sRNA-Seq data, since their conservative role in regulation of many abiotic stresses (Pilon, 2017). sRNA-Seq analysis revealed that miR398b and miR408 are regulated differently in *A. halleri* and *A. thaliana.* These miRNAs are able to modulate levels of non-essential Cu-proteins such as Copper/Zinc superoxide dismutases (CSDs) and Laccases (LAC) and are two of the most conserved and fundamental Cu-miRNAs in plants (Ma *et al.,* 2015; Pilon, 2017). In addition, the overexpression of these miRNAs or their targets have been reported to have a very strong impact on plant growth under stressful conditions (Ma *et al.,* 2015; Leng *et al.,* 2017), confirming the potential role of these two multi-stress responsive miRNAs as tool for genetic modification of crops (Zhang *et al.,* 2017; Tang and Chu, 2017). The involvement of these Cu-miRNAs in response to nutrient deficiency, metal exposure, oxidative stress response and other stresses has also been widely reported (Lu *et al.,* 2013; Wang *et al.,* 2014; Leng *et al.,* 2017; Jalmi *et al.,* 2018). In our experiment, both miRNAs resulted to be down-regulated in *A. halleri* in comparison with *A. thaliana*, suggesting different regulation mechanisms in these two plant species. Real Time RT-PCR and Northern Blot analysis were performed to confirm the results obtained by sRNA-Seq analysis.

Although there are three *MIR398* loci (*MIR398a, MIR398b,* and *MIR398c*) in *Arabidopsis*, only miR398b was taken into account for these analyses, given its major role in Cu-homeostasis regulation compared to

the others (Sunkar *et al.,* 2006). Constitutive lower levels of miR398b expression were found in *A. halleri* than Zn-treated and untreated *A. thaliana*, which displayed a positive modulation of miR398b expression upon Zn excess (**Fig.4.6**) consistently with Cd exposure (Smeets *et al.,* 2013; Qui *et al.,* 2016). Interestingly, overexpression of SODs in *Arabidopsis* and tobacco was observed to increase Cd and Cu tolerance as compared to its WT (Leng *et al.,* 2017; Li *et al.,* 2017), confirming their essential role in ROS detoxification brought about by stress. From this data it is possible to speculate that the constitutive down-regulation of miR398b in *A. halleri* could be related to a greater expression of *CSD1* and *CSD2* (Sunkar *et al.,* 2006) which would allow it to tolerate greater amounts of Zn/Cd in the growth medium and respond to oxidative stress, as also reported in other plant species (Jozefczak *et al.,* 2015; Leng *et al.*, 2017; Li *et al.,* 2017).

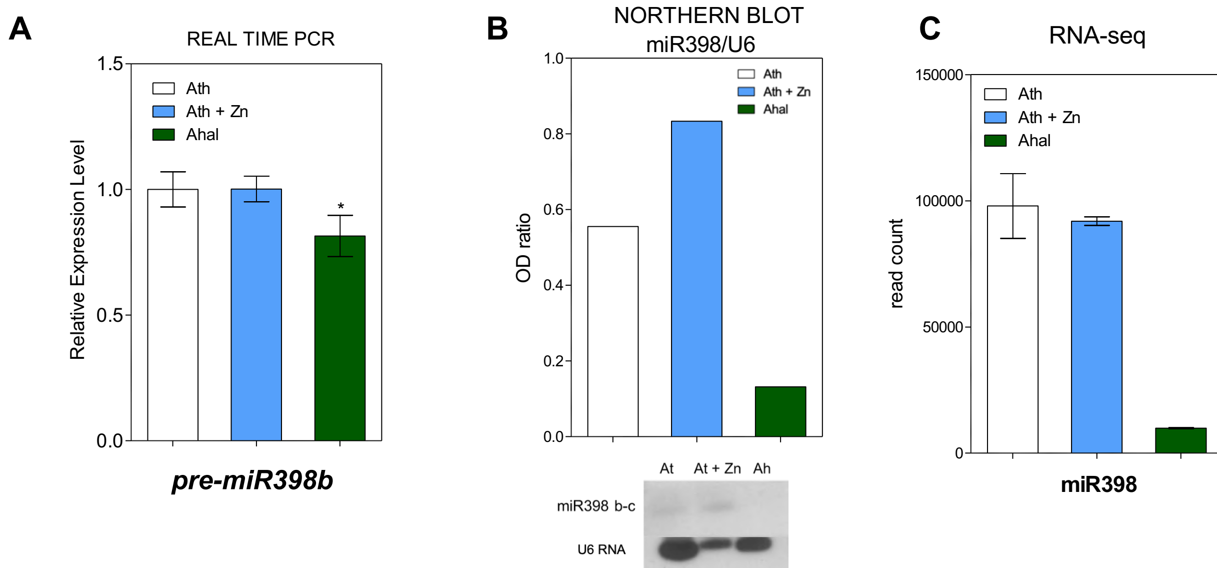

Figure 4.6: Relative expression of *pre-miR398b* **(A)** in *A. thaliana, A. thaliana* + Zn and *A. halleri* resulting from Real Time RT-PCR and Northern Blot **(B)** data validation of the sRNA-Seq experiment.

miR408 is a conserved small RNA molecule that plays a role in the plant's response to several types of abiotic stresses, including cold, metals, salinity, drought, oxidative stress and Cu shortage (Zhang and Li, 2013; Ma *et al.,* 2015; He *et al.,* 2016). As previously reported, miR408 is involved in the vegetative growth and plant adaptation to environmental cues (Zhang and Li, 2013; Carriò-Segui *et al.,* 2019). Constitutive expression of miR408 in *Arabidopsis* and other plant species increased their biomass and seed yield (Pan *et al.,* 2018; Song *et al.,* 2018). Moreover, the modulation of miR408 expression *in planta* strongly affected the response to Fe deficiency (Carriò-Segui *et al.,* 2019), indicating the involvement of this miRNA in the regulation of mineral nutrition. miR408, as well as the other Cu-miRNAs, is also responsive to Cd and Zn (Gielen *et al.,* 2016; Zeng *et al.,* 2019).

The data from the sRNA-Seq analysis were confirmed by Real Time RT-PCR performed on *pre-miR408:* in particular, constitutive lower levels of miR408 expression was found in *A. halleri* compared to *A. thaliana*, either treated or untreated with Zn (**Fig 4.7**). Moreover, miR408 resulted to be up-regulated under Zn excess condition in *A. thaliana*, in agreement with the data obtained in rice subjected to Zn treatment (Dong *et al.*, 2018). These results seem to confirm the possible competition between Cu and Zn (Gielen *et al.,* 2016; Dong *et al.,* 2018) and the involvement of Cu-miRNAs in the regulation of mineral nutrition (Shahbaz and Pilon, 2019). In particular, high levels of Zn in the medium can modulate Cu-related genes in order to maintain adeguate levels of Cu for essential processes or induce a general stress response *in planta* in which miR408 can participate (Ma *et al.,* 2015). However, it must also be considered that Laccases, targeted by miR408, take part in the formation of the plant cell wall (Zhao *et al.,* 2013; Schuetz *et al.,* 2014), a process which seems to be required for the response to a variety of stressful conditions, including heavy metal exposure (Loix *et al.,* 2017; Carriò-Segui *et al.,* 2019). It has been demonstrated that Fe concentration in the growth medium is responsible for the modulation of *LMCO* genes *in planta*, with a consequent effect on the lignification of the cell wall, which can directly interact with these elements (Carriò-Segui *et al.,* 2019). Moreover, miR408 also regulates the expression levels of an ascorbate oxidase, which is required for the response to ROS (see Yang and Chen, 2013). Since Fe and Zn seem to compete one to each other in plants (Xie *et al.,* 2019), it is possible to speculate that variable concentration of Zn in the medium can modify, similarly to Fe, the expression of miR408 and, thus, the expression of *LMCO* genes in order to better respond to these environmental changes (Abdel-Ghany and Pilon, 2008; Carriò-Segui *et al.,* 2019). Therefore, a different modulation of miR408 could also indicate a distinct ability of *A. halleri* to respond to high amounts of metals in the growth medium, mediated by a general remodulation of the Cu homeostasis, the developmental profile and the oxidative stress response.

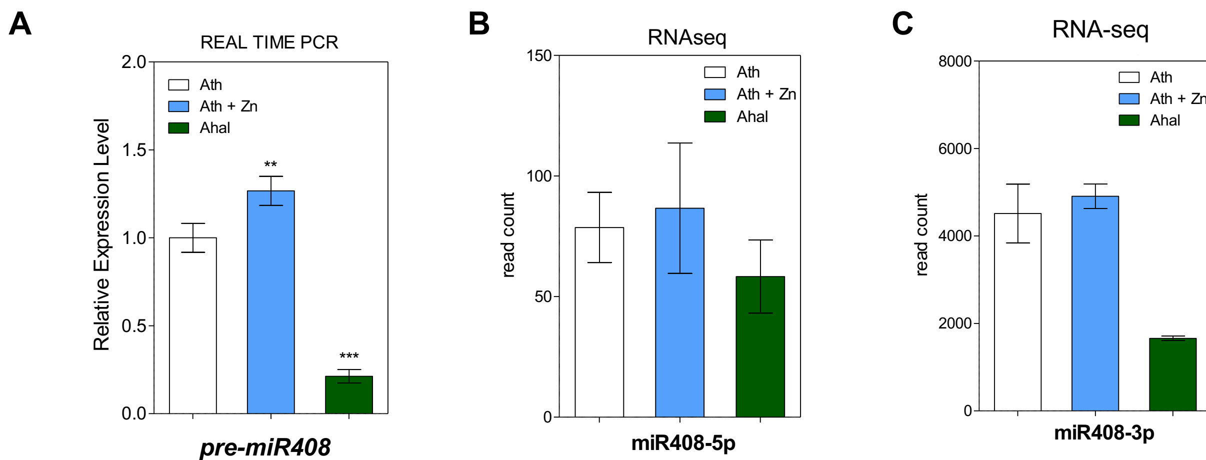

Figure 4.7: Relative expression of *pre-miR408* **(A)** in *A. thaliana, A. thaliana* + Zn and *A. halleri* resulting from Real Time RT-PCR and Northern Blot **(B)** data validation of the sRNA-Seq experiment.

4.3 Study of the roles of miR398b and miR408 in response to Zn in *Arabidopsis*

Although various microRNAs have been observed to be involved in the response to metal stress (Jalmi *et al.,* 2018), no information is available on their role in hyperaccumulator plants. Nevertheless, the putative involvement of these molecules, including Cu-miRNAs, in the strategies to avoid metal toxicity has been recently explored in various plant species (Gielen *et al.,* 2016; Pilon *et al.,* 2017; Schulten and Krämer, 2018).

The expression levels of miR398b and miR408 were investigated in *A. thaliana* and *A. halleri* treated with excess Zn. A Real Time RT-PCR analysis was performed on *pre-miR398b* and *pre-miR408* from the shoots of *A. thaliana* and *A. halleri.* These plants were grown under controlled conditions for 2 weeks and then treated with excess Zn (20 µM $ZnSO_4$ for *A. thaliana;* 100 µM of $ZnSO_4$ for *A. halleri*).

After 6 and 24 hours of exposure, the RNA was extracted from the shoots of all plants and retro-transcribed into cDNA and Real Time RT-PCR was performed using specific primers.

As shown in **Fig. 4.8**, both *pre-miR398b* and *pre-miR408* were induced in the presence of high Zn concentrations in the two species, following the same regulation pattern also described by other authors (Han *et al.,* 2016; Dong *et al.,* 2018; Gao *et al.,* 2019; Zeng *et al.,* 2019). Interestingly, miR398b in *A. halleri* was significantly modulated after 6 hours of treatment, whereas in *A. thaliana* this response was only evident after 24 hours. These data clearly indicate that excess Zn can induce a Cu-deficiency response in *Arabidopsis,* as well as for other plant species (Zeng *et al.,* 2019), which usually results in a regulated modulation of Cu-miRNAs to preserve adequate Cu levels in the cell for essential proteins (Pilon, 2017).

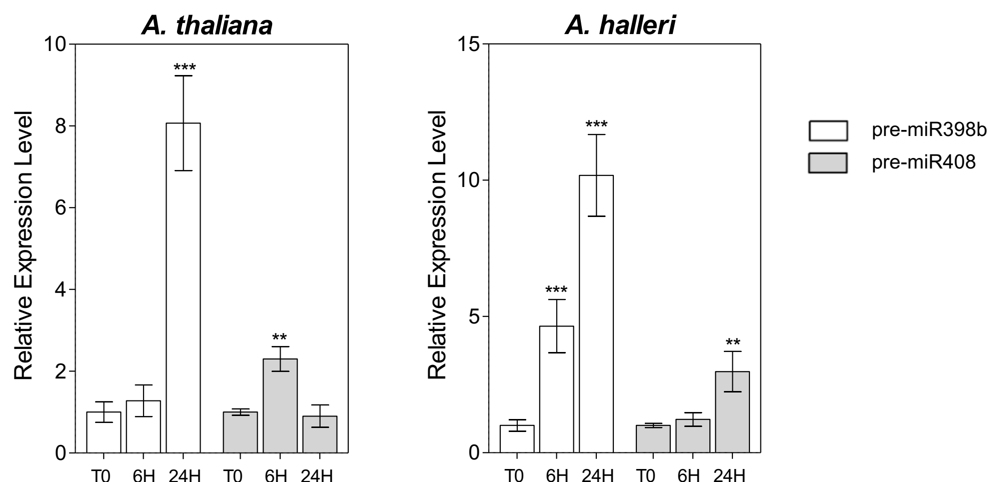

Figure 4.8: Real-Time RT-PCR measuring the expression of pre-*MIR398b* and pre-*MIR408* against that of the housekeeping gene *Actin2*. Plants of *A. thaliana* and *A. halleri* and were grown in hydroponics and subjected to excess Zn for 6 and 24 hours. T0 is time zero. Statistically significant variations, according to ANOVA (n = 3), are marked by asterisks. Bars correspond to standard errors, calculated as in Muller *et al.* (2002).

4.4 Expression analysis of miR398b

In order to better investigate the modulation of miR398b in the two *Arabidopsis* species exposed to excess Zn*,* the promoter sequence upstream of their miR398b precursors was amplified from their genomic DNA and then fused to the *GUS* reporter gene as described in Section 3.5.

4.4.1 Identification and analysis of the DNA motifs in pMIR398B of *A. thaliana* and *A. halleri*

A bioinformatic analysis on the promoters of *A. thaliana* and *A. halleri* miR398b was performed to understand the regulation patterns of this miRNA in both the non-accumulating and hyperaccumulator plant species, using the PLACE database tool (http://www.dna.affrc.go.jp/PLACE; Higo *et al.,* 1999) (**Fig. S7**). The promoter sequences of pAtMIR398b and pAhMIR398b were therefore analyzed with the Signal Scan Search tool in the PLACE database. Various *cis*-active elements responding to a number of biotic and abiotic stresses, such as cold (LTRE, Low Temperature Responsive Element), nutrient deficiency, presence of pathogens, as well as ones sensitive to ABA (ABRE, ABA responsive element), or light-regulated were found, confirming the importance of miR398 in response to different stresses *in planta* (Zhu *et al.,* 2011). In addition, some motifs for tissue localization were also identified. In particular, root-specific motifs were found in both promoters. The highly-conserved ATATT motif in particular, responsible for the expression in the vasculature (Elmayan and Tepfer, 1995), was present at much higher levels in the AhMIR398b promoter than that of AtMIR398b, suggesting that miR398b may be regulated differently in this Zn/Cd hyperaccumulator plant. These data also indicate that miR398b, as well as other nutrient-responsive miRNAs (Sunkar *et al.,* 2006), can be translocated from root to shoot in order to regulate nutrient uptake and cell homeostasis (Kehr *et al.,* 2013). Cu-related genes and Cu-miRNAs are both modulated by the activity of the transcription factor SPL7, which is sensitive to the conserved GTAC motif (CURE; copper-response element) and it is responsible for the regulation of Cu-miRNAs under normal and stressful conditions (Yamasaki *et al.,* 2009; Pilon, 2017; Schulten and Krämer, 2018). The modulation of Cu-related genes under Cd stress has been recently described in *Arabidopsis* (Gayomba *et al.,* 2013; Gielen *et al.,* 2016). Given that Cd and Zn are quite similar (Tkalec *et al.,* 2014; Gielen *et al.,* 2016) it is possible that excess Zn may induce the same regulation of Cu-related genes mediated by SPL7. Several CURE DNA motifs were found in the promoter region of both pAtMIR398b and pAhMIR398b (**Fig. 4.9**), although more abundantly in *A. halleri*, as well as sulphur-responsive elements (SUREs). These motifs have been shown to respond to S-concentration in *Arabidopsis* (Nakashita *et al.,* 2005) by modulating various S-responsive miRNAs (Huang *et al.,* 2010; Liang *et al.,* 2010) even under heavy metal exposure. This would suggest that miR398b may also respond to this nutrient deficiency condition.

