

Characterisation of the *Arabidopsis* *thaliana*

Metallothionein gene family in response to nickel with the aid of specific mutants

Ana Filipa Sousa Afonseca

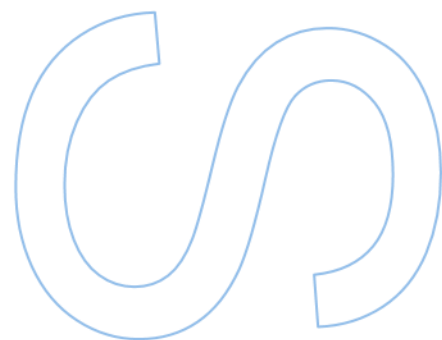
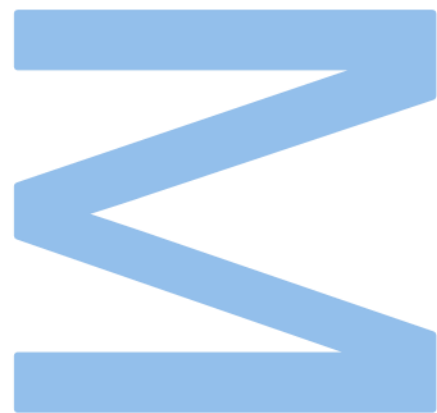
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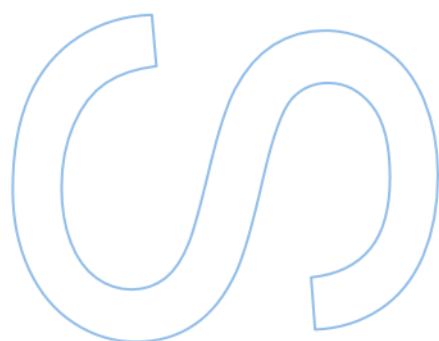
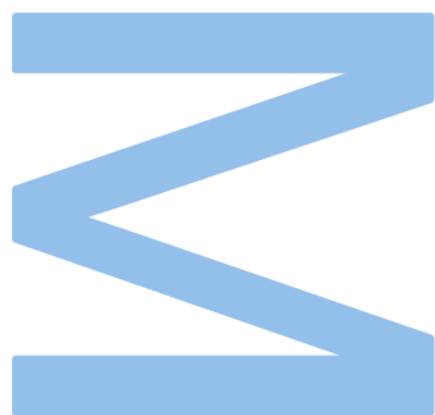
Supervisor

Jorge Teixeira, Assistant Professor, Faculdade de Ciências da
Universidade do Porto

Co-supervisor

Luís Gustavo Pereira, Assistant Professor, Faculdade de Ciências
da Universidade do Porto





Dedicated to my grandpa,

I miss you every day.

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“I have always known there is no mountain you cannot climb, one step at a time”

— Taylor Jenkins Reid

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Resumo

A poluição ambiental levou a um aumento da concentração de metais pesados na natureza, levando ao desenvolvimento de estratégias para prevenir o seu impacto no ambiente e nos humanos. As metalotioninas são proteínas pequenas, ricas em cisteínas, com baixa massa molecular, responsáveis pela destoxificação de metais pesados nas células. Estas proteínas, codificadas por uma família multigénica, conseguem quelatar os metais pesados, regular a homeostasia celular e o processo de destoxificação, prevenindo que estes interfiram com o funcionamento da célula. Foi demonstrado que *Arabidopsis thaliana* expressa 8 genes que codificam metalotioninas e, apesar de serem estudados há décadas, ainda não existe muito conhecimento em relação à resposta individual destes a alguns metais pesados. Assim, é pretendido estudar a resposta da família multigénica das metalotioninas à exposição a níveis elevados de níquel em *A. thaliana* selvagens e em mutantes com a expressão silenciada em certas metalotioninas para avaliar a participação destas a este stress. Sementes de *A. thaliana* forma colocadas em meio MS com concentrações crescentes de Ni de forma a perceber os efeitos de exposição a este metal a longo-prazo. Depois de 21 dias de exposição, a expressão dos genes *AtMTs* foi analisada através de RT-qPCR em diferentes órgãos da planta: raízes, folhas jovens e folhas maduras. Alguns parâmetros relativos ao stress oxidativo das plantas também foram estudados, como a concentração de pigmentos fotossintéticos, de peróxido de hidrogénio e de glutatona reduzida. Adicionalmente, para testar a mobilidade destas proteínas dentro da planta, iniciou-se o processo de clonagem para a obtenção de proteínas de fusão com *AtMT1A* e *AtMT2B* e a proteína repórter mNeonGreen. A análise da expressão de genes demonstrou que os 8 genes reagem diferencialmente às várias concentrações de Ni e de uma forma dependente do órgão. Foi notado que a expressão de MT1A, MT1C e MT3 aumentou nas raízes, enquanto as respostas nas folhas oscilaram mais visto que MT1B e MT1C aumentaram nas folhas jovens, e MT1A, MT2A, MT2B e MT3 diminuíram nas folhas jovens e MT1A, MT2A e MT2B diminuíram nas folhas maduras. Estes resultados sugerem fortemente que o aumento da expressão das metalotioninas nas raízes, especialmente MT1A, MT1C e MT3, será importante na redução da toxicidade de Ni. Entretanto, a redução na expressão de metalotioninas nas folhas pode estar ligada à potencial translocação das metalotioninas das raízes. Uma vez que a investigação em relação ao impacto do Ni em *A. thaliana* está em fase inicial, os resultados obtidos