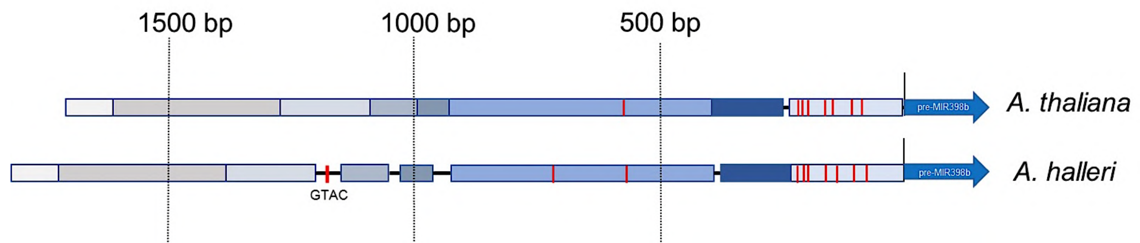

Figure 4.9 *In silico* analysis of *A. thaliana* and *A. halleri* miR398b promoters. CURE motifs (GTAC) are depicted in red.

4.4.2 Expression pattern of AtMIR398b

In order to clarify the expression pattern of miR398b in both *A. thaliana* and *A. halleri*, *GUS* expression was determined using transgenic lines of *A. thaliana* expressing pAtMIR398b::*GUS* and pAhMIR398b::*GUS* and grown under different conditions.

Both *A. thaliana* and *A. halleri* control plants (**Fig. 4.10**) displayed a ubiquitous expression of miR398b, with GUS signals in the shoots (leaves), the vascular root-shoot tissues and also the roots, in keeping with the results observed by Sunkar and colleagues (2006). AtMIR398b was expressed in both primary and lateral roots, especially young lateral primordia, but not in the apex of the primary root. This expression pattern is similar to that of the SPL7 TF (Araki *et al.,* 2018), which is known to directly regulate miR398b and other Cu-related genes (Ding and Zhu, 2009; Yamasaki *et al.,* 2009), thus confirming a direct interaction between miR398b and SPL7.

In order to understand the expression of miR398b under excess Zn conditions and verify whether Cu and Zn compete against each other as previously proposed (Remans *et al.,* 2012; Gielen *et al.,* 2016), transgenic *A. thaliana* plants expressing pAtMIR398b::*GUS* were grown in the presence of excess Zn, Cu, or both for 6 hours and a GUS assay was then performed. Compared to their controls, high levels of miR398b::*GUS* expression were found in the roots and shoots of Zn-treated plants, thus confirming the up-regulation of this miRNA by excess Zn (**Fig. 4.11;** Remans *et al.,* 2012). Interestingly, Zn exposure gave rise to a strong GUS signal in the trichomes, which are known to be one of the most important sink organs for metal storage under stress conditions (Sarret *et al.,* 2009). An explanation for the high expression levels of miR398b observed in the roots could be that Zn, like other micronutrients such as Cu, is directly taken up from the rhizosphere. Therefore, if Cu balance and sensing is under the control of SPL7, which is also expressed in roots (Araki *et al.,* 2018), it is possible to speculate that excess Zn may modulate the expression of this TF and consequently miR398b, in order to economize the Cu levels under Cu-limited conditions.

The effect of high levels of Cu on miR398b expression was investigated in *Arabidopsis* (Sunkar *et al.,* 2006; Smeets *et al.,* 2013) and more recently in grapevine (Leng *et al.,* 2017). Under excess Cu conditions, which induce oxidative stress, these authors observed a marked down-regulation of miR398b, with an increase

in CuSOD levels to avoid ROS toxicity (Sunkar *et al.,* 2006). In this work, a very strong reduction in miR398b expression was found in both the shoots and the roots of Cu-treated *A. thaliana* transgenic lines expressing pAtMIR398b::*GUS* (**Fig. 4.12**), consistently with previous data (Sunkar *et al.,* 2006; Smeets *et al.,* 2013; Leng *et al.,* 2017). In the roots in particular, no signal was detected, suggesting that 6 hours of treatment are sufficient to induce oxidative stress *in planta*, consequently reducing the expression levels of miR398 and increasing those of SODs. Interestingly, Zn deficiency is responsible for maintaining a higher level of ROS defence in rice (Lee *et al.,* 2017). Therefore, high Cu levels in the medium could also induce Zn deficiency conditions in plants, leading to a reduction in the expression of miR398b.

As previously said, one treatment provided for a combination of excess Cu and Zn: this was done in order to verify a possible competition between these two elements and try to understand the biological processes which occur in plants under stressful conditions. miR398b expression was slightly reduced in both roots and shoots, although a weak GUS signal was maintained in the lateral root primordia (**Fig. 4.13**), indicating that high levels of Zn and Cu in the growth medium can probably induce oxidative stress after 6 hours of treatment with a reduction of miR398b expression (Sunkar *et al.,* 2006).

It is therefore possible to speculate that high levels of Zn in the growth medium may induce Cu-starved conditions, with a positive modulation of miR398b and a down-regulation of the expression levels of SODs. Conversely, high concentrations of Cu and both Zn and Cu in the medium induce the opposite, with plants strongly reducing the expression of miR398b, presumably to increase SODs levels and respond to the oxidative stress (Sunkar *et al.,* 2006; Li *et al.,* 2017).

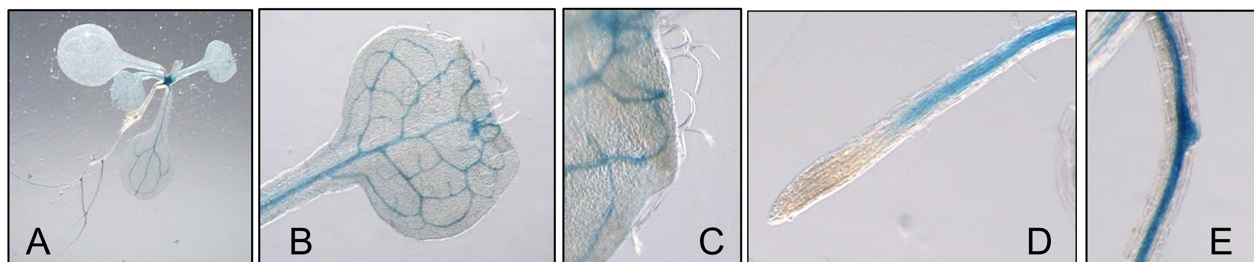

Figure 4.10: GUS expression in transgenic pAtMIR398b::*GUS A. thaliana* plants grown in MS medium: A) whole plant, B) leaves, C) thricomes, D) primary root and E) LR primordia.

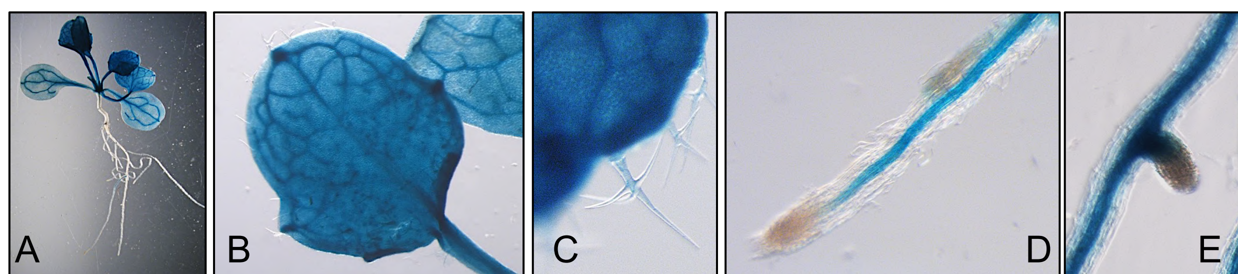

Figure 4.11: GUS expression in transgenic pAtMIR398b::*GUS A. thaliana* plants grown in MS medium containing Zn excess (6 hours of treatment): A) whole plant, B) leaves, C) trichomes, D) primary root and D) lateral root.

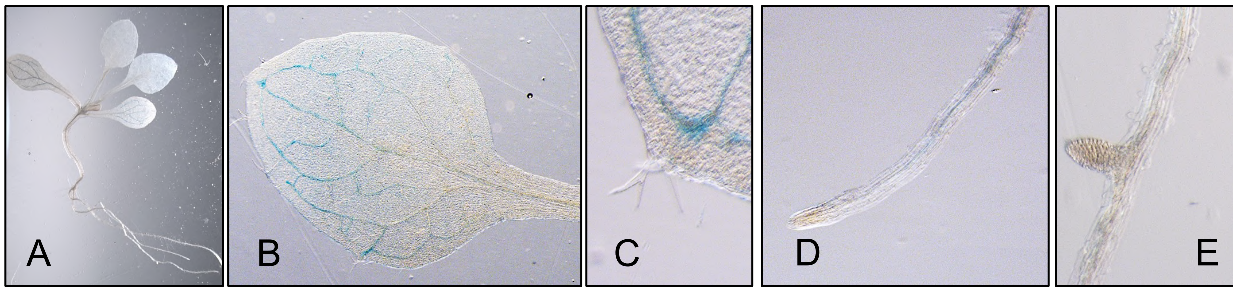

Figure 4.12: GUS expression in transgenic pAtMIR398b::*GUS A. thaliana* plants grown in MS medium containing Cu excess (6 hours of treatment): A) whole plant, B) leaves, C) trichomes, D) primary root and D) lateral root.

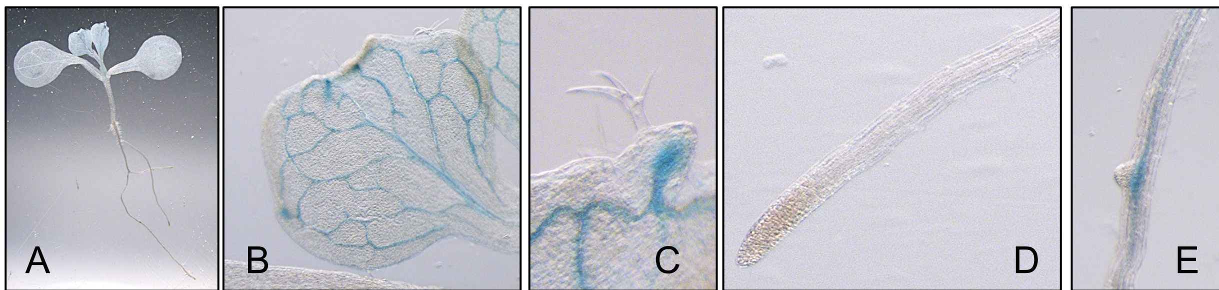

Figure 4.13: GUS expression in transgenic pAtMIR398b::*GUS A. thaliana* plants grown in MS medium containing Zn and Cu excess (6 hours of treatment): A) whole plant, B) leaves, C) trichomes, D) primary root and D) lateral root.

4.4.3 Expression pattern of AhMIR398b

As for pAtMIR398b, the same experimental design was followed to study the expression pattern of miR398 in *A. halleri*. Transgenic lines expressing pAhMIR398b::*GUS* were generated and GUS assays performed considering Zn and Cu as metals of interest. As in *A. thaliana*, AhMIR398b was expressed in both shoots and roots and displayed the same general pattern of tissue expression found for AtMIR398b, thus confirming its conservative role in plants. The only real difference was that in *A. halleri*, miR398b was also expressed in the root cap (**Fig. 4.14**). The GUS signal was however much stronger than AtMIR398b in all tissues, suggesting possible differences in miR398b expression.

Interestingly, the GUS signal detected in the trichomes of plants grown under standard conditions was lower than that of pAtMIR398b, and miR398b was only found in these same tissues at high concentrations of Zn. This result can be explained by considering that the expression levels of miR398b depend on the activity of SPL7 (Beauclair *et al.,* 2010; Pilon *et al.,* 2017). The promoter sequence of miR398b in *A. halleri* is characterized by the presence of many more CURE-responsive elements (GTAC motifs) rather than the same sequence in *A. thaliana*. Therefore, it can be presumed that the stronger miR398b expression found in *A. halleri* is directly linked to the more abundant presence of these *cis*-acting elements, making this miRNA much more sensitive to Cu levels than AtMIR398b.

Under excess Zn conditions (**Fig. 4.15**), miR398 was strongly up-regulated in both roots and shoots, especially in the primary and secondary roots and the trichomes. Like pAtMIR398b, in the presence of excess Cu (**Fig. 4.16**), transgenic lines expressing pAhMIR398b::*GUS* reduced miR398b expression, although notable levels of GUS were also detected in the primary roots (root cap) and the lateral ones. This different expression pattern in roots may be explained by considering the presence of much more root-specific motifs and CURE-responsive elements in pAhMIR398b promoter sequence rather than pAtMIR398b, possibly determining the different sensibility of this miRNA to the Cu levels in the growth medium (Araki *et al.,* 2018). Since *A. halleri* is a Zn/Cd hyperaccumulator plant species (Becher *et al.,* 2004; Hammond *et al.,* 2006; van de Mortel *et al.,* 2006), we should also consider that *A. halleri* and *A. thaliana* can respond differently to metal exposure and display distinct tolerance to excess metal levels. It is therefore possible that miR398b, which regulates the expression of SODs, can display a different expression pattern from that in *A. thaliana*. Under high Cu and Zn levels (**Fig. 4.17**) pAhMIR398b expression was mostly reduced in the shoots (leaves and trichomes), whereas it remained considerable in the roots. Interestingly, a weak GUS signal was also detected in the trichomes, confirming a direct link between the expression pattern of miR398b and the presence of Zn in the growth medium (Remans *et al.,* 2012). In brief, the general pattern of miR398b expression in *A. halleri* was quite similar to that of *A. thaliana* miR398b, although in the hyperaccumulator plant miR398b was also expressed in the root cap and preserved higher levels of gene expression than *A. thaliana* even when subjected to oxidative stress.

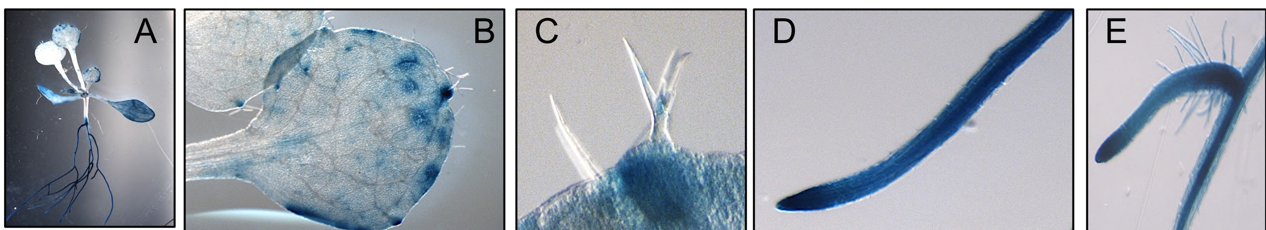

Figure 4.14: GUS expression in transgenic pAhMIR398b::*GUS A. thaliana* plants grown in MS medium: A) whole plant, B) leaves, C) thricomes, D) primary root and E) lateral root.

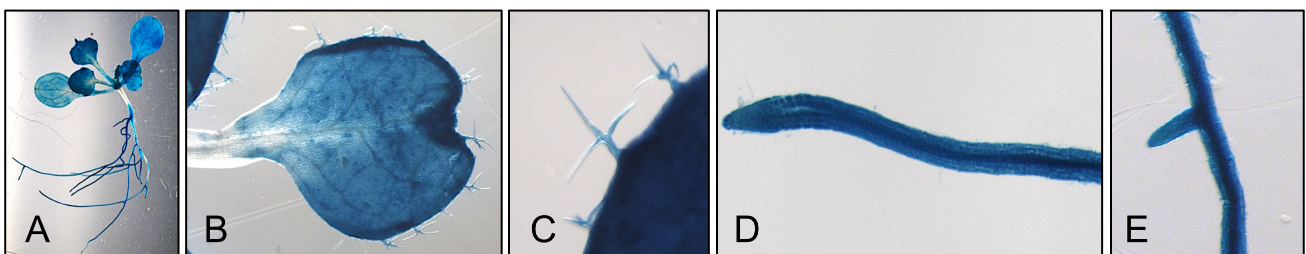

Figure 4.15: GUS expression in transgenic pAhMIR398b::*GUS A. thaliana* plants grown in MS medium containing Zn excess (6 hours of treatment): A) whole plant, B) leaves, C) trichomes, D) primary root and E) lateral root.

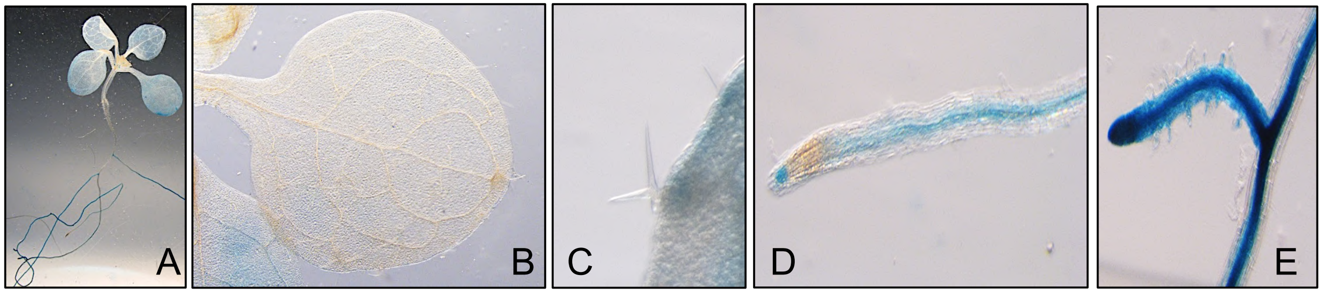

Figure 4.16: GUS expression in transgenic pAhMIR398b::*GUS A. thaliana* plants grown in MS medium containing Cu excess (6 hours of treatment): A) whole plant, B) leaves, C) trichomes, D) primary root and E) lateral root.

Figure 4.17: GUS expression in transgenic pAhMIR398b::*GUS A. thaliana* plants grown in MS medium containing Zn and Cu excess (6 hours of treatment): A) whole plant, B) leaves, C) trichomes, D) primary root and E) lateral root.

4.5 Expression analysis of miR408

Promoter sequences of MIR408 were obtained from genomic DNA of both *A. thaliana* and *A. halleri* ecotype I16 (Section 4.5). The amplicons were then aligned and analyzed using the bioinformatic software MEME (http://meme.nbcr.net/meme; Bailey *et al.*, 2009) for the determination of conserved motifs (**Fig. S7**).

4.5.1 Identification and analysis of DNA motifs in the pMIR408 of *A. thaliana* and *A. halleri*

Like for pMIR398b, a bioinformatics analysis was performed on the promoter of miR408 from the gDNA of *A. thaliana* and *A. halleri*, to obtain more information on the regulation pattern of this small RNA molecule. In this case too, several *cis*-active elements responsible for the adaptation to different abiotic stresses were found in the pMIR408 of both *A. thaliana* and *A. halleri*, confirming the role of miR408 in the adaptation to several environmental factors (Wang *et al.,* 2014; Ma *et al.,* 2015; Zhao *et al.,* 2015). The most notable DNA motifs found in the promoter sequence of MIR408 are linked to responses to low-temperature (LTRE), hormones such as ABA (ABRE, ABA responsive element), nutrient deficiency (SURE and CURE), oxidative stress and light. The tissue localization of miR408 expression in

A. thaliana has only been partially reported (Zhang and Li, 2013), but there is no information on the expression of this miRNA in the Zn/Cd hyperaccumulator plant *A. halleri*. From the bioinformatics analysis, it emerged that many DNA motifs responsible for tissue localization were identified in both pAtMIR408 and pAhMIR408 (**Fig. 4.18**). In particular, root-specific motifs were found in the promoter sequences of both *A. thaliana* and *A. halleri* but were more abundant in the non-accumulating species.
As described for *A. thaliana*, the ATATT motifs, responsible for the expression in the vasculature (Elmayan and Tepfer, 1995), were more numerous in the pAtMIR408 promoter sequence than that of pAhMIR408, suggesting that in general miR408 could also be transported across the entire plant as well as the other nutrient-responsive miRNAs (Liang *et al.,* 2015; Paul *et al.,* 2015; Noman *et al.,* 2019).
The promoter analysis on pMIR408 revealed the presence of many CURE motifs (GTAC motifs) in both sequences, but much more abundantly in *A. halleri*. As well as CURE motifs, also sulphur-responsive elements (SUREs) were identified in pMIR408; moreover, unlike pMIR398b, both pAtMIR408 and pAhMIR408 displayed Fe-responsive elements (IRO2OS), indicating a possible modulation of these Cu-responsive genes under Fe-starvation conditions (Waters *et al.,* 2012). This is an interesting find, because the literature describes a modulation of Fe-responsive genes, such as FSD1 (Abdel-Ghany *et al.,* 2005; Schulten and Krämer, 2018) linked to the Cu levels in plants, suggesting that there is a complex mechanism behind the regulation of nutrient homeostasis in *Arabidopsis*. Because the promoter of FeSOD1 is characterized by the presence of GTAC motifs, low levels of Cu in the plant cell have been observed to modulate SPL7 TFs and Cu-responsive genes, hence reducing CuSOD levels and increasing FeSOD1 ones (Abdel-Ghany *et al.,* 2005; Yamasaki *et al.,* 2009; Schulten and Krämer, 2018). It is therefore possible that a modulation of miR408 may occurs under Cu-deficient conditions, which can be determined by Fe concentration levels in the plant cells, also responsible for the lignification of the cell wall (Curie and Mari, 2017; Carrió-Seguí *et al.,* 2019). Moreover, phosphate starvation responsive elements (P1BS) were also only found in pAtMIR408, indicating its possible modulation by P-starvation as also recently demonstrated in wheat (Bai *et al.,* 2018).

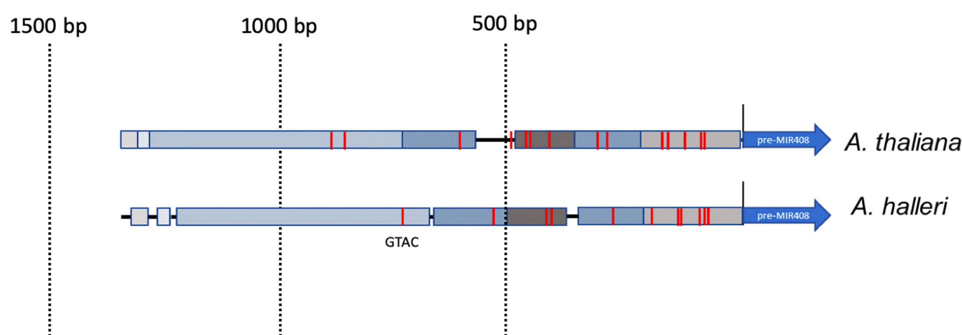

Figure 4.18: *In silico* analysis of miR408 promoters in *A. thaliana* and *A. halleri*. CURE motifs (GTAC) are depicted in red.

4.5.2 Expression pattern of pAtMIR408

In order to study the regulation of miR408 under excess Zn conditions, transgenic *A. thaliana* plants expressing pAtMIR408::*GUS* were generated and GUS expression was tested in plants exposed to Zn, Cu and a combination of both for 6 hours.

Under standard conditions (**Fig. 4.19**), AtMIR408 was expressed in both shoots and roots, but at different levels; the GUS signal was much stronger in the roots than the shoots, and was mainly localized in the lateral roots and the root cap. This result is in keeping with what was observed by Zhang and Li (2013), who studied the expression of AtMIR408 in the roots and at whole plant level. Zhang *et al.* (2014) observed that miR408 expression levels in shoots are down-regulated in the presence of adequate Cu levels, but up-regulated at high concentrations of this micronutrient, confirming the fine modulation of this miRNA by Cu concentration. The low expression levels of miR408 may also suggest that the medium used in this experiment (MS) probably contains adequate levels of Cu, thus negatively modulating the expression of miR408 (Zhang *et al.,* 2014). The promoter analysis of pMIR408 revealed the presence of different *cis*-acting elements linked to its expression in the roots, consistently with the GUS-staining data and previous works on this topic (Yamasaki *et al.,* 2009; Araki *et al.,* 2018).

Under excess Zn conditions (**Fig. 4.20**), a slight up-regulation of miR408 expression was found in both shoots (leaves and trichomes) and (primary and lateral) roots, confirming the general modulation pattern observed for miR398b, as also demonstrated by Real Time RT-PCR (Section 4.3). The same positive modulation of miR408 was also found in rice under Zn-resupply, confirming the role of miRNAs in the regulation of Zn and Cu nutrition (Pilon *et al.,* 2017; Zeng *et al.,* 2019). Low levels of miR408 expression were detected in the shoots (leaves and trichomes) of pAtMIR408-expressing transgenic lines of *A. thaliana* exposed to high concentrations of Cu, although high expression levels of this miRNA were preserved in both primary and lateral roots (**Fig. 4.21**). This result can be explained by considering the versatile role of this small RNA molecule in response to several abiotic stresses in plants (Ma *et al.,* 2015), including oxidative stress, which is induced by the high concentration of Cu. However, a reduction of Zn availability caused by excess levels of Cu in the medium cannot be excluded (Zeng *et al.,* 2019).

A combination of Cu and Zn in the growth medium reduced the expression of miR408 in the shoots: no signal was detected in the trichomes, whereas conversely, both primary and lateral roots displayed a strong GUS signal (**Fig. 4.22**). Although an oxidative stress response is possible under these conditions, it is more plausible that high Zn concentrations at the rhizosphere would compete with Cu (Remans *et al.,* 2012; Zeng *et al.,* 2019). SPL7 TF is responsible for the primary process of Cu-sensing that obviously occurs mainly in the roots rather than the shoots (Yamasaki *et al.,* 2009), although the response in other tissues could be different. Moreover, the regulation of Cu concentrations seems to be also mediated by other molecules, suggesting that this process may also occurs at post-transcriptional level (Yamasaki *et al.,* 2009).

Although it can be speculated that high levels of Zn and Cu may induce a Cu-deficiency-like response, we cannot exclude an oxidative stress response as well, more evident in the shoots, in which miR398b and miR408 would play a more active role.

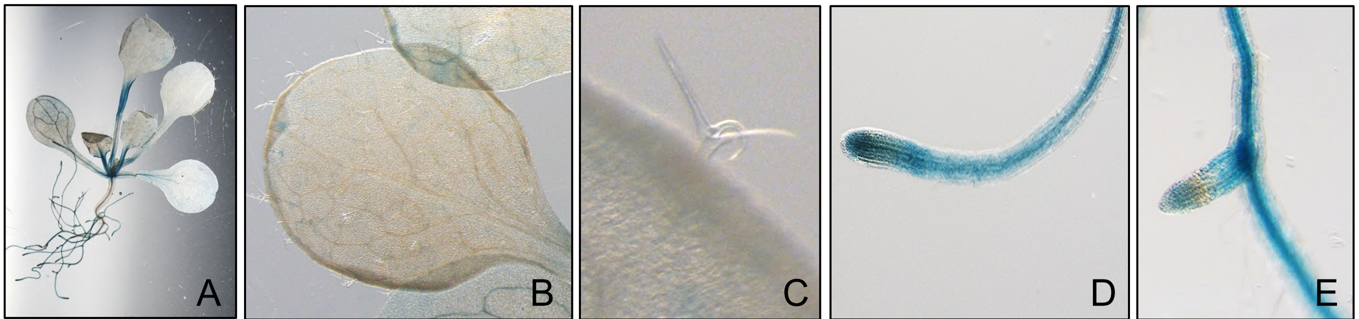

Figure 4.19: GUS expression in transgenic pAtMIR408::*GUS A. thaliana* plants grown in MS medium A) whole plant, B) leaves, C) trichomes, D) primary root and E) lateral root.

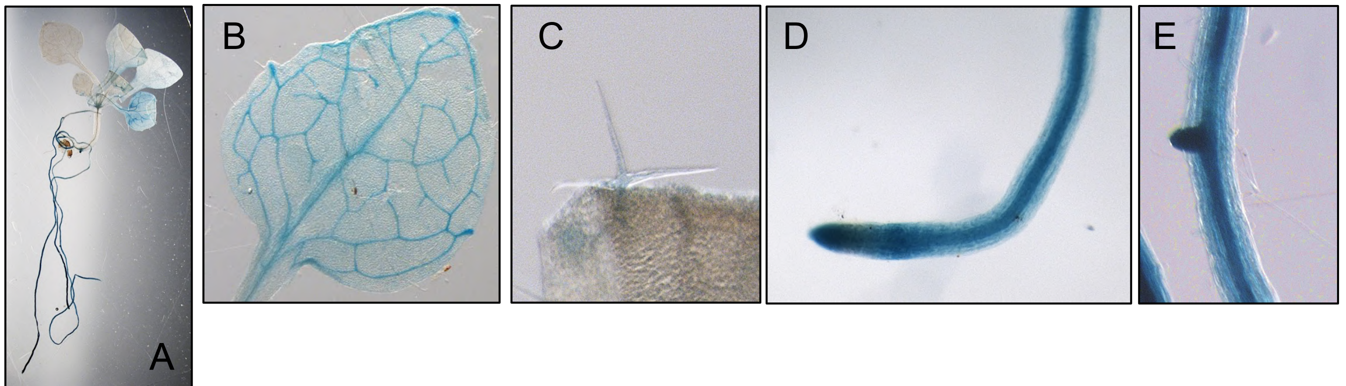

Figure 4.20: GUS expression in transgenic pAtMIR408::*GUS A. thaliana* plants grown in MS medium containing Zn excess (6 hours of treatment): A) whole plant, B) leaves, C) trichomes, D) primary root and E) lateral root.

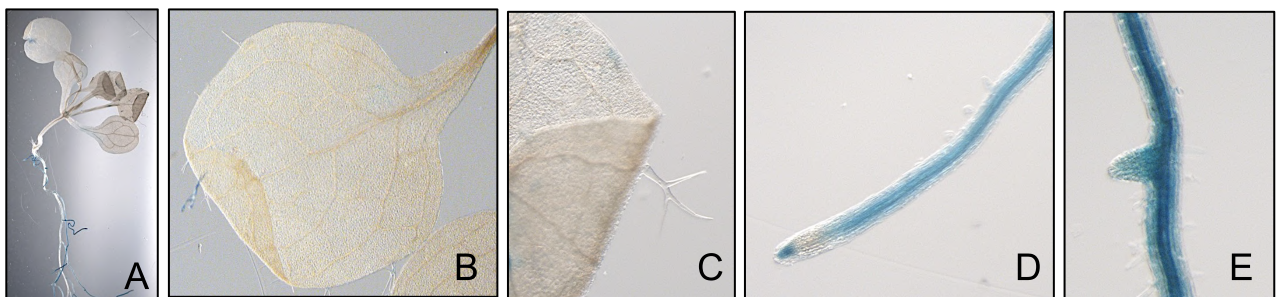

Figure 4.21: GUS expression in transgenic pAtMIR408::*GUS A. thaliana* plants grown in MS medium containing Cu excess (6 hours of treatment): A) whole plant, B) leaves, C) trichomes, D) primary root and E) lateral root.

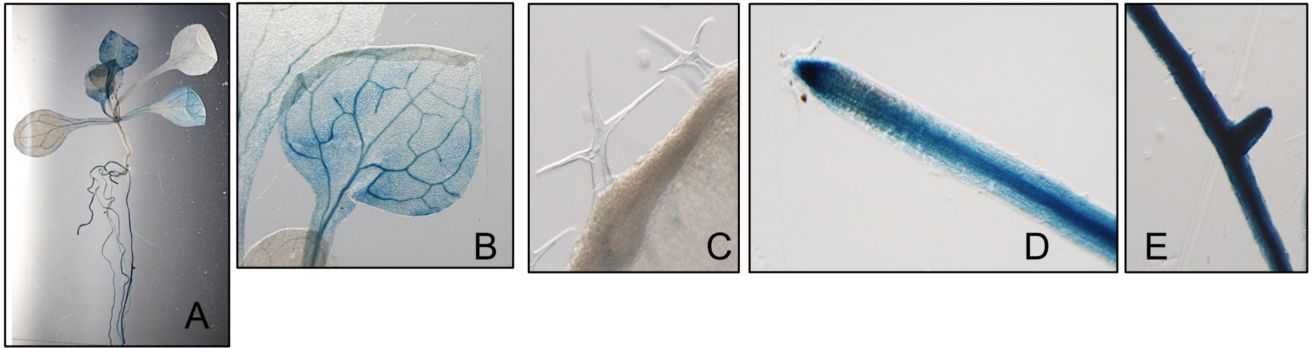

Figure 4.22: GUS expression in transgenic pAtMIR408::*GUS A. thaliana* plants grown in MS medium containing Cu and Zn excess (6 hours of treatment): A) whole plant, B) leaves, C) trichomes, D) primary root and E) lateral root.