contribuem significativamente para a compreensão dos mecanismos de defesa das plantas contra metais pesados.

Palavras-chave: Expressão Génica, Stress Oxidativo, RTq-PCR, Contaminação por Metais Pesados.

Abstract

Environmental pollution has increased the concentration of heavy metals in the environment, leading to the development of strategies to prevent their impact on the ecosystems and humans. Metallothioneins are small Cys-rich proteins, with low molecular mass, responsible for the detoxification of heavy metals in cells. These proteins, encoded by a multigenic family, are able to chelate heavy metals, regulate cellular homeostasis and the detoxification process, preventing heavy metals from interfering with cell functioning. It was shown that *Arabidopsis thaliana* expresses 8 metallothionein genes and, despite being studied for decades, there is still not much knowledge regarding their individual response to some heavy metals. Thus, it is intended to study the response of the multigenic family of metallothioneins to the exposure to high levels of nickel in wild *A. thaliana* and in mutants with silenced expression of certain metallothioneins to assess their response in this stress. Seeds of *A. thaliana* were placed in MS medium with increasing concentrations of Ni in order to understand the effects of a long-term exposure to this metal. After 21 days of exposure, the expression of the *AtMTs* genes was analysed through RT-qPCR in different plant organs: roots, young leaves and mature leaves. Some parameters regarding oxidative stress of the plants were also pursued, including the concentration of photosynthetic pigments, of hydrogen peroxide and of reduced glutathione. Furthermore, to see if these proteins have mobility within the plant, the cloning process to obtain chimeric *AtMT1A* and *AtMT2B* fusion proteins with the mNeonGreen reporter protein was initiated. The gene expression analysis showed that the 8 genes reacted differentially to the varying concentrations of Ni and in an organ-specific way. It was noted that the expression of MT1A, MT1C and MT3 increased in the roots, while the response in the leaves was more fluctuating as MT1B and MT1C increased in young leaves, and MT1A, MT2A, MT2B and MT3 decreased in young leaves and MT1A, MT2A and MT2B decreased in mature leaves. These results strongly suggest that the increase in the expression of MTs in the roots, especially MT1A, MT1C and MT3, could be of great importance in reducing Ni toxicity. Meanwhile, the reduction of the expression of metallothioneins in the leaves could be linked to eventual translocations of metallothioneins from the roots. Since the research regarding the impact of Ni in *A. thaliana* is at an early stage, these results significantly contribute for the understanding of the defence mechanisms of plants against this metallothioneins.

Keywords: Gene Expression, Oxidative Stress, RT-qPCR, Heavy Metal Contamination

Table of Contents

List of Tables	ix
List of Figures	x
List of Abbreviations	xiii
1. Introduction.....	1
1.1. Environmental Pollution	1
1.1.1. Heavy Metals and their impact in the environment.....	1
1.1.2. Nickel (Ni) as a Contaminant	3
1.2. Plant strategies to deal with HMs toxicity	4
1.2.1. Metallothioneins.....	5
1.2.2. Oxidative Stress	7
1.3. <i>Arabidopsis thaliana</i> Heynh	8
1.4. Objectives.....	11
2. Materials and Methods.....	12
2.1. Medium Preparation and Seed Germination	12
2.2. Mutant Seeds	13
2.2.1. Seed disinfection	14
2.3. Mutants' Genotyping.....	14
2.4. DNA Extraction	14
2.5. RNA Extraction	15
2.6. DNA and RNA Quantification.....	16
2.7. Metallothionein Sequences retrieval	16
2.7.1. Primer Design.....	17
2.8. PCR Optimization for the designed primer pairs	18
2.8.1. Amplification of Metallothioneins' cDNAs for Cloning	18
2.9. Reverse Transcription (cDNA Synthesis).....	19
2.10. RT-qPCR	19