4.5.3 Expression pattern of pAhMIR408

The expression pattern of miR408 of *A. halleri* was evaluated with GUS assay using *A. thaliana* transgenic lines expressing pAhMIR408::*GUS*, which were grown in the presence or absence of Zn and Cu.

As shown in **Fig. 4.23,** under normal conditions, *GUS* expression was mainly detected in shoots, and the leaf vasculature in particular, although a weak signal was also found in the trichomes. miR408 was principally expressed in the roots, in keeping with what observed for *A. thaliana* pMIR408, even though the signal was much stronger in *A. halleri* than the non-hyperaccumulating plant. This may reflect a different sensitivity of miR408 for Cu and other metals in the growth medium, as also suggested by the presence of several metal-responsive *cis*-acting elements in the promoter sequence.

Under excess Zn conditions (**Fig. 4.24**), a reduction in miR408 expression was found in the leaf vasculature, but compared to the control, an intense GUS signal was detected in the trichomes and in the primary and lateral roots, suggesting a different sensitivity of miR408 to metals between *A. halleri* and *A. thaliana.* As observed for pAtMIR408, a high Cu concentration in the growth medium, which according to some authors may induce oxidative stress, reduced the expression of miR408 in both the shoots and the roots (**Fig. 4.25**) (Sunkar *et al.,* 2006; Leng *et al.,* 2017). The same response was also found for miR398b, which resulted to be repressed under oxidative stress conditions in order to avoid toxicity caused by high levels of ROS. This data confirms the coordinate role of these Cu-related genes in Cu management in plants (Pilon, 2017).

Finally, the expression of pAhMIR408 was also studied at high levels of both Cu and Zn. GUS signal was detected in shoots, with a low intensity in the leaves (vasculature and trichomes), but mainly in the roots, although less intense than that observed in plants grown under the other experimental conditions (**Fig. 4.26**). This pattern is similar to that found for pAtMIR408, although *A. halleri* seems to maintain a basal level of miR408 expression in trichomes, therefore suggesting that miR408 and miR398b may be involved in response to metal stress in *Arabidopsis* species (Gielen *et al.,* 2016; Pilon *et al.,* 2017).

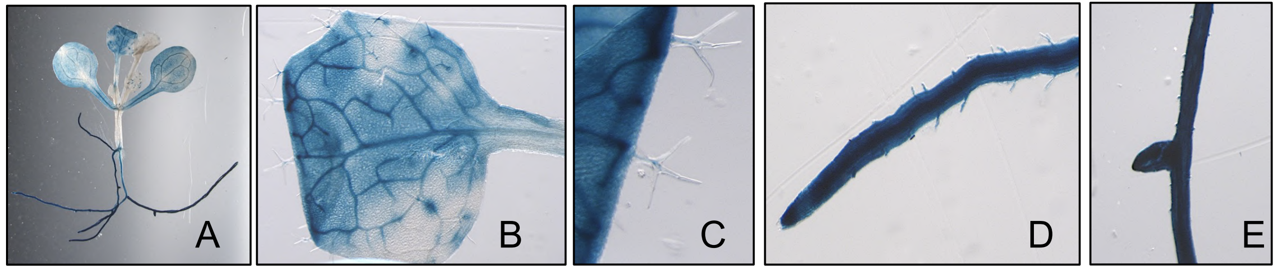

Figure 4.23: GUS expression in transgenic pAhMIR408::*GUS A. thaliana* plants grown in MS medium: A) whole plant, B) leaves, C) trichomes, D) primary root and E) lateral root.

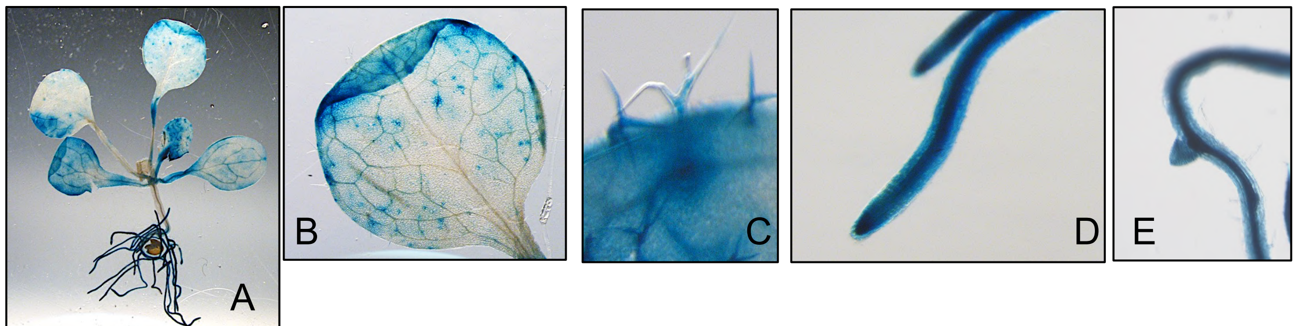

Figure 4.24: GUS expression in transgenic pAhMIR408::*GUS A. thaliana* plants grown in MS medium containing Zn excess (6 hours of treatment): A) whole plant, B) leaves, C) trichomes, D) primary root and E) lateral root.

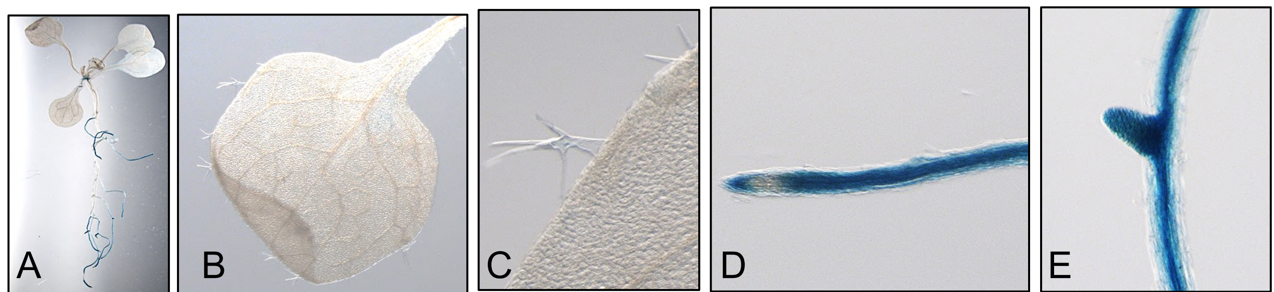

Figure 4.25: GUS expression in transgenic pAhMIR408::*GUS A. thaliana* plants grown in MS medium containing Cu excess (6 hours of treatment): A) whole plant, B) leaves, C) trichomes, D) primary root and E) lateral root.

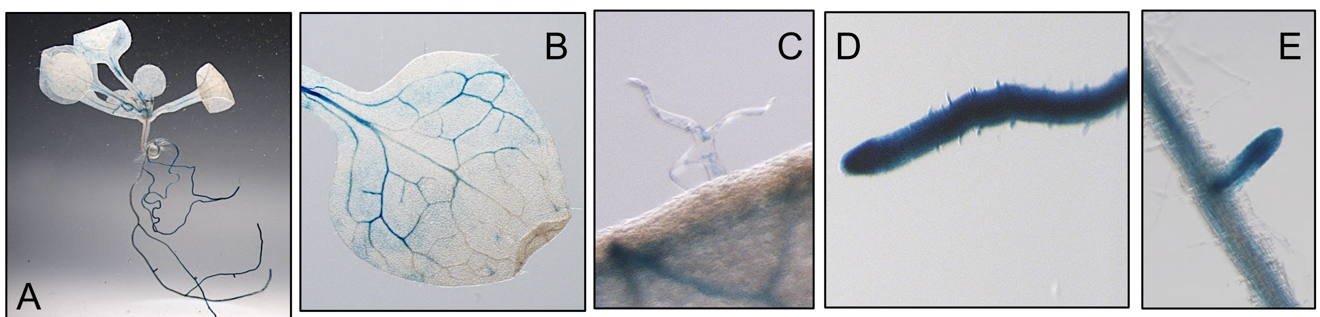

Figure 4.26: GUS expression in transgenic pAhMIR408::*GUS A. thaliana* plants grown in MS medium containing Zn and Cu excess (6 hours of treatment): A) whole plant, B) leaves, C) trichomes, D) primary root and E) lateral root.

4.6 Generation of 35S::***pre-AhMIR408*** transgenic lines

The biological role of miR408 in *A. thaliana* and other plant species has been clarified using overexpressing lines (Pan *et al.,* 2018; Song *et al.,* 2018). In general, high constitutive levels of miR408 increased plant biomass and seed yield, with a general remodulation of the response to several environmental stresses (Ma *et al.,* 2015; Song *et al.,* 2018; Carrió-Seguí *et al.,* 2019) confirming its role as a general regulator of the plant's response to abiotic stresses.

To better understand the possible involvement of this small RNA in response to excess metal, we generated *A. thaliana* transgenic lines overexpressing the *pre-MIR408* of *A. halleri,* since no information is available in the literature regarding the role of miR408 in this hyperaccumulator species. Interestingly, the complete stem-loop structure of *pre-MIR408* of *A. halleri* seems to be much more similar to the corresponding sequence of *A. lyrata* rather than *A. thaliana* (data not shown)*,* possibly suggesting a different biogenesis and maturation in the Zn/Cd hyperaccumulator plant.

4.6.1 Phenotypic characterization of *A. thaliana* plants overexpressing *pre-MIR408* under Zn excess

The possible involvement of miR408 in response to excess Zn was investigated by monitoring the tolerance of *A. thaliana* WT and three lines overexpressing *pre-MIR408* (#1, #2 and #3) grown *in vitro* under conditions of Zn sufficiency (0.7 µM Zn), as well as its mild (50 µM Zn) or severe (100 µM Zn) excess (**Fig. 4.27**). Tolerance to this metal was determined by measuring the root length after 10 days of treatment.

Compared to the WT, all three transgenic lines displayed a significant reduction in root length when subjected to mild and severe Zn excess, but greater shoot biomass. This feature was also observed under standard conditions, although in this case no differences were evident in their root length.

Several authors have described the regulation of miR408 in *Arabidopsis* plants under a variety of abiotic stresses (Zheng *et al.,* 2014; Ma *et al.,* 2015; Carriò-Segui *et al.,* 2019); heavy metal stress in particular seems to induce a Cu-deficiency-like condition (Yamasaki *et al.,* 2007; Ma *et al.,* 2015; Gielen *et al.,* 2016), and this aspect was also evident in our experiments. As previously proposed (Gayomba *et al.,* 2013), it is possible that high concentrations of Zn in the growth medium may modulate Cu-related genes to increase the flow of this micronutrient to PC, regulated by the activity of miR408. On the other hand, high concentrations of Zn can also cause oxidative stress in plants, and hence modulate miR398 and other Cu-related genes to avoid metal toxicity (Remans *et al.,* 2012; Schulten and Krämer, 2018).

Since our work revealed that miR398b and miR408 are positively regulated under excess Zn conditions, it can be presumed that high levels of Zn can induce Cu-starvation in plants (Hafeez *et al.,* 2013; Zhang *et al.,* 2019). However, the high levels of miR408 in the transgenic lines could also disturb the Cu balance and homeostasis regulation, overall affecting more negatively these lines than the WT.

The impact of overexpressing miR408 in *Arabidopsis* transgenic plants grown at low Fe availability has been recently studied (Carriò-Segui *et al.,* 2019); these conditions were reported to induce the opposite response brought about by low Cu levels or oxidative stress, indicating a competition between Cu and Fe (Dong *et al.,* 2018). In plants, variable concentrations of Fe in the growth medium have been reported to induced oxidative stress responses and disturb the activity of lignin peroxidation, which is regulated by the interaction between miR408 and its target genes (Carrió-Seguí *et al.,* 2019). In particular, Fe deficiency normally reduced miR408 levels in the medium, thus leading an increase levels of Laccases in plant cell (Carrió-Seguí *et al.,* 2019) and promoting lignification, also under oxidative stress conditions (Loix *et al.,* 2017). Nevertheless, high expression of miR408 *in planta* may change this process, hence reducing the capacity of plants to tolerate Fe deficiency conditions (Carrió-Seguí *et al.,* 2019).

Because Fe and Zn may compete one to each other in plants (Grotz and Guerinot, 2006), it is possible that excess Zn levels in the growth medium could induce Fe-deprivation condition, thus activating the modulation of the expression of MIR408-target genes with a consequent imbalance of i) Cu homeostasis, ii) oxidative stress responses and iii) lignification processes. In addition, miR408 can modulate the expression levels of an ascorbate oxidase and, therefore, levels of Ascorbate in the cell, a molecule which is directly involved in the response to oxidative stress (Bielen *et al.,* 2013; Yang and Chen, 2013). Therefore, it is possible to assume that in the overexpressing lines high levels of miR408 can disturb all these biological processes, hence leading to a reduction in growth compared to WT plants.

Moreover, we have also to consider that an increase in the flow of Cu to the PC, induced by high levels of miR408 (Song *et al.,* 2017), could decrease the availability of Cu for other biological processes, including the expression of Cu-SODs, which are normally required for the response to oxidative stress induced by excess metals.

All these assumptions may explain the observed phenotype for 35S::*pre-AhMIR408* transgenic plants under Zn treatment, although many aspects need to be clarified.

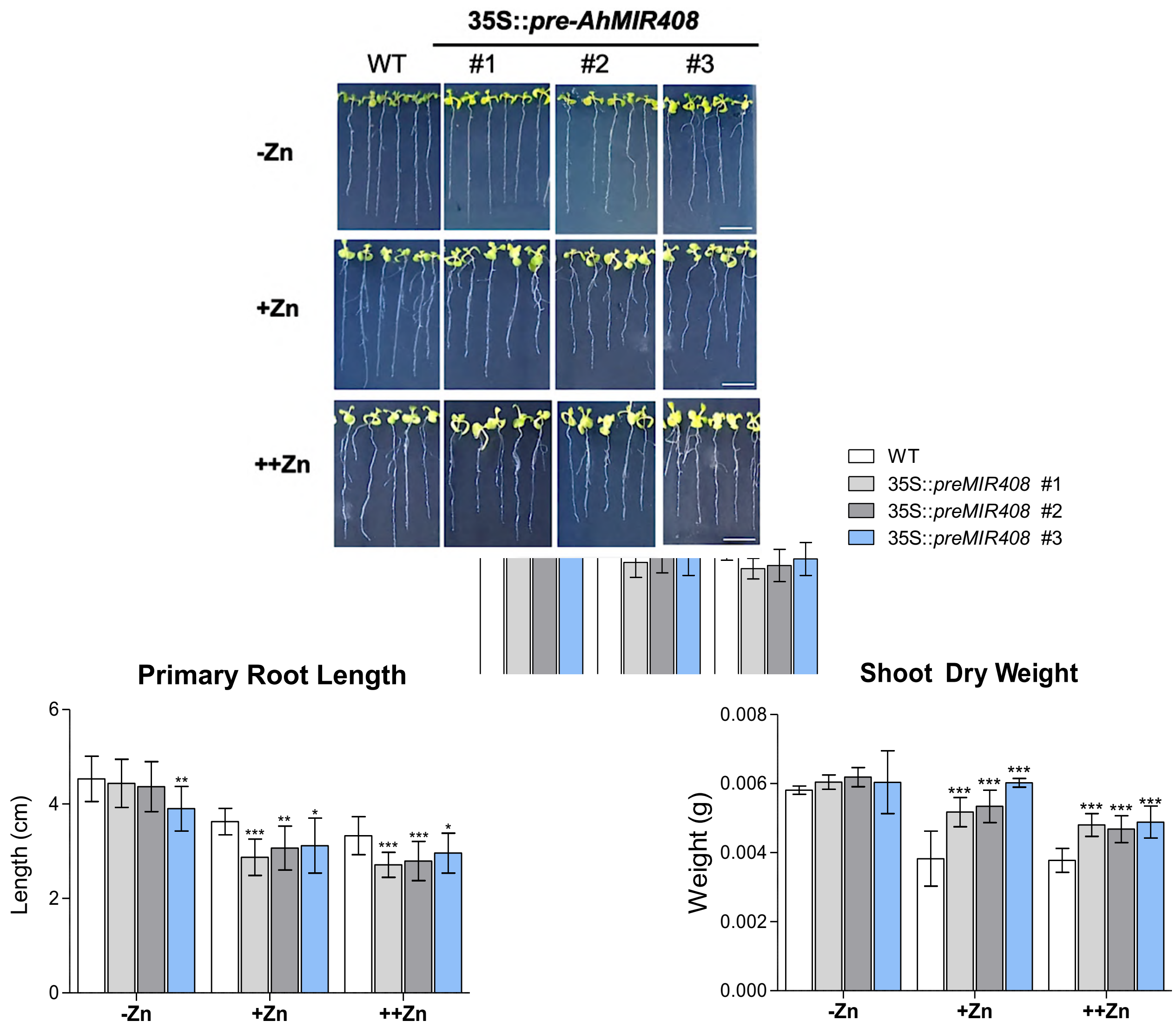

Figure 4.27: Primary root length and shoot dry weight in three lines overexpressing *pre-AhMIR408* and the WT at different Zn concentrations. Values are means ± SD (n≥15). Scale bars = 1 cm.