2.11.	Cloning of the full-length cDNAs of <i>AtMT1</i> and <i>AtMT2B</i>	20
2.11.1.	DNA Purification from the gel	21
2.11.2.	Restriction and Ligation of the insert and vector	21
2.11.3.	<i>Escherichia coli</i> DH5 α Transformation	22
2.11.4.	Culture Medium for plating bacteria.....	23
2.11.5.	Culture of Bacteria	23
2.11.6.	Transformed <i>E. coli</i> Stocks	23
2.11.7.	Plasmid Purification.....	23
2.12.	Biochemical Parameters	24
2.12.1.	Quantification of Hydrogen Peroxide (H ₂ O ₂).....	24
2.12.2.	Extraction and Quantification of Photosynthetic Pigments.....	24
2.12.3.	Quantification of Reduce Glutathione (GSH)	25
2.13.	Biometric Parameters.....	26
2.14.	Statistical Analysis	26
3.	Results	27
3.1.	Plant Growth.....	27
3.2.	Mutants and Genotypes.....	30
3.3.	Primer design and PCR optimization	31
3.4.	Primers and PCR optimization for the amplification of insert fragments for posterior cloning	36
3.5.	RNA Quality	36
3.6.	Confirmation of the expression of the MT genes in the different organs of <i>A. thaliana</i> WT by qualitative RT-PCR	38
3.7.	RT-qPCR	41
3.7.1.	Primer Validation and qPCR optimization	41
3.7.2.	Gene Expression study of the MTs	42
3.8.	Biochemical parameters	46
3.8.1.	Effect of the increasing concentrations of Ni in the H ₂ O ₂ levels.....	46

3.8.2.	Effect of the increasing concentrations of Ni in the Photosynthetic pigments levels.....	47
3.8.3.	Effect of the increasing concentrations of Ni in the GSH levels.....	48
3.9.	Effect of the increasing concentrations of Ni in biometric parameters	48
3.10.	Genetic transformations (<i>E. coli</i> and higher plants)	49
4.	Discussion	51
5.	Conclusion.....	60
6.	References	62
7.	Supplements.....	75

List of Tables

Table 1- Two-digit code used for the collected samples and biological replicates.	13
Table 2 - Reference genes used for the RT-qPCR, with the respective 5' and 3' primer sequences, the size of the amplicon (bp) and the reference from where the primers where obtained.	20
Table 3 - Germination rate of <i>A. thaliana</i> WT when exposed to increasing concentration of Ni - 0 mg/L; 12.5 mg/L; 25 mg/L; 50 mg/L.	27
Table 4 - Germination rate of <i>A. thaliana</i> WT when exposed to increasing concentration of Ni - 0 mg/L; 2.5 mg/L; 5 mg/L; 7.5 mg/L; 10 mg/L.	28
Table 5 - Germination rate of <i>A. thaliana</i> WT and knockout “mutants”.	30
Table 6- Primers designed to be used in RT-qPCR, Forward and Reverse sequences (5'→3'), the size of the amplicon (bp) when using gDNA as template and the starting T_a tested.....	32
Table 7 -Sequence of the forward and reverse primers used in RT-qPCR, the final T_a for optimal results and reference of the primers found in the literature.	34
Table 8 - Sequence of the forward and reverse primers used to amplify the insets to be used for cloning and the optimal annealing temperature to be used in PCR. The added restriction sites for <i>Xba</i> I in the forward- and <i>Sal</i> I in the reverse primers are underlined.	36
Table 9 - RNA quantification and purity results. YL – Young leaves; ML – Mature leaves; R – Roots. 0-1 to 0-3 – control; 1-1 to 1-3 – 2.5 mg/L Ni; 2-1 to 2-3 – 5 mg/L Ni; 3-1 to 3-3 – 7.5 mg/L Ni; and 4-1 to 4-3 – 10 mg/L Ni.	37
Table 10 - List of genes and primer sequences used in the qPCR analysis, and the corresponding efficiency (E), correlation coefficients (R^2); slope, Melting Temperature and the tissue used.....	42

List of Figures

Figure 1 - Schematic illustration of sources of heavy metals in agricultural soils and their toxic effect to plants and humans. Adapted from Ahmed et al., (2021).....	2
Figure 2 - Schematic representation of the four types of MT structures, according to Cobbett & Goldsbrough (2002), along with the number of Cys residues present in each type.....	6
Figure 3- <i>Arabidopsis thaliana</i> Heynh, (Porto, 2023).....	10
Figure 4-A. <i>thaliana</i> WT grown in MS medium with increasing concentrations of Ni 0 mg/L (A, B); 12.5 mg/L (C, D, E); 25 mg/L (F, G, H); 50 mg/L (I, J, K), one month after exposure.....	28
Figure 6 - <i>A. thaliana</i> WT grown in 1x MS medium with increasing concentrations of Ni - 0 mg/L (A); 2.5 mg/L (B); 5 mg/L (C); 7.5 mg/L (D); 10 mg/L (E), 7 days post-germination, approximately.	29
Figure 8 - <i>A. thaliana</i> WT (A) and knockout mutants (B, C, D) in 1x MS medium, 7 days post-germination. A- WT, B – MT1A, C-MT2B, D-MT3.....	30
Figure 9 - Example of the result of the genotyping of the mutants with the knockdown gene MT1A (A) and MT2B (B). Lane 1 corresponds to the PCR using the forward and reverse primers for the MT in question; lane 2 corresponds to the PCR using the primers forward of the MT and the primer for the T-DNA; and lane 3 corresponds to the results of the PCR using the primer reverse of the MT and the primer for the T-DNA. The PCRs were performed using gDNA extracted from the leaves of mutant plants. The gene ruler used- GRS Ladder 50bp, grisp.	31
Figure 10 – Agarose gel electrophoresis with the results from the first optimization PCR using gDNA from WT <i>A. thaliana</i> and testing the primers MT1AB, MT1B, MT1C, MT2A, MT3, MT4A and MT4B (left to right, mentioned above the corresponding lanes). The gene ruler- GRS Ladder 50bp, grisp.	33
Figure 11 - Agarose gel electrophoresis with the results of the optimized PCR using gDNA of WT <i>A. thaliana</i> for each set of primer pairs designed. The MT for each amplicon is mentioned above the corresponding lane (MT1AB, MT1B, MT1C, MT2A, MT3, MT4A and MT4B, - left to right). The gene ruler used- GRS Ladder 50bp, Grisp.....	35
Figure 12 - Agarose gel electrophoresis gel with the results of the optimized PCR using gDNA from WT <i>A. thaliana</i> for the new pairs of primers for MT1C, MT2B (left) and MT1A (right). The gene ruler used- GRS Ladder 50bp, grisp.	35
Figure 13 - Agarose gel electrophoresis with the results of the optimized PCR using cDNA from WT <i>A. thaliana</i> as template for the primer pairs MT1A+RE (A) and MT2B+RE	