5. CONCLUSIONS

Environmental contamination caused by an excess of heavy metals represents one of the major problems needing to be solved in the 21st century, since it has a negative impact on crop yield (Valko *et al.,* 2005; Fang *et al.,* 2013). Among the different mechanisms that plants adopt to respond to nutrient deficiency and heavy metal stress, the emerging role of small non-coding RNAs in these processes represented a great interest for the scientific community in the past years (Liang *et al.,* 2015; Paul *et al.,* 2015; Noman *et al.*, 2019). In particular, the advancement of high-throughput sequencing technologies has allowed to better understand the roles of small regulatory molecules in distinct biological processes (Ma *et al.,* 2015; Adams *et al.,* 2018; Peng *et al.,* 2018). Among the different classes of sRNAs present in plants, the category of miRNAs represents one of the most interesting. miRNAs are small endogenous RNA molecules (20-24 nc) which represent master regulators of gene expression by RNAi (Wang *et al.,* 2019). In particular, they play essential roles in the regulation of plant development (D'ario *et al.,* 2017) and response to several biotic and abiotic stresses (Pegler *et al.,* 2019). In *A. thaliana, B. napus*, maize, barley and other crops most of the targets of these conserved metal responsive miRNAs are involved in many biological processes, including nutrient allocation and assimilation, oxidative stress response, phytohormone signaling and sRNA biogenesis (Shriram *et al.,* 2016; de Vries *et al.,* 2018; Noman *et al.,* 2019). RNA interference (RNAi) is increasingly becoming a new biotechnological reality for the improvement of certain crop traits in order to provide more adaptability to adverse environmental conditions (Khare *et al.,* 2018; Zhou *et al.,* 2020), although a GMO-RNAi free technique was recently proposed (Worrall *et al.,* 2019; Dalakouras *et al.,* 2020). Several approaches can be used for crop improvement, including overexpression or downregulation of a specific miRNA, overexpression of target genes or expression a mutant version of those (Gupta, 2015; Basso *et al.,* 2019). For examples, overexpression of miR397 in rice increased grain size and overall yield in transgenic plants, and the same result was also obtained expressing a mutant version of *SPL14/16* normally controlled by miR156 (see the review of Gupta, 2015). These data confirm the importance of manipulating gene expression of miRNAs and/or their target genes in plants of agronomic interest to obtain an improvement of one or more specific traits. Moreover, the possible applications of miRNA-based delivery system for the treatment of human diseases have been recently reported (Fu *et al.,* 2019), suggesting a cross-kingdom regulation of gene expression mediated by plant miRNAs.

The aim of this research work was to identify and study putative miRNAs with a role in the response to Zn excess in *Arabidopsis*, taking the Zn/Cd hyperaccumulator plant *A. halleri* into account. Since Zn is essential as a cofactor for the activity of numerous enzymes, deficiency conditions of this element are a very serious problem for the agriculture (Broadley *et al.,* 2007). Therefore, plants have evolved various strategies in order to improve the bioavailability of essential elements, including Zn: the most important

are i) the production of metal chelating agents (Ma, 2000; Nigam *et al.,* 2001; Abadía *et al.,* 2002), ii) the induction of changes in the soil composition, iii) the modification of their root architecture (Robinson *et al.,* 2006) and iv) the storage functions. It is important to note that excessive Zn levels in the growth medium are harmful for the plant growth and development, which tend to avoid metal toxicity in many ways (Emamverdian *et al.,* 2015).

Different miRNAs have been recently confirmed to be directly involved in the regulation of Zn nutrition and response to the other metals, including the toxic element Cd (Shi *et al.,* 2013; Zeng *et al.,* 2019; Zhou *et al.,* 2019); however, no information is actually available regarding the possible involvement of these sRNAs in hyperaccumulator and hypertolerant plants, thus representing an interesting field for the research.

In this study, several approaches were applied to elucidate the functions of miRNAs in response to excess Zn. At first, sRNA-Seq analyses were performed on the shoots of *A. thaliana, A. thaliana* treated with excess Zn (500 μM) for 1 week and *A. halleri* ecotype I16 (Lombardy, Italy). 100 miRNAs, belonging to several conserved families, were found to be differently modulated in the hyperaccumulator plant with respect to *A. thaliana* (both treated and untreated). On the other hand, only few miRNAs were significantly modulated by the Zn treatment in *A. thaliana*, suggesting that the concentration of this metal used in this experiment was probably not strong enough to induce a more extended modulation of miRNAs. Most of the miRNAs expressed differently in *A. halleri* have conserved roles in plant development and response to different stresses, although many others are part of the complex signaling network which is required for mineral absorption and homeostasis regulation of many essential nutrients (Yang and Chen, 2013; Noman *et al.,* 2019; Pegler *et al.,* 2019). Some metal-responsive miRNAs resulted to be modulated in the same manner in Zn-treated *A. thaliana* and *A. halleri* (see Jalmi *et al.,* 2018), also suggesting the conservative roles of these molecules in response to metal stress, although *A. halleri* may possess different constitutive mechanisms regulating miRNA-target interactions, possibly being correlated with its metal hypertolerance and hyperaccumulation trait.

Some miRNAs, which emerged to be potentially involved in the response to mineral deficiency and metal stress, including Zn nutrition (Shi *et al.,* 2013; Jalmi *et al.,* 2018; Zeng *et al.,* 2019), were selected and subjected to Northern Blot analysis and Real Time RT-PCR for the experimental validation.

Two functional groups of miRNAs were considered: those involved in plant development (miR157, miR159 and miR390) and many others involved in the regulation of nutrient homeostasis (miR395, miR398 and miR408).

miR157, miR159 and miR390 are conserved miRNAs with a role in shoot and root development, representing fundamental molecules for the morphological adaptation in response to a series of stimuli (Barciszewska-Pacak *et al.,* 2015; He *et al.,* 2018; Jalmi *et al.,* 2018). These miRNAs play critical roles for the adaptation to adverse environmental conditions by regulating the activity of TFs (Ding *et al.,* 2016;

He *et al.,* 2018; Millar *et al.,* 2019), that include the classes of SPL and WKRY, required for plant development and response to stresses (Phukan *et al.,* 2016; Shulten *et al.,* 2019). In addition, miR159 seems to be involved in the regulation of the expression levels of the ABC metal transporters, which participate to the response to heavy metal stress in plants (see Yang and Chen, 2013), supporting the characteristic of miR159 as conserved metal responsive miRNA. The other class of miRNAs which was considered in this experiment comprise molecules with a documented role in regulation of nutrient homeostasis (miR395, miR398 and miR408). These miRNAs participate in the homeostasis regulation network of Cu and S homeostasis in plants, which seems to be not only related to the response to excess metal, but also fundamental for Zn mineral nutrition (Liang *et al.,* 2015; Pilon, 2017; Shahzad *et al.,* 2018). Particularly, miR159 and miR395 takes part in the system for regulating the detoxification of metals induced by phytochelatins (Yang and Chen, 2013) and miR398b and miR408 are responsible for the regulation of the expression levels of CuSODs and an ascorbate peroxidase, which are required for ROS detoxification (Pilon, 2017). In our experiment, miR159, miR390, miR395 and miR408 resulted to be up-regulated in *A. thaliana* upon exposure to Zn (Section 4.1-4.2), consistently with previous results (Remans *et al.,* 2012; Fan *et al.,* 2018), while miR157 was down-regulated, following the same pattern brought about Cd exposure (Gao *et al.,* 2019). High expression levels of miR159, miR395 and the Cu-related miRNAs (miR398b and miR408) under Zn treatment may indicate a strong connection between Cu, S and Zn homeostasis *in planta* (Grotz and Guerinot, 2006; Liang *et al.,* 2015; Paul *et al.,* 2015), in which these miRNAs play key roles. In particular, miR159 and miR395 seem to be directly involved in the regulation of the production of phytochelatins, with an important role in response to Cd stress (Cobbett and Goldsbrough, 2002), hence confirming this hypothesis. Moreover, the modulation pattern observed for miR157 and miR390 seem to confirm that the regulation of plant development, mediated by the activity of TFs such as SPL and MYB, could represents a strategy adopted by many plant species in order to cope with different environmental stresses, due to a modulation of root and shoot development (Noman and Aqueel, 2017; Samad *et al.,* 2017; Jalmi *et al.,* 2018). In the Zn/Cd hyperaccumulator plants, constitutive higher expression of miR159 and miR395 were found in comparison to Zn-treated and untreated *A. thaliana* plants, and the opposite results were observed for miR157, miR390, miR398b and miR408, which displayed constitutive lower levels of expression. Therefore, it is possible that *A. halleri* may respond in a different manner to high levels of metals compared with *A. thaliana*, and that this phenomenon could also be connected with the activity of these metal responsive miRNAs, normally involved in the regulation of plant development and mineral nutrition respectively. In particular, the modulation of miR395, miR398b and miR408, and their targets (SOD and many sulphur responsive genes) seems to indicate that the regulation of Cu and S homeostasis can be required for the adaptation of *A. halleri* to excess Zn/Cd levels. Studies on *A. thaliana*, *B. napus* and rice have shown a modulation of these miRNAs upon Cd exposure (Zhang *et al.,* 2013; Gielen *et al.,* 2016), thus supporting that these molecules could

respond in the same manner *in planta* in order to avoid Zn excess conditions by requiring a complex and finely regulated regulatory network which comprise i) oxidative stress response, ii) regulation of mineral nutrition and iii) changes in the developmental profile. However, it must also be taken into consideration that these two plants also have a diverse profile of development, and that consequently these differences in the expression of some miRNAs could also be correlated with this phenomenon.

In the second part of this work, miR398b and miR408 were selected for further investigations, given their proposed role as metal-responsive miRNAs (Yamasaki *et al.,* 2009; Gayomba *et al.,* 2013; Pilon, 2017). In particular, Cu-miRNAs are involved in the regulation of Zn homeostasis in plants (Zeng *et al.,* 2019), as well as their modulation upon Cd exposure (Gao *et al.,* 2019; Jian *et al.,* 2019), thus representing interesting candidates for Zn excess response *in planta.* In addition, these two molecules also represent conserved multi-stress responsive miRNAs and, therefore, promising targets for genetic improvement of crops (Zhang *et al.,* 2017; Song *et al.,* 2018).

miR398b and miR408 were significantly up-regulated in *A. thaliana* and *A. halleri* plants treated with Zn excess for several hours, following the same regulation pattern also observed in other research works (Remans *et al.,* 2012; Gielen *et al.,* 2016). In particular, a modulation of Cu-miRNAs was also proved in *A. thaliana* and other plants upon Cd treatment (Gielen *et al.,* 2016; Zhou *et al.,* 2019), hence supporting a possible conservative response mechanism to metal stress mediated by Cu-miRNAs.

The same modulation pattern of miR398b and miR408 was also observed in *A. halleri* plants under Zn excess, although the expression levels of these molecules were much greater in comparison to *A. thaliana.* In order to understand if miR398b and miR408 can be differentially regulated in these two species, the promoter sequences of both miRNAs were then amplified from the genomic DNA of *A. thaliana* and *A. halleri* and fused to the *GUS* reporter gene. A bioinformatic analysis revealed the presence of many *cis*-DNA motifs, required for the responses to abiotic stresses, metal homeostasis regulation and tissue localization, in all promoter sequences, although many differences were found between *A. thaliana* and *A. halleri.* In particular, both miR398b and miR408 of the Zn/Cd hyperaccumulator plant *A. halleri* displayed many more CURE responsive elements (GTAC motifs) that directly interact with the SPL7 transcription factor, one of the most important regulators of Cu homeostasis *in planta* (Bernal *et al.,* 2012; Pilon *et al*., 2017; Araki *et al.,* 2018). Cu-related genes can also participate in the regulation of Zn homeostasis *in planta*, hence suggesting their possible involvement in mineral nutrition (see Zhen *et al.,* 2019). GUS assay was therefore performed on *A. thaliana* transgenic lines expressing pAtMIR398::*GUS*, pAhMIR398b::*GUS*, pAtMIR408::*GUS*, pAhMIR408::*GUS*, which were grown under different conditions, in order to obtain more information about the involvement of miR398b and miR408 in response to variable concentration of Zn and Cu. Both molecules were ubiquitously expressed in both roots and shoots, confirming previous investigations (Sunkar *et al.,* 2006; Zhang and Li, 2013). Furthermore, miR398b in *A. halleri* was also expressed in the root cap and more abundantly in all the

tissues examined than in *A. thaliana*, confirming the data obtained also from Real Time RT-PCR analysis (Section 4.3). After exposure to Zn, both miR398b and miR408 of *A. thaliana* and *A. halleri* were up-regulated, miR398b in particular, confirming that Zn can compete with Cu also activating the same response normally found at low Cu supply (Yamasaki *et al.,* 2007; Remans *et al.,* 2012; Pilon *et al.,* 2019). Interestingly, the GUS signal was detected in trichomes, which are the fundamental site for Zn and Cd storage in the plant cell (Zhao *et al.,* 2000; Sarret *et al.,* 2009), confirming the involvement of these Cu-miRNAs in response to Zn excess. A reduced miR398b and miR408 expression was found in the shoots after exposure to Cu, in keeping with other investigations (Smeets *et al.,* 2013; Leng *et al.,* 2017), confirming that high levels of Cu may induce oxidative stress *in planta*. However, notable levels of miR398b and miR408 were preserved in the roots, an organ in which SPL7 is particularly sensitive to Cu, indicating that Cu homeostasis could also depend on the activity of other molecules (Yamasaki *et al.,* 2009). Finally, the effect of the combined excess of Zn and Cu in the same medium was assessed on the modulation of miR398b and miR408 of both *A. thaliana* and *A. halleri*. Both miRNAs were strongly down-regulated in the shoots of transgenic lines, although high levels of gene expression were detected in the roots. All together these data strongly indicate that the modulation of Cu-responsive genes may principally depend on i) the concentration of Zn and Cu in the medium and ii) the sensitivity of the miRNAs to Cu and Zn. In particular, Zn excess seems to induce a Cu-starvation condition in plants, leading an activation of both miR398b and miR408 in *A. thaliana* and *A. halleri*, presumably to optimize Cu homeostasis regulation and increase the flux of Cu towards fundamental processes (Pilon *et al.,* 2017; Shahbaz and Pilon, 2019). The different GUS signal found under these stressful conditions in *A. thaliana* and *A. halleri* may also reflect a different sensitiveness of the Cu-responsive miRNAs, which could be related to the presence of various CURE-responsive elements in their promoter sequences.

In the final part of this work the effect of overexpressing the stem-loop precursor of *A. halleri* miR408 on Zn tolerance was evaluated by producing *A. thaliana* transgenic lines and growing them in the presence of excess levels of this element. Over the past years, the use of transgenic lines overexpressing a miRNA and/or its target molecule or knockout mutants of target genes were extensively applied in order to obtain more information about the role of miRNAs in response to different types of stimuli (Zhang *et al.,* 2013; Ma *et al.,* 2015; Patel *et al.,* 2016), thus representing a powerful system for studying the function of a specific molecule. In particular, the prediction of the target/s for certain miRNAs is a fundamental step to better elucidate the biological role of miRNA-target interaction in many regulatory networks *in planta,* which could also be exploited for genetic engineering (Xu *et al.,* 2019). For this reason, several *in silico* and computational tools have been developed and used for many research groups in recent years, including psRNATarget (https://plantgrn.noble.org/psRNATarget/home), and the predicted target/s for miRNAs were validated by degradome analysis and sequencing (Ferdous *et al.,* 2017; Xiu *et al.,* 2019).