(B) using the optimal annealing temperature for each pair. The gene ruler used- GRS Ladder 50bp, grisp.....	36
Figure 13 - RNA quality accessed by a 0.8% (w/v) gel electrophoresis of 300 ng of the total RNA extracted. A-Young leaves RNA. B- Mature leaves RNA. C- Roots RNA.	38
Figure 14 - Typical agarose gel electrophoresis with the results of the PCR for each set the original primers pairs (MT1AB, MT1B, MT1C, MT2A, MT3, MT4A and MT4B, -left to right) using cDNA of roots (A) and shoots (B) from WT <i>A. thaliana</i> . The gene ruler used were A – GRS Ladder 50 bp, grisp; B -MassRuler DNA Ladder Mix, ready-to-use, Thermo Scientific.	39
Figure 15 – Typical agarose gel electrophoresis with the results of the PCR for each set the original pairs of primers (MT1AB, MT1B, MT1C, MT2A, MT3, MT4A and MT4B, -left to right) using cDNA of young leaves (A) and mature leaves (B) from WT <i>A. thaliana</i> . The gene ruler used MassRuler DNA Ladder Mix, ready-to-use, Thermo Scientific.	40
Figure 16 - Typical agarose gel electrophoresis with PCR products using cDNA from WT <i>A. thaliana</i> from the different organs and the MTs primers at the corresponding annealing temperature. A- MT1A and MT1B. B -MT1C and MT2A. C- MT2B and MT3. D- MT4A and MT4B. ML-Mature leaves; YL- Young leaves; R-Roots. The gene ruler used- GRS Ladder 50 bp, grisp.	41
Figure 17 - Relative Normalized expression of the MT1A (A), MT1B (B), MT1C (C), MT2A (D), MT2B (E), MT3 (F), MT4A (G) AND MT4B (H) in young leaves of plants exposed to increasing concentrations of Ni- 2.5, 5, 7.5, 10 mg/L. Asterisks represent significant differences at $p < 0.05$ between the control and treatments.	43
Figure 18 - Relative Normalized expression of the MT1A (A), MT1B (B), MT1C (C), MT2A (D), MT2B (E), MT3 (F), MT4A (G) AND MT4B (H) in mature leaves of plants exposed to increasing concentrations of Ni- 2.5, 5, 7.5, 10 mg/L. Asterisks represent significant differences at $p < 0.05$ between the control and treatments.	44
Figure 19 - Relative Normalized expression of the MT1A (A), MT1B (B), MT1C (C), MT2A (D), MT2B (E), MT3 (F), MT4A (G) AND MT4B (H) in roots of plants exposed to increasing concentrations of Ni- 2.5, 5, 7.5, 10 mg/L. Asterisks represent significant differences at $p < 0.05$ between the control and treatments.	45
Figure 20 - Example of a gel electrophoresis analysis of the RT-qPCR for MT2A, using the cDNA of young leaves (A, B) from one of the technical replicates from each of the biological samples and NTC. 0-1 to 0-3 – control; 1-1 to 1-3 – 2.5 mg/L Ni; 2-1 to 2-3 – 5 mg/L Ni; 3-1 to 3-3 – 7.5 mg/L Ni; and 4-1 to 4-3 – 10 mg/L Ni.	46

Figure 21 - Average + SD levels of Hydrogen Peroxide, expressed as nmole/g FW in the aerial part (AP) (A) and in the roots (R) (B) of plants exposed to increasing concentrations of Ni – 0, 2.5, 5, 7.5, 10 mg/L.....	46
Figure 22 - Average + SD levels of Chlorophyll a in young leaves (A) and mature leaves (E); Chlorophyll b in young leaves (B) and mature leaves (F); β -Carotenes in young leaves (C) and mature leaves (G); and Lutein in young leaves (D) and mature leaves (H), expressed as mg/g FW in plants exposed to the different concentrations of Ni – 0, 2.5, 5, 7.5, 10 mg/L.....	47
Figure 23 - Average + SD levels of GSH in aerial part of the plant (AP) (A) and in the roots (R) (B) of plants exposed to increasing concentrations of Ni – 0, 2.5, 5, 7.5, 10 mg/L.	48
Figure 24 - Average + SD total leaf area, expressed in pixels, of young leaves and mature leaves of plants exposed to increasing concentrations of Ni – 0, 2.5, 5, 7.5, 10 mg/L.	48
Figure 25 – Average + SD root length in cm of plants exposed to increasing concentrations of Ni – 0, 2.5, 5, 7.5, 10 mg/L.....	49
Figure 26 - Petri dishes with LB medium with ampicillin and X-GAL. A-Transformed cells with the ligation of MT1A and pSK with blunt ends, created by <i>EcoR321</i> ; B- Transformed cells with the ligation of MT2B and pSK with blunt ends, created by <i>EcoR321</i>	50
Figure 27 - (A) <i>E coli</i> . colony resulting from the transformation with the sticky end ligation between pMDC83 and MT1A, using the restriction enzymes <i>XbaI</i> and <i>SalI</i> , in culture medium with kanamycin. (B) Electrophoresis of the mini-prep from the colony in (A); the gene ladder used is GeneRuler 1 kb DNA Ladder, thermoscientific; 1- pMDC83; 2- miniprep pMDC83 + MT1A digested with <i>XbaI</i> and <i>SalI</i>	50