In addition, target mimicry analysis and the expression of miRNA-resistant targets have represented fundamental approaches to study the biological features of these molecules *in planta* (Franco-Zorilla *et al.,* 2007; Gautam and Gupta, 2020).
miR408 is multi-stress responsive miRNA plays fundamental roles in promoting the plant vegetative growth under normal and stressful condition (Ma *et al.,* 2015), also representing a potential candidate to increase crop yield and resistance to environmental stressful conditions (Song *et al.,* 2018; Wu *et al.,* 2019). In our experiments, the transgenic 35S::*pre-AhMIR408* overexpressing lines displayed a lower tolerance to excess Zn compared to WT plants, hence confirming the correlation between Cu and Zn homeostasis. In addition, these data may also indicate that high levels of miR408 expression could modify the ability of plants to respond to metal excess, probably due to a possible alteration in the mineral nutrition and the formation of the cell wall, which also have been proposed to play a role in response to Fe deficiency conditions (Ma *et al.,* 2015; Carrió-Seguí *et al.,* 2019). Particularly, excess Zn levels in the medium could change Cu distribution in cell of 35S::*pre-AhMIR408* transgenic lines, with a negative impact on the oxidative stress response and modulation of plant development also mediated by Laccases (Zhao *et al.,* 2013; Schuetz *et al.,* 2014; Loix *et al.,* 2017). In fact, overexpression of *OsLAC10* in *A. thaliana* increased the tolerance of transgenic plants to excessive Cu levels (Liu *et al.,* 2017), thus confirming the involvement of lignin peroxidation in response to heavy metal stress (Loix *et al.,* 2017).

In conclusion, this work confirms the importance of sRNA-Seq technology in the modern scientific research field on ncRNAs, in order to better elucidate the biological roles of sRNAs *in planta* (Xu *et al.,* 2019). All the experiments performed indicated that several metal-conserved miRNAs, targeting TFs, metal transporters, nutrient responsive genes and other molecules, could be directly involved in response to excess Zn in *A. thaliana* and may be differentially regulated in *A. halleri*, hence suggesting their possible role in determining the Zn hyperaccumulation/hypertolerant trait in this species. Moreover, the conservative roles of miR398b and miR408 as Zn responsive miRNAs in plants make them ideal candidates for the genetic improvement of crops to increase the tolerance to heavy metals/nutrient deficiency conditions, also allowing to shed more light on the role of miRNAs in the complex network of Zn homeostasis regulation *in planta* (Zhang *et al.,* 2017; Jalmi *et al.,* 2018; Noman *et al.,* 2019).

6. SUPPLEMENTARY MATERIAL

Figure S1: List of miRNAs significatively up-regulated in shoots of *A. halleri* vs *A. thaliana* as obtained by sRNA-Seq experiment.

Family name	miRNA name	Gene bank accession	miRbase accession	Sequence 5'-3'	Length (nt)	Counts *At*	Counts *Ah*	Log FC	P value
miR156	ath-miR156h	AT5G55835	MIMAT0001013	UGACAGAAGAAAGAGAGCAC	20	12,3	52,7	2,1	2,82E-08
miR158	ath-miR158a-5p	AT5G10745	MIMAT0031873	CUUUGUCUACAAUUUUGGAAA	21	39,3	410,0	3,34	8,73E-64
miR159	ath-miR159a	AT1G73687	MIMAT0000177	UUUGGAUUGAAGGGAGCUCUA	21	33693,0	43778,7	0,35	0,022840794
	ath-miR159b-3p	AT1G18075	MIMAT0000207	UUUGGAUUGAAGGGAGCUCUU	21	23909	32745	0,43	0,002581773
	ath-miR159c	AT2G46255	MIMAT0001015	UUUGGAUUGAAGGGAGCUCCU	21	737,3	11333,7	3,91	1,39E-140
miR165	ath-miR165a-5p	AT1G01183	MIMAT0031879	GGAAUGUUGUCUGGAUCGAGG	21	35,0	109,3	1,6	3,61E-10
miR166	ath-miR166b-3p	AT3G61897	MIMAT0031897	UCGGACCAGGCUUCAUUCCCC	21	836356,3	1065693,7	0,22	0,034720113
	ath-miR166c	AT5G08712	MIMAT0000907	UCGGACCAGGCUUCAUUCCCC	21	837084,7	1065693,7	0,33	0,034720113
	ath-miR166d	AT5G08717	MIMAT0000908	UCGGACCAGGCUUCAUUCCCC	21	837084,7	1065693,7	0,33	0,034720113
	ath-miR166e-3p	AT5G41905	MIMAT0000909	UCGGACCAGGCUUCAUUCCCC	21	835179,0	1065693,7	0,33	0,036086854
	ath-miR166f	AT5G43603	MI0000980	UCGGACCAGGCUUCAUUCCCC	21	835179,0	1065693,7	0,33	0,03493543
	ath-miR166g	AT5G63715	MI0000981	UCGGACCAGGCUUCAUUCCCC	21	835907,3	1065693,7	0,33	0,03493543
miR169	ath-miR169f-3p	AT3G14385	MIMAT0031898	GCAAGUUGACCUUGGCUCUGC	21	0	135,3	10,33	5,70E-46
	ath-miR169g-3p	AT4G21595	MIMAT0000912	UCCGGCAAGUUGACCUUGGCU	21	0	51,0	8,95	6,61E-22
miR170	ath-miR170-5p	AT5G66045	MIMAT0031887	UAUUGGCCUGGUUCACUCAGA	21	31,7	82,7	1,37	5,51E-09
miR171	ath-miR171a-3p	AT3G51375	MIMAT0000202	UGAUUGAGCCGCGCCAAUAUC	21	7,6	47,0	2,58	7,16E-09
	ath-miR171a-5p	AT3G51375	MIMAT0031888	AGAUAUUAGUGCGGUUCAAUC	21	31,7	82,7	1,37	8,20E-09
	ath-miR171b-3p	AT1G11735	MIMAT0000920	UUGAGCCGUGCCAAUAUCACG	21	60,7	89,7	0,56	0,018975546
	ath-miR171b-5p	AT1G11735	MIMAT0031899	AGAUAUUAGUGCGGUUCAAUC	21	13,7	89,7	1,24	0,001376928

Figure S1 *(continues)*: List of miRNAs significatively up-regulated in shoots of *A. halleri* vs *A. thaliana* as obtained by sRNA-Seq experiment.

Family name	miRNA name	Gene bank accession	miRbase accession	Sequence 5'-3'	Length (nt)	Counts *At*	Counts *Ah*	Log FC	P value
miR171	ath-miR171c-3p	AT1G62035	MIMAT0000921	UUGAGCCGUGCCAAUAUCACG	21	60,7	89,7	0,57	0,017110029
	ath-miR171c-5p	AT1G62035	MIMAT0031900	AGAUAUUGGUGCGGUUCAAUC	21	11	36	1,70	4,11E-06
miR319	ath-miR319a	AT4G23713	MIMAT0000511	UUGGACUGAAGGGAGCUCCCU	21	686,7	1182,0	0,76	8,49E-06
	ath-miR319b	AT5G41663	MIMAT0000512	UUGGACUGAAGGGAGCUCCCU	21	778,7	1524,7	0,95	8,40E-08
	ath-miR395c	AT1G26985	MIMAT0000940	CUGAAGUGUUUGGGGGGACUC	21	0	36,3	8,40	7,83E-22
	ath-miR395f	AT1G69797	MIMAT0000943	CUGAAGUGUUUGGGGGGACUC	21	0	36,3	8,40	6,62E-22
miR399	ath-miR399b	AT1G63005	MIMAT0000952	UGCCAAAGGAGAGUUGCCCUG	21	69,3	452,7	2,66	3,64E-39
	ath-miR399c-3p	AT5G62162	MIMAT0000953	UGCCAAAGGAGAGUUGCCCUG	21	72,3	452,7	2,62	3,07E-38
miR472	ath-miR472-5p	AT1G12294	MIMAT0032014	AUGGUCGAAGUAGGCAAAAUC	20	22	195,0	3,10	1,31E-47
miR848	ath-miR848	AT5G13887	MIMAT0004270	UGACAUGGGACUGCCUAAGCUA	21	9,3	104,0	3,46	1,30E-27

Figure S2: List of miRNAs significatively down-regulated in shoots of *A. halleri* vs *A. thaliana* as obtained by sRNA-Seq experiment.

Family name	miRNA name	Gene bank accession	miRbase accession	Sequence 5'-3'	Length (nt)	Counts *At*	Counts *Ah*	Log FC	P value
miR156	ath-miR156d-5p	AT5G10945	MIMAT0000169	UGACAGAAGAGAGUGAGCAC	20	15353	6252,3	-1,25	2,29E-18
miR157	ath-miR157a-3p	AT1G66783	MIMAT0031870	GCUCUCUAGCCUUCUGUCAUC	21	106,0	35,0	-1,57	3,32E-12
	ath-miR157a-5p	AT1G66783	MIMAT0000172	UUGACAGAAGAUAGAGAGCAC	21	16298,3	2521,3	-2,71	3,85E-65
	ath-miR157b-3p	AT1G66795	MIMAT0031871	GCUCUCUAGCCUUCUGUCAUC	21	106,3	35,0	-1,57	3,55E-12
	ath-miR157b-5p	AT1G66795	MIMAT0000173	UUGACAGAAGAUAGAGAGCAC	21	16298,3	2521,3	-2,71	1,95E-65
	ath-miR157c-3p	AT1G18217	MIMAT0031872	GCUCUCUAUACUUCUGUCACC	21	1888,0	168,7	-3,51	3,67E-92
	ath-miR157c-5p	AT1G18217	MIMAT0000174	UUGACAGAAGAUAGAGAGCAC	21	16295,3	2521,3	-2,70	1,79E-64
	ath-miR157d	AT1G48742	MIMAT0000175	UGACAGAAGAUAGAGAGCAC	20	1585,0	370,3	-2,12	5,44E-38
miR158	ath-miR158b	AT1G55591	MIMAT0001014	CCCCAAAUGUAGACAAAGCA	20	212,0	142,3	-0,59	0,000894708
miR160	ath-miR160a-5p	AT2G39175	MIMAT0000178	UGCCUGGCUCCCUGUAUGCCA	21	148,0	94,6	-0,68	0,000128041
	ath-miR160c-3p	AT2G46845	MIMAT0031875	CGUACAAGGAGUCAAGCAUGA	21	22,3	0	-7,70	5,69E-14
	ath-miR160c-5p	AT2G46845	MIMAT0000180	UGCCUGGCUCCCUGUAUGCCA	21	148	94,7	-0,68	0,000145873
miR162	ath-miR162a-5p	AT5G08185	MIMAT0031876	UGGAGGCAGCGGUUCAUCGAUC	22	127,3	49,0	-1,39	6,42E-12
miR163	ath-miR163	AT1G66725	MIMAT0000184	UUGAAGAGGACUUGGAACUUCGAU	24	163,3	56,7	-1,50	2,77E-10
miR166	ath-miR166a-5p	AT2G46685	MIMAT0031880	GGACUGUUGUCUGGCUCGAGG	21	614	224,7	-1,74	2,27E-13
	ath-miR166b-5p	AT3G61897	MIMAT0031881	GGACUGUUGUCUGGCUCGAGG	21	614	224,7	-1,74	1,71E-13
miR167	ath-miR167a-3p	AT3G22886	MIMAT0031883	GAUCAUGUUCGCAGUUUCACC	21	285,0	102,0	-1,45	1,00E-10
	ath-miR167a-5p	AT3G22886	MIMAT0000196	UGAAGCUGCCAGCAUGAUCUA		1476	995	-0,57	0,002590014
	ath-miR167b	AT3G63375	MIMAT0000197	UGAAGCUGCCAGCAUGAUCUA		1473,7	994,7	-0,57	0,002590014

Figure S2 *(continues)*: List of miRNAs significatively down-regulated in shoots of *A. halleri* vs *A. thaliana* as obtained by sRNA-Seq experiment.

Family name	miRNA name	Gene bank accession	miRbase accession	Sequence 5'-3'	Length (nt)	Counts A*t*	Counts *Ah*	Log FC	P value
miR168	ath-miR168a-3p	AT4G19395	MIMAT0031884	UUCCCGACCUGCACCAAGCGA	21	2070,7	1423,7	-0,56	0,001742754
	ath-miR168a-5p	AT4G19395	MIMAT0000198	UCGCUUGGUGCAGGUCGGGAA	20	7801,7	6190,3	-0,34	0,042052775
	ath- miR168b-3p	AT5G45307	MIMAT0031885	CCCGCCUUGCAUCAACUGAAU	20	482,0	278,3	-0,79	0,000102239
	ath-miR168b-5p	AT5G45307	MIMAT0000199	UCGCUUGGUGCAGGUCGGGAA	21	7727,3	6120,3	-0,34	0,04098578
miR172	ath-miR172b-5p	AT5G04275	MIMAT0000204	GCAGCACCAUUAAGAUUCAC	20	44,0	0	-8,71	1,78E-24
	ath-miR172e-5p	AT5G59505	MIMAT0001019	GCAGCACCAUUAAGAUUCAC	20	46,7	0	-8,78	1,01E-25
miR319c	ath-miR319c	AT2G4085	MIMAT0001016	UUGGACUGAAGGGAGCUCCUU	21	163,3	100,3	-0,73	5,34E-05
miR390	ath-miR390a-5p	AT2G38325	MIMAT0000931	AAGCUCAGGAGGGAUAGCGCC	21	2263	864,0	-1,39	7,16E-12
	ath-miR390b-5p	AT5G58465	MIMAT0000932	AAGCUCAGGAGGGAUAGCGCC	21	2263	864,0	-1,39	6,89E-12
miR391	ath-miR391-3p	AT5G60408	MIMAT0031904	ACGGUAUCUCUCCUACGUAGC	20	191,7	0	-10,82	1,53E-77
	ath-miR391-5p	AT5G60408	MIMAT0000933	UUCGCAGGAGAGAUAGCGCCA	21	369	0	-11,77	1,48E-116
miR393	ath-miR393a-3p	AT2G39885	MIMAT0031905	AUCAUGCUAUCUCUUUGGAUU	21	82,3	13,0	-2,58	6,20E-08
	ath-miR393a-5p	AT2G39885	MIMAT0000934	UCCAAAGGGAUCGCAUUGAUCC	22	327,7	189,0	-0,81	3,43E-05
	ath-miR393b-5p	AT3G55734	MIMAT0000935	UCCAAAGGGAUCGCAUUGAUCC	22	327,7	189,0	-0,81	4,42E-05
miR398	ath-miR398a-3p	AT2G03445	MIMAT0000948	UGUGUUCUCAGGUCACCCCUU	21	97814,3	9904,7	-3,32	2,07E-103
	ath-miR398a-5p	AT2G03445	MIMAT0031910	AAGGAGUGGCAUGUGAACACA	21	176,7	0	-10,70	2,26E-78
	ath-miR398b-3p	AT2G03445	MIMAT0000949	UGUGUUCUCAGGUCACCCCUG	21	97937,3	9907,0	-3,32	2,63E-104
	ath-miR398b-5p	AT2G03445	MIMAT0031911	AGGGUUGAUAUGAGAACACAC	21	407,3	0	-11,91	6,42E-124
	ath-miR398c-3p	AT5G14565	MIMAT0000950	UGUGUUCUCAGGUCACCCCUG	21	97937,3	9907,0	-3,25	3,77E-103
	ath-miR398c-5p	AT5G14565	MIMAT0031912	AGGGUUGAUAUGAGAACACAC	21	407,3	0	-11,91	3,84E-123
miR400	ath-miR400	AT1G32582	MIMAT0001001	UAUGAGAGUAUUAUAAGUCAC	21	38,0	10,7	-1,68	0,001871535
miR403	ath-miR403-3p	AT2G37275	MIMAT0001004	UUAGAUUCACGCACAAACUCG	21	13578,3	8985,7	-0,63	0,000158077

Figure S2 *(continues)*: List of miRNAs significatively down-regulated in shoots of *A. halleri* vs *A. thaliana* as obtained by sRNA-Seq experiment.