List of Abbreviations

ACT2	ACTIN 2 GENE
AP	AERIAL PART
DTT	DITHIOTHREITOL
EDTA	ETHYLENEDIAMINETETRAACETIC ACID
FW	FRESH WEIGHT
GS	GLUTATHIONE
GSH	REDUCE GLUTATHIONE
HEPES	(4-(2-HYDROXYETHYL)-1-PIPERAZINEETHANESULFONIC ACID)
HM(S)	HEAVY METAL(S)
LB	LURIA BROTH BASE
LUT	LUTEIN
ML	MATURE LEAVES
MT(S)	METALLOTHIONEIN(S)
MT1	METALLOTHIONEIN TYPE I
MT2	METALLOTHIONEIN TYPE II
MT3	METALLOTHIONEIN TYPE III
MT4	METALLOTHIONEIN TYPE IV
PC(S)	PHYTOCHELATIN(S)
R	ROOTS
RE	RESTRICTION ENZYME
RT	ROOM TEMPERATURE
PVPP	POLYVINYLPOLYPYRROLIDONE
SAND	SAND FAMILY GENE
SB	SODIUM BORIC ACID
SD	STANDARD DEVIATION
SN	SUPERNATANT
T _A	ANNEALING TEMPERATURE
T _M	MELTING TEMPERATURE
WT	<i>WILD-TYPE</i>
YL	YOUNG LEAVES
YLS8	YELLOW-LEAF-SPECIFIC GENE 8

1. Introduction

1.1. Environmental Pollution

The increase of world population, the necessity of producing enough food, along with the industrialization and urbanization, lead to an increased accumulation of pollutants in the environment (Ghous et al., 2022; Houshmandfar & Moraghebi, 2011; Kiran et al., 2022; Sall et al., 2020). The economic growth, coupled with the development of agriculture and industry, for example, has negatively impacted ecosystems, and both anthropogenic and natural resources are responsible for the release of toxic substances into ecosystems (Alengebawy et al., 2021).

Environmental pollutants are toxic substances that can vary from metals, such as, mercury (Hg), arsenic (As), cadmium (Cd), aluminium (Al), lead (Pb), and many others, to chemical substances, including pesticides, fungicides and insecticides (Alengebawy et al., 2021). The overexposure and uncontrolled use of these substances resulted in their accumulation in the food chain and impacted the ecosystems negatively, since these substances are not easily degradable or eliminated (Alengebawy et al., 2021; Ghous et al., 2022; Radočaj et al., 2020).

Soil pollution by heavy metals (HMs) is a significant issue due to their toxicity and persistence in ecosystems, as they are not degradable, meaning they cannot be decomposed either by chemical or physical procedures, they remain in the soil for a long time, and account for a considerable portion of soil pollutants (Ahmed et al., 2021; Pan et al., 2018; Tumanyan et al., 2020). HM presence can impact the soil negatively since they can alter its properties and chelate specific organic contaminants, influencing its quality and turning agricultural lands barren (Ahmed et al., 2021; Pan et al., 2018). The HM contamination has severely impacted agricultural lands, becoming an alarming and serious environmental issue (Ahmed et al., 2021; Ghous et al., 2022; Sall et al., 2020). Hence, the need to research and improve different techniques to resolve this problem has been highlighted by researchers and several organizations.

1.1.1. Heavy Metals and their impact in the environment

HMs are a heterogenous group of elements with a variety of chemical properties and are characterized for weighing more than 5 g/cm³ (Kiran et al., 2022; Zenk, 1996). HMs are organically present in the Earth's crust and natural disasters, such as soil erosion, flooding, volcanic activity, and others, are the main source of HM in agricultural soils.

However, anthropogenic activities, for instance, mining, leather industries, sewage, automobiles, landfills, animal manure, and many others, are the main motive behind HMs contamination in the ecosystems (Ahmed et al., 2021; Kiran et al., 2022; Zenk, 1996).

Despite some HMs being essential for most species, such as, molybdenum (Mo), manganese (Mn), copper (Cu), nickel (Ni), iron (Fe), zinc (Zn), since they are needed for catalytic and structural properties of many proteins, when they are present in a higher concentration than the required, they can become toxic and inhibit plant development (Ahmed et al., 2021; W.-J. Guo et al., 2008; Radočaj et al., 2020). These higher concentrations of HMs, either essential or non-essential, negatively affect agricultural crops and consequently human health. Once HMs are incorporated into plant systems, they contaminate the food chain, posing a significant risk to food quality and human health safety (Ahmed et al., 2021; Radočaj et al., 2020).

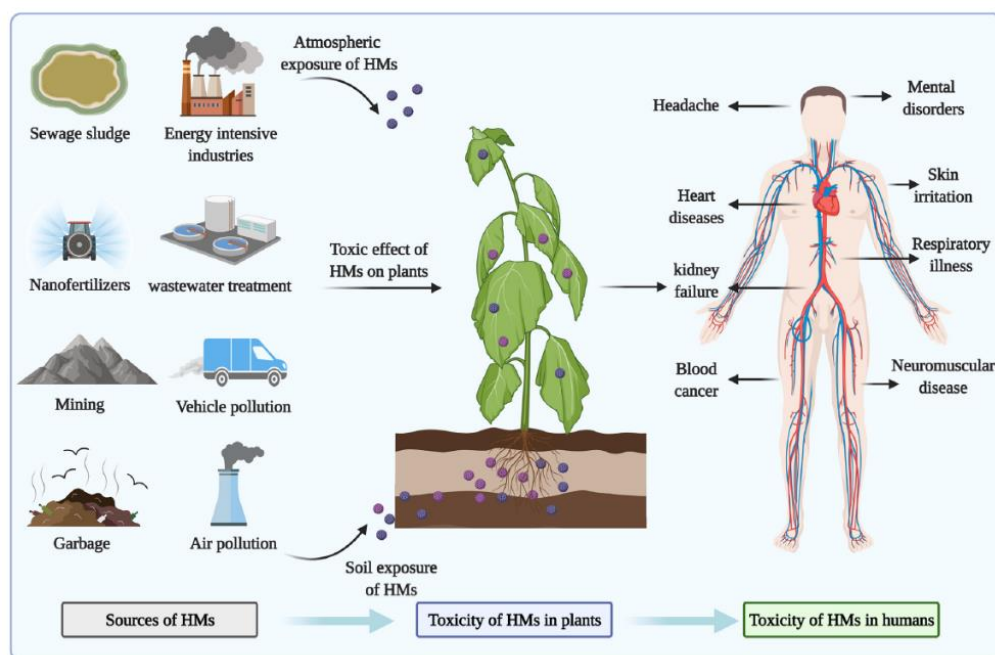


Figure 1 - Schematic illustration of sources of heavy metals in agricultural soils and their toxic effect to plants and humans. Adapted from Ahmed et al., (2021).

The fact that HMs are non-biodegradable and water-soluble, allows them to easily enter plants, and consequently humans, through the food chain (Kiran et al., 2022). They enter the food chain due to being dissolved in the water which is then absorbed along with other nutrients from the soil (Ahmed et al., 2021).

In plants, HMs affects the uptake of essential nutrients from soil, for example, high concentrations of Cr in the soil form insoluble complexes, masking the absorption site

(Ahmed et al., 2021). Once absorbed, they can interact directly with several biochemical and physiological processes, such as, the photosynthetic apparatus, modulating the donor site of enzymes involved in oxidation-reduction reaction and the enzymes involved in the Calvin cycle (Rai et al., 2016), but also deteriorate cell organelles and reduce plant growth (Gunalan et al., 2018). Overall, HMs are able to bind and interfere with molecular targets and compete for binding sites, altering cell metabolism or pathway signalling events (Chen et al., 2021).

HMs are easily absorbed by the root of the plant and accumulate, in some cases, in the edible part of the plant. This results in the over-ingestion of the contaminants by animals, including humans (Chen et al., 2021; Ghous et al., 2022). Many studies have shown that HMs impact humans negatively, as its intake causes a reduction of essential minerals, which, consequently, results in malnutrition (Ghous et al., 2022), furthermore, most metals are carcinogenic affecting human growth and development (Sall et al., 2020).

1.1.2. Nickel (Ni) as a Contaminant

Nickel is a nutritionally essential trace metal for a number of species, from animals to microorganisms, including plants. Therefore, its deficiency or toxicity can occur when the organism is exposed to insufficient or excessive amounts of Ni, respectively (Cempel & Nikel, 2006; Kiran et al., 2022).

Nickel is the 24th most abundant element in the Earth's crust and makes up 3% of the Earth's composition. This element naturally occurs in various mineral forms and has many industrial and commercial applications. The progress of industrialization has led to the increase emission of Ni into the ecosystems and, even though Ni is a minor essential metal, its concentration in areas of anthropogenic development may become toxic to the surrounding living organisms (Buxton et al., 2019; Cempel & Nikel, 2006).

Nickel is naturally released from the environment through actions like windblown and weathering of rocks, and wildfires. However, anthropogenic activities like casting and battery manufacturing factories, fossil fuel burning and fertiliser applications also release Ni, increasing its concentration in the ecosystems (Buxton et al., 2019; Ferraz et al., 2012; Kiran et al., 2022).