Family name	miRNA name	Gene bank accession	miRbase accession	Sequence 5’-3’	Length (nt)	Counts A*t*	Counts *Ah*	Log FC	P value
miR408	ath-miR408-3p	AT2G47015	MIMAT0001011	AUGCACUGCCUCUUCCCUGGC	21	4513	1659,7	-1,46	1,21E-17
	ath-miR408-5p	AT2G47015	MIMAT0031915	ACAGGGAACAAGCAGAGCAUG	21	78,6	58,3	-0,47	0,048272055
miR472	ath-miR472-3p	AT1G12294	MIMAT0003931	UUUUUCCUACUCCGCCCAUACC	22	115,7	0	-10,10	6,07E-45
miR5640	ath-miR5640		MIMAT0022401	UGAGAGAAGGAAUUAGAUUCA	21	332	0	-11,08	1,24E-89
miR5642	ath-miR5642a		MIMAT0022403	UCUCGCGCUUGUACGGCUUU	20	16,3	0	-7,25	2,53E-10
	ath-miR5642b		MIMAT0022435	UCUCGCGCUUGUACGGCUUU	20	16,3	0	-7,25	2,04E-10
miR5651	ath-miR5651		MIMAT0022422	UUGUGCGGUUCAAAUAGUAAC	21	30,7	0	-8,19	2,02E-17
miR775	ath-miR775	AT1G78206	MIMAT0003934	UUCGAUGUCUAGCAGUGCCA	20	136	0	-10,33	4,27E-71
miR822	ath-miR822-5p	AT5G03552	MIMAT0004239	UGCGGGAAGCAUUUGCACAUG	21	90,3	0	-9,73	4,96E-45
miR823	ath-miR823	AT3G13724	MIMAT0004240	UGGGUGGUGAUCAUAUAAGAU	21	39,7	0	-8,55	1,57E-25
miR824	ath-miR824-3p	AT4G24415	MIMAT0032024	CCUUCUCAUCGAUGGUCUAGA	21	673,3	531,3	-0,36	0,037443509
miR825	ath-miR825	AT2G26211	MIMAT0004241	UUCUCAAGAAGGUGCAUGAAC	21	36,3	7,3	-2,2	1,48E-05
miR827	ath-miR827	AT3G59884	MIMAT0004243	UUAGAUGACCAUCAACAAACU	20	86,0	63,0	-0,48	0,028467055
mIR840	ath-miR840-5p		MIMAT0004262	ACACUGAAGGACCUAAACUAAC	22	128,0	0	-10,23	1,44E-57
miR841	ath-miR841a-3p	AT4G13564	MIMAT0032022	AUUUCUAGUGGGUCGUAUUCA	21	814,0	0	-12,91	1,41E-143
	ath-miR841a-5p	AT4G13564	MIMAT0004263	UACGAGCCACUUGAAACUGAA	21	1022,7	0	-13,24	1,41E-143
	ath-miR841b-3p		MIMAT0017742	CAAUUUCUAGUGGGUCGUAUU	21	813,3	0	-12,04	1,41E-143
	ath-miR841b-5p		MIMAT0017741	UACGAGCCACUGGAAACUGAA	21	46	0	-8,77	6,62E-22
miR846	ath-miR846-3p		MIMAT0004269	UUGAAUUGAAGUGCUUGAAUU	21	189,3	96,0	-1,06	4,55E-07
	ath-miR846-5p	AT1G61226	MIMAT0032023	CAUUCAAGGACUUCUAUUCAG	21	28	0	-8,07	2,23E-15
miR847	ath-miR847	AT1G07051	MIMAT0004278	UCACUCCUCUUCUUCUUGAUG	21	101,7	0	-9,91	1,52E-48

Figure S2 *(continues):* List of miRNAs significatively down-regulated in shoots of *A. halleri* vs *A. thaliana* as obtained by sRNA-Seq experiment.

Family name	miRNA name	Gene bank accession	miRbase accession	Sequence 5'-3'	Length (nt)	Counts A*t*	Counts *Ah*	Log FC	P value
miR850	ath-miR850	AT4G13493	MIMAT0004272	UAAGAUCCGGACUACAACAAAG	22	688,7	0	-12,67	3,24E-111
miR861	ath-miR861-3p	AT3G48201	MIMAT0004306	GAUGGAUAUGUCUUCAAGGAC	21	66,7	0	-9,29	1,74E-29
miR863	ath-miR863-3p	AT4G13494	MIMAT0004310	UUGAGAGCAACAAGACAUAAU	21	31,7	0	-8,22	1,16E-18

Figure S3: List of miRNAs significatively up-regulated in shoots of *A. halleri* vs *A. thaliana*+ Zn as obtained by sRNA-Seq experiment.

Family name	miRNA name	Gene bank accession	miRbase accession	Sequence 5'-3'	Length (nt)	Counts At + *Zn*	Counts *Ah*	Log FC	P value
miR156	ath-miR156h	AT5G55835	MIMAT0001013	UGACAGAAGAAAGAGAGCAC	20	19	52,7	1,49	1,10E-05
miR158	ath-miR158a-5p	AT5G10745	MIMAT0031873	CUUUGUCUACAAUUUUGGAAA	21	39,3	410,0	3,45	4,30E-60
miR159	ath-miR159a	AT1G73687	MIMAT0000177	UUUGGAUUGAAGGGAGCUCUA	21	35621,3	43778,7	0,29	9,58E-05
	ath-miR159b-3p	AT1G18075	MIMAT0000207	UUUGGAUUGAAGGGAGCUCUU	21	26685,3	32745,0	0,29	0,0002897
	ath-miR159c	AT2G46255	MIMAT0001015	UUUGGAUUGAAGGGAGCUCCU	21	745,3	11333,7	3,93	2,04E-199
miR165	ath-miR165a-5p	AT1G01183	MIMAT0031879	GGAAUGUUGUCUGGAUCGAGG	21	36,7	109,3	1,51	2,69E-10
miR166	ath-miR166b-3p	AT3G61897	MIMAT0031897	UCGGACCAGGCUUCAUUCCCC	21	913492,7	1065693,7	0,22	0,033065
	ath-miR166c	AT5G08712	MIMAT0000907	UCGGACCAGGCUUCAUUCCCC	21	914259,333	1066861,7	0,22	0,0338895
	ath-miR166d	AT5G08717	MIMAT0000908	UCGGACCAGGCUUCAUUCCCC	21	914259,333	1066861,7	0,22	0,0358494
	ath-miR166e-3p	AT5G41905	MIMAT0000909	UCGGACCAGGCUUCAUUCCCC	21	912253	1064912,7	0,22	0,0338895
	ath-miR166f	AT5G43603	MI0000980	UCGGACCAGGCUUCAUUCCCC	21	912251,7	1064911,7	0,22	0,035884
	ath-miR166g	AT5G63715	MI0000981	UCGGACCAGGCUUCAUUCCCC	21	913019,3	1066081,3	0,22	0,033065
miR169	ath-miR169f-3p	AT3G14385	MIMAT0031898	GCAAGUUGACCUUGGCUCUGC	21	0	135,3	9,87	4,79E-44
	ath-miR169g-3p	AT4G21595	MIMAT0000912	UCCGGCAAGUUGACCUUGGCU	21	0	51	8,49	1,24E-21
miR170	ath-miR170-5p	AT5G66045	MIMAT0031887	UAUUGGCCUGGUUCACUCAGA	21	36,7	82,7	1,25	1,95E-06
miR171	ath-miR171a-3p	AT3G51375	MIMAT0000202	UGAUUGAGCCGCGCCAAUAUC	21	7,0	47,0	2,57	3,58E-08
	ath-miR171a-5p	AT3G51375	MIMAT0031888	AGAUAUUAGUGCGGUUCAAUC	21	36,7	82,7	1,25	2,48E-06
	ath-miR171b-3p	AT1G11735	MIMAT0000920	UUGAGCCGUGCCAAUAUCACG	21	43,7	89,7	1,03	1,39E-05
	ath-miR171b-5p	AT1G11735	MIMAT0031899	AGAUAUUAGUGCGGUUCAAUC	21	14,3	33	1,19	0,0020922
	ath-miR171c-3p	AT1G62035	MIMAT0000921	UUGAGCCGUGCCAAUAUCACG	21	43,7	89,7	1,03	2,02E-05
	ath-miR171c-5p	AT1G62035	MIMAT0031900	AGAUAUUGGUGCGGUUCAAUC	21	5,7	36	2,36	1,12E-07

Figure S3 *(continues)*: List of miRNAs significatively up-regulated in shoots of *A. halleri* vs *A. thaliana* + Zn as obtained by sRNA-Seq experiment.

Family name	miRNA name	Gene bank accession	miRbase accession	Sequence 5'-3'	Length (nt)	Counts At + *Zn*	Counts *Ah*	Log FC	P value
miR319	ath-miR319a	AT4G23713	MIMAT0000511	UUGGACUGAAGGGAGCUCCCU	21	656,3	1182,0	0,85	1,42E-08
	ath-miR319b	AT5G41663	MIMAT0000512	UUGGACUGAAGGGAGCUCCCU	21	739,3	1524,7	1,05	2,54E-11
miR394	ath-miR394a	AT1G20375	MIMAT0000936	UUGGCAUUCUGUCCACCUCC	20	26,0	76,0	1,67	1,68E-08
	ath-miR394b-5p	AT1G76135	MIMAT0000937	UUGGCAUUCUGUCCACCUCC	20	26,0	77,0	1,69	7,71E-09
miR395	ath-miR395b	AT1G26975	MIMAT0000939	CUGAAGUGUUUGGGGGGACUC	21	2,0	36,3	3,66	1,34E-10
	ath-miR395c	AT1G26985	MIMAT0000940	CUGAAGUGUUUGGGGGGACUC	21	2,0	36,3	3,66	2,94E-10
	ath-miR395f	AT1G69797	MIMAT0000943	CUGAAGUGUUUGGGGGGACUC	21	2,0	36,3	3,65	1,45E-10
miR399	ath-miR399b	AT1G63005	MIMAT0000952	UGCCAAAGGAGAGUUGCCCUG	21	109	452,0	2,10	9,14E-32
	ath-miR399c-3p	AT5G62162	MIMAT0000953	UGCCAAAGGAGAGUUGCCCUG	21	109	456,0	2,1	1,86E-31
miR472	ath-miR472-5p	AT1G12294	MIMAT0032014	AUGGUCGAAGUAGGCAAAAUC	20	26,3	195,0	2,79	1,14E-33
miR848	ath-miR848	AT5G13887	MIMAT0004270	UGACAUGGGACUGCCUAAGCUA	21	3,3	104,0	4,46	2,47E-37

Figure S4: List of miRNAs significatively down-regulated in shoots of *A. halleri* vs *A. thaliana* + Zn as obtained by sRNA-Seq experiment.

Family name	miRNA name	Gene bank accession	miRbase accession	Sequence 5'-3'	Length (nt)	Counts *At* +Zn	Counts *Ah*	Log FC	P value
miR156	ath-miR156d-5p	AT5G10945	MIMAT0000169	UGACAGAAGAGAGUGAGCAC	20	14109,7	6252,3	-1,1	3,71E-42
miR157	ath-miR157a-3p	AT1G66783	MIMAT0031870	GCUCUCUAGCCUUCUGUCAUC	21	97,3	35,0	-1,49	1,99E-08
	ath-miR157a-5p	AT1G66783	MIMAT0000172	UUGACAGAAGAUAGAGAGCAC	21	4695,0	2521,3	-2,54	2,49E-170
	ath-miR157b-3p	AT1G66795	MIMAT0031871	GCUCUCUAGCCUUCUGUCAUC	21	97,3	35,0	-1,42	1,90E-08
	ath-miR157b-5p	AT1G66795	MIMAT0000173	UUGACAGAAGAUAGAGAGCAC	21	14695,0	2521,3	-2,54	1,07E-171
	ath-miR157c-3p	AT1G18217	MIMAT0031872	GCUCUCUAUACUUCUGUCACC	21	1605,3	168,7	-2,54	6,28E-143
	ath-miR157c-5p	AT1G18217	MIMAT0000174	UUGACAGAAGAUAGAGAGCAC	21	14692,0	2521,3	-2,70	1,45E-165
	ath-miR157d	AT1G48742	MIMAT0000175	UGACAGAAGAUAGAGAGCAC	20	1275,0	370,3	-1,78	2,83E-42
miR158	ath-miR158b	AT1G55591	MIMAT0001014	CCCCAAAUGUAGACAAAGCA	20	203,7	142,3	-0,52	0,00171
miR160	ath-miR160a-5p	AT2G39175	MIMAT0000178	UGCCUGGCUCCCUGUAUGCCA	21	157,3	94,6	-0,71	0,0020363
	ath-miR160c-3p	AT2G46845	MIMAT0031875	CGUACAAGGAGUCAAGCAUGA	21	15,3	0	-6,69	1,79E-08
	ath-miR160c-5p	AT2G46845	MIMAT0000180	UGCCUGGCUCCCUGUAUGCCA	21	157,3	94,7	-0,71	0,0018661
miR162	ath-miR162a-5p	AT5G08185	MIMAT0031876	UGGAGGCAGCGGUUCAUCGAUC	22	161,3	49,0	-1,72	3,37E-16
miR163	ath-miR163	AT1G66725	MIMAT0000184	UUGAAGAGGACUUGGAACUUCGAU	24	258,3	52,7	-2,15	4,94E-30
miR166	ath-miR166a-5p	AT2G46685	MIMAT0031880	GGACUGUUGUCUGGCUCGAGG	21	760	224,7	-1,74	1,86E-31
	ath-miR166b-5p	AT3G61897	MIMAT0031881	GGACUGUUGUCUGGCUCGAGG	21	760	224,7	-1,74	1,18E-32
	ath-miR166e-5p	AT5G41905	MIMAT0000190	GGAAUGUUGUCUGGCACGAGG	21	33	16,7	-1,02	0,0096391
miR167	ath-miR167a-3p	AT3G22886	MIMAT0031883	GAUCAUGUUCGCAGUUUCACC	21	264,7	102,0	-1,35	8,08E-13

Figure S4 *(continues)*: List of miRNAs significatively down-regulated in shoots of *A. halleri* vs *A. thaliana* + Zn as obtained by sRNA-Seq experiment.

Family name	miRNA name	Gene bank accession	miRbase accession	Sequence 5'-3'	Length (nt)	Counts *At* +Zn	Counts *Ah*	Log FC	P value
miR168	ath-miR168a-3p	AT4G19395	MIMAT0031884	UUCCCGACCUGCACCAAGCGA	21	2426,7	1423,7	-0,77	2,34E-14
	ath-miR168a-5p	AT4G19395	MIMAT0000198	UCGCUUGGUGCAGGUCGGGAA	20	9223,0	6190,3	-0,57	8,40E-08
	ath- miR168b-3p	AT5G45307	MIMAT0031885	CCCGCCUUGCAUCAACUGAAU	20	586,3	278,3	-1,05	8,24E-13
	ath-miR168b-5p	AT5G45307	MIMAT0000199	UCGCUUGGUGCAGGUCGGGAA	21	9139,0	6120,3	-0,57	8,20E-08
miR172	ath-miR172b-5p	AT5G04275	MIMAT0000204	GCAGCACCAUUAAGAUUCAC	20	71,6	0	-8,92	6,19E-33
	ath-miR172e-5p	AT5G59505	MIMAT0001019	GCAGCACCAUUAAGAUUCAC	20	72	0	-8,92	5,96E-34
miR390	ath-miR390a-5p	AT2G38325	MIMAT0000931	AAGCUCAGGAGGGAUAGCGCC	21	2522,3	864,0	-1,54	1,20E-25
	ath-miR390b-5p	AT5G58465	MIMAT0000932	AAGCUCAGGAGGGAUAGCGCC	21	2522,3	864,0	-1,54	4,43E-26
miR391	ath-miR391-3p	AT5G60408	MIMAT0031904	ACGGUAUCUCUCCUACGUAGC	20	255,7	0	-10,77	1,14E-97
	ath-miR391-5p	AT5G60408	MIMAT0000933	UUCGCAGGAGAGAUAGCGCCA	21	255,7	0	-11,57	9,78E-140
miR393	ath-miR393a-3p	AT2G39885	MIMAT0031905	AUCAUGCUAUCUCUUUGGAUU	21	106,0	13,0	-2,98	3,20E-10
	ath-miR393a-5p	AT2G39885	MIMAT0000934	UCCAAAGGGAUCGCAUUGAUCC	22	328,0	189,0	-0,78	1,15E-06
	ath-miR393b-5p	AT3G55734	MIMAT0000935	UCCAAAGGGAUCGCAUUGAUCC	22	328,0	189,0	-0,78	1,69E-06
miR398	ath-miR398a-3p	AT2G03445	MIMAT0000948	UGUGUUCUCAGGUCACCCCUU	21	91783,7	9904,7	-3,21	0
	ath-miR398a-5p	AT2G03445	MIMAT0031910	AAGGAGUGGCAUGUGAACACA	21	272,3	0	-10,85	3,13E-67
	ath-miR398b-3p	AT2G03445	MIMAT0000949	UGUGUUCUCAGGUCACCCCUG	21	91914,7	9907,0	-3,21	0
	ath-miR398b-5p	AT2G03445	MIMAT0031911	AGGGUUGAUAUGAGAACACAC	21	487,3	0	-11,69	3,21E-148
	ath-miR398c-3p	AT5G14565	MIMAT0000950	UGUGUUCUCAGGUCACCCCUG	21	91914,7	9907,0	-3,21	0
	ath-miR398c-5p	AT5G14565	MIMAT0031912	AGGGUUGAUAUGAGAACACAC	21	487,3	0	-11,69	8,19E-148
miR400	ath-miR400	AT1G32582	MIMAT0001001	UAUGAGAGUAUUAUAAGUCAC	21	33,3	10,7	-1,53	0,0121335
miR403	ath-miR403-3p	AT2G37275	MIMAT0001004	UUAGAUUCACGCACAAACUCG	21	11119,3	8985,7	-0,30	0,0189201

Figure S4 *(continues):* List of miRNAs significatively down-regulated in shoots of *A. halleri* vs *A. thaliana* + Zn as obtained by sRNA-Seq experiment.

Family name	miRNA name	Gene bank accession	miRbase accession	Sequence 5'-3'	Length (nt)	Counts *At* +Zn	Counts *Ah*	Log FC	P value
miR408	ath-miR408-3p	AT2G47015	MIMAT0001011	AUGCACUGCCUCUUCCCUGGC	21	4907,3	1659,7	-1,56	9,69E-63
	ath-miR408-5p	AT2G47015	MIMAT0031915	ACAGGGAACAAGCAGAGCAUG	21	86,7	58,3	-0,61	0,0399468
miR472	ath-miR472-3p	AT1G12294	MIMAT0003931	UUUUUCCUACUCCGCCCAUACC	22	99,7	0	-9,41	2,60E-34
miR5640	ath-miR5640		MIMAT0022401	UGAGAGAAGGAAUUAGAUUCA	21	332	0	-11,15	4,87E-81
miR5642	ath-miR5642a		MIMAT0022403	UCUCGCGCUUGUACGGCUUU	20	17,7	0	-6,9	3,14E-10
	ath-miR5642b		MIMAT0022435	UCUCGCGCUUGUACGGCUUU	20	17,7	0	-6,9	2,15E-10
miR5651	ath-miR5651		MIMAT0022422	UUGUGCGGUUCAAAUAGUAAC	21	40,3	0	-8,16	7,90E-18
miR775	ath-miR775	AT1G78206	MIMAT0003934	UUCGAUGUCUAGCAGUGCCA	20	129	0	-9,77	5,16E-55
miR822	ath-miR822-5p	AT5G03552	MIMAT0004239	UGCGGGAAGCAUUUGCACAUG	21	57,3	0	-8,69	3,79E-23
miR823	ath-miR823	AT3G13724	MIMAT0004240	UGGGUGGUGAUCAUAUAAGAU	21	38,0	0	-8,07	3,79E-23
miR824	ath-miR824-3p	AT4G24415	MIMAT0032024	CCUUCUCAUCGAUGGUCUAGA	21	677,7	531,3	-0,35	0,0031007
miR825	ath-miR825	AT2G26211	MIMAT0004241	UUCUCAAGAAGGUGCAUGAAC	21	30,3	7,3	-1,93	0,0004222
miR827	ath-miR827	AT3G59884	MIMAT0004243	UUAGAUGACCAUCAACAAACU	20	164,7	63,0	-0,48	1,62E-13
mIR840	ath-miR840-5p		MIMAT0004262	ACACUGAAGGACCUAAACUAAC	22	172,7	0	-10,23	6,34E-66
miR841	ath-miR841a-3p	AT4G13564	MIMAT0032022	AUUUCUAGUGGGUCGUAUUCA	21	792,0	0	-12,4	1,15E-155
	ath-miR841a-5p	AT4G13564	MIMAT0004263	UACGAGCCACUUGAAACUGAA	21	931,7	0	-12,63	4,83E-189
	ath-miR841b-3p		MIMAT0017742	CAAUUUCUAGUGGGUCGUAUU	21	791,0	0	-12,04	2,99E-151
	ath-miR841b-5p		MIMAT0017741	UACGAGCCACUGGAAACUGAA	21	46	0	-8,32	2,39E-24
miR846	ath-miR846-3p		MIMAT0004269	UUGAAUUGAAGUGCUUGAAUU	21	193,3	96,0	-1,04	3,72E-06
	ath-miR846-5p	AT1G61226	MIMAT0032023	CAUUCAAGGACUUCUAUUCAG	21	193,3	0	-7,77	6,54E-17
miR847	ath-miR847	AT1G07051	MIMAT0004278	UCACUCCUCUUCUUCUUGAUG	21	92,7	0	-9,31	1,64E-40

Figure S4 *(continues):* List of miRNAs significatively down-regulated in shoots of *A. halleri* vs *A. thaliana* + Zn as obtained by sRNA-Seq experiment.

Family name	miRNA name	Gene bank accession	miRbase accession	Sequence 5'-3'	Length (nt)	Counts *At* +Zn	Counts *Ah*	Log FC	P value
miR850	ath-miR850	AT4G13493	MIMAT0004272	UAAGAUCCGGACUACAACAAAG	22	1034,3	0	-12,78	3,33E-183
miR861	ath-miR861-3p	AT3G48201	MIMAT0004306	GAUGGAUAUGUCUUCAAGGAC	21	56,3	0	-8,60	4,32E-30
miR863	ath-miR863-3p	AT4G13494	MIMAT0004310	UUGAGAGCAACAAGACAUAAU	21	19,3	0	-7,11	4,48E-11

Figure S5*:* List of miRNAs significatively up-regulated in shoots of *A. halleri* vs *A. thaliana* + Zn as obtained by sRNA-Seq experiment.

Family name	miRNA name	Gene bank accession	miRbase accession	Sequence 5'-3'	Length (nt)	Counts *At*	Counts *At* +Zn	Log FC	P value
miR163	ath-miR163	AT1G66725	MIMAT0000184	UUGAAGAGGACUUGGAACUUCGAU	20	163,3	258,3	0,62	0,001895092
miR398	ath-miR398a-5p	AT2G03445	MIMAT003190	AAGGAGUGGCAUGUGAACACA	21	17677	272,3	0,57	0,041657482
miR827	ath-miR827	AT3G59884	MI0005383	UUUGGAUUGAAGGGAGCUCUA	21	86,0	164,7	0,90	5,08E-05
miR850	ath-miR850	AT4G13493	MI0005405	UAAGAUCCGGACUACAACAAAG	21	688,7	1034,3	0,55	0,040309585

Figure S6: List of miRNAs significatively up-regulated in shoots of *A. halleri* vs *A. thaliana* + Zn as obtained by sRNA-Seq experiment.

Family name	miRNA name	Gene bank accession	miRbase accession	Sequence 5'-3'	Length (nt)	Counts *At*	Counts *At* +Zn	Log FC	P value
MiR167	ath-miR167a-5p	AT3G22886	MI0000208	UGAAGCUGCCAGCAUGAUCUA	24	1476	1035,3	-0,55	6,81E-05
	ath-miR167b	AT3G63375	MI0000209	UGAAGCUGCCAGCAUGAUCUA	21	1473,67	1033	-0,55	6,81E-05
	ath-miR167d	AT1G31173	MI0000975	UGAAGCUGCCAGCAUGAUCUGG	22	1293,7	883,7	-0,59	6,81E-05
MiR822	ath-miR822-5p	AT5G03552	MI0005379	UGCGGGAAGCAUUUGCACAUG	21	90,3	57,3	-0,68	0,040309585

Figure S7: List of the main *cis*-regulatory elements found in the *A. thaliana* and *A. halleri MIR398b* and *MIR408* promoters.

Group	Site Name	Sequence	Description	patMIR398b	pahMIR398b	patMIR408	pahMIR408
Inr	INRNTPSADB	YTCANTYY	Inr (initiator) elements found in the tobacco *psaDb* gene promoter without TATA boxes; light-responsive transcription of *psaDb* depends on Inr, but not on TATA box.	795(+); 809(-); 1063(-)	892(-); 906 (-); 1541(-)	72(+); 166(-)	228(-); 999(-)
ABRE	ABRERATCAL	MACGYGB	ABRE (ABA responsive element)-related sequence or Repeated sequence motifs identified in the upstream regions of 162 *A. thaliana* Ca2+-responsive up-regulated genes		1445(+)	374 (-); 438 (-); 1137 (-); 1138(+);	436(-); 500(-);1175(-); 1176(+)
	DRECRTCOREAT	RCCGAC	Core motif of DRE/CRT (dehydration-responsive element/C-repeat) *cis*-acting element found in many genes in *A. thaliana* and in rice.	309(+)	303(+)	283(+)	345(+)
bZIP	DPBFCOREDCDC3	ACACNNG	A novel class of bZIP transcription factors, DPBF-1 and 2 (*Dc3* promoter-binding factor-1 and 2), binding core sequence, found in the carrot *Dc3* gene promoter, whose expression is normally embryo-specific and can also be induced by ABA.	878(+); 1005(+); 1701(-)	974(+); 1735(+); 1795(-)	6(+); 1319(-)	6(+); 1365(-)
LTRE	LTRECOREATCOR15	CCCGAC	Core of low temperature responsive element (LTRE) of cor15a gene in Arabidopsis. A portion of repeat-C (C-repeat) which is repeated twice in cor15a promoter (Baker etal., 1994); ABA responsiveness; Involved in cold induction ofBN115 gene from winter Brassica napus; LTRE	310(+)	223(+); 304(+); 831(+)	284(+); 379(-)	346(+); 633(+)
	GT1GMSCAM4	GAAAAA	GT-1 motif found in the promoter of soybean (*Glycine max*) CaM isoform, *SCaM-4*. It plays a role in pathogen- and salt-induced *SCaM-4* gene expression.	503(-); 523(-); 1657(-)	472(+); 1507(-); 1653(+); 1750(-);	69(-); 246(+); 689(-)	73(-); 784(-); 988(-)
	GT1CONSENSUS	GRWAAW	Consensus GT-1 binding site in many light-regulated genes, e.g., RBCS from many species,	256(+); 374(-); 469(-); 503(-); 523(-); 1077(+); 1078(+); 1100(-); 1251(-); 1394(-); 1554(+); 1613(-); 1657(-)	251(+); 297(-); 367(-); 454(-); 472(+); 486(-); 715(+); 767(+); 1175(+); 1193(+); 1197(-); 1507(-); 1508(+); 1652(+); 1653(+); 1707(-); 1750(-)	69(-); 245(+); 246(+); 689(+); 967(-)	73(-); 784(-); 785(-); 979(-); 988(-); 1210(-); 1357(+)
I-box	IBOX	GATAA	Conserved sequence upstream of light-regulated genes of both monocots and dicots	382(-); 862(+)	375(-); 958(+); 1326(-)	68(+); 136(-)	118(-); 772(-)
MYB	MYB	WAACCA	MYB recognition site found in the promoters of the dehydration-responsive gene *rd22* and many other genes in *A. thaliana*.	961(-); 1331(+); 1793(+)	56(+); 591(-); 1182(-); 1536(+)	278(+); 686(-); 722(-); 1022(+); 1194(+)	340(+); 807(-); 972(-) 1034(+)

Figure S7 *(continues)***:** List of the main *cis*-regulatory elements found in the *A. thaliana* and *A. halleri MIR398b* and *MIR408* promoters.

Group	Site Name	Sequence	Description	patMIR398b	pahMIR398b	patMIR408	pahMIR408
	MYBCORE	CNGTTR	Binding site for all animal MYB and at least two plant MYB proteins AtMYB1 and AtMYB2, both isolated from *A. thaliana*.	0(-); 189(-); 352(+); 560(+); 673(+); 1579(+)	185(-); 691(-); 1677(+);	252(-); 357(-); 682(+); 694(+)	314(-); 419(-); 639(+); 746(+); 858(+)
	MYB2CONSENSUSAT	YAACKG	Recognition site found in the promoters of the dehydration-responsive gene rd22 and many other genes in Arabidopsis; Y=C/T; K=G/T	560(-); 673(-); 1579(-)	1677(-)	252(+); 357(+); 1233(-)	314(+); 419(+)
	DRECRTCOREAT	RCCGAC	Core motif of DRE/CRT (dehydration-responsive element/C-repeat) *cis*-acting element found in many genes in *A. thaliana* and in rice.	309(+)	303(+)	283(+)	345(+)
MYC	MYCCONSENSUSAT	CANNTG	Binding site of AtMYC2 (previously known as rd22BP1); binding site of ICE1 (inducer of CBF expression 1), that regulates the transcription of *CBF*/*DREB1* genes in cold stress	560(+/-); 879(+/-); 1006(+/-); 1715(+/-)	975 (+/-); 1475 (+/-); 1808(+/-)	357(+/-);658(+/-); 824(+/-); 951(+/-);1138(+/-);1233(+/-);	419(+/-);721(+/-); 897(+/-); 1176(+/-);
WRKY	WBOXNTERF3	TGACY	W box" found in the promoter region of a transcriptional repressor ERF3 gene in tobacco; May be involved in activation of ERF3 gene by wounding	531(-); 703(+); 1029(-)	518(-); 628(+); 934(-);1379(+); 1590(-)	152(-); 211(-); 600(+); 875(-); 911(-)	214(-); 273(-); 663(+)
	WRKY71OS	TGAC	"A core of TGAC-containing W-box" of, e.g., Amy32b promoter. Binding site of rice WRKY71, a transcriptional repressor of the gibberellin signaling pathway.	496(-); 532(-); 703(+); 1030(-); 1367(-)	519(-); 566(-); 628(+); 924(-); 935(-); 1379(+); 1473(-); 1480(-); 1591(-)	/	/
Root motif	ROOTMOTIFTAPOX1	ATATT	The sequence is conserved in the SE2 (stem element) of different plant root-specific genes; SE2 is responsible for expression in the vasculature.	114(-); 115(+); 819(-); 838(+); 1336(-); 1435(-); 1501(-);	110(-); 113(+); 728(+); 741(-); 916(-); 948(+); 1431(-); 1489(-); 1550(-); 1919(+)	114(-); 115(+); 560(-); 709(-); 710(+);887(-); 888(+); 923(-); 946(+); 975(-); 1082(-); 1248(-); 1302(-); 1303(+)	622(-); 1095(-); 1349(-); 1350(+)
Light-regulation	SORLIP1AT	GCCAC	One of Sequences Over-Represented in Light-Induced Promoters (SORLIPs) in *A. thaliana*, computationally identified phyA-induced motifs	1480(+); 1688(+)	/	629(-); 1141(-)	692(-)
	SORLIP2AT	GGGCC	One of Sequences Over-Represented in Light-Induced Promoters (SORLIPs) in *A. thaliana*, computationally identified phyA-induced motifs.	396(+); 565(+); 584(-)	828(+)		171(-)

Figure S7 *(continues)***:** List of the main *cis*-regulatory elements found in the *A. thaliana* and *A. halleri MIR398b* and *MIR408* promoters.

Group	Site Name	Sequence	Description	patMIR398b	pahMIR398b	patMIR408	pahMIR408
Nutrent homeostasis	CURECOR	GTAC	GTAC is the core of a CuRE (copper-response element)	1155(+/-); 1542(+/-); 1573(+/-); 1613(+/-); 1638(+/-); 1679(+/-); 1708(+/-)	559(+/-); 1108(+/-); 1270(+/-); 1665(+/-); 1682(+/-);1696(+/-); 1733(+/-); 1758(+/-); 1800(+/-); 1830(+/-)	369(+/-); 404(+/-); 755(+/-); 957(+/-);1052(+/-);1058(+/-); 1077(+/-); 1111(+/-); 1122(+/-); 1272(+/-);1297(+/-);1327(+/-); 1343(+/-)	431(+/-); 466(+/-); 828(+/-); 1063(+/-); 1069(+/-);1090(+/-); 1149(+/-); 1263(+/-); 1296(+/-); 1319(+/-);1344(+/-); 1373(+/-); 1389(+/-)
	SURECOREATSULTR11	GAGAC	Core of sulfur-responsive element (SURE) found in the promoter of SULTR1;1 high-affinity sulfate transporter gene in Arabidopsis. SURE contains auxin response factor (ARF) binding sequence.	219(+); 443(-); 1138(+); 1788(+)	215(+); 433(-); 612(+); 1235(+); 1394(-); 1451(-);	171(+)	233(+); 412(+)
	IRO2OS	CACGTGG	OsIRO2-binding core sequence. Transcription factor OsIRO2 is induced exclusively by Fe deficiency.	/	/	1137(-); 1138(+)	1175(-); 1176(+)
bZIP	P1BS	GNATATNC	PHR1-binding sequence found in the upstream regions of phosphate starvation responsive genes from several plant species.	/	/	565(-); 565(+); 708(-); 708(+); 1247(-); 1247(+)	

IUPAC notation for degenerated bases: N = A/C/G/T; B = C/G/T; D = A/G/T; H = A/C/T; V = A/C/G; K = G/T; M = A/C; R = A/G; Y = C/T; S = C/G; W =A/T

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