High concentrations of Ni in the ecosystems can affect the plant in different capacities, from the development to the metabolic pathways. Ni is accumulated in roots,

which affects their growth, its toxicity can reduce crop yield and decrease the biomass. This metal can also disrupt the uptake and metabolism of Fe, which in turn causes chlorosis and necrosis, inhibits chlorophyll production and disrupts chloroplast structure (Kiran et al., 2022; Sreekanth et al., 2013). Overall, Ni can affect the morphology, inhibit the photosynthesis and transpiration and affect mineral nutrition and enzyme activity (Ahmad & Ashraf, 2012; Hassan et al., 2019; Sreekanth et al., 2013).

The increased intake of Ni has an impact in human health (Dubey et al., 2021; Ghous et al., 2022; Pan et al., 2018; Radočaj et al., 2020). This ubiquitous metal is frequently associated with allergic skin reactions, as it is a common cause for allergic contact dermatitis but the exposure to higher quantities of Ni from daily diet, has also been responsible for skin allergies. Hypersensitivity to this HM has shown to trigger asthma, conjunctivitis and inflammatory reactions. It has also been shown that once inhaled Ni is extremely carcinogenic, causing chronic bronchitis and reducing lung functionality. However, the most affected organ by Ni exposure is the kidney, leading to kidney injury (Buxton et al., 2019; Cempel & Nikel, 2006; Kiran et al., 2022).

1.2. Plant strategies to deal with HMs toxicity

To ameliorate the toxic effects of HMs or the excess of essential elements, plants employ various strategies (Peterson & Oliver, 2006; Zenk, 1996). According to the chemical and physical properties of metals, plants approaches can be distinguished between: production of reactive oxygen species (ROS), by auto-oxidation and Fenton reaction; blocking of essential functional groups in biomolecules; and displacement of essential metal ions from biomolecules (Solanki & Dhankhar, 2011).

A key process to maintain cellular homeostasis in plants is the neutralization and sequestration of excessive free metal ions (Nualla-ong et al., 2020). Plants use different types of organic molecules to decrease metal availability including organic acids, such as malate and citrate; low molecular weight proteins and peptides, like metallothioneins (MTs) and phytochelatins (PCs), respectively (Filiz et al., 2019; Peterson & Oliver, 2006). MTs and PCs are responsible for the chelation process, which consists in the capture and binding of free metal ions to these molecules, resulting in the decrease of metal ions reactivity and solubility (Nualla-ong et al., 2020).

In the presence of HMs in the intracellular space, the production of MTs and PCs are induced (Dubey et al., 2021; Giritch et al., 1998; Sekhar et al., 2011). Both types of

peptides are used by higher plants. The first, MTs, compose a gene-encoded protein family; the second, PCs, are enzymatically formed from glutathione (Zhigang et al., 2006).

1.2.1. Metallothioneins

Eukaryotes have specialized peptides responsible for the detoxification of HMs present in the cells (Sekhar et al., 2011). MTs are cysteine- (Cys) -rich proteins with a low molecular weight (4-14 kDa), and are found in a wide variety of species (Giritch et al., 1998; Sekhar et al., 2011; Xiong et al., 2018). This superfamily of evolutionarily conserved proteins have the capacity to bind HMs via the thiol groups of their Cys residues (Zhu et al., 2009) and it has been proved that they have a wide range of functional roles (Dubey et al., 2021; Zhu et al., 2009). However, the main role of these small proteins involves their ability to chelate metals, regulating cellular metal homeostasis and the detoxification process, providing protection against HM toxicity and oxidative stress (Dubey et al., 2021; Giritch et al., 1998; Jin et al., 2014; Xiong et al., 2018).

MTs in animals, besides having an important role in the homeostasis and detoxification, are also involved in several other physiological processes, such as, regulating cell growth and proliferation, and ROS scavenging (Zhu et al., 2009). In mammalian genomes, these genes are differentially regulated and probably have overlapping but distinct functions (Giritch et al., 1998).

In plants, MTs are also important in seed germination and in the response to abiotic stresses (Dubey et al., 2021; Gu et al., 2015; Sekhar et al., 2011), they are additionally implicated in some important development processes, for instance, fruit ripeness, root development and suberization (Jin et al., 2014; Zhu et al., 2009).

According to the distribution of the Cys residues in the amino- and carboxy-terminal regions, the MTs can be classified into 4 types: type I (MT1), type II (MT2); type III (MT3); and type IV (MT4) (Giritch et al., 1998; Gu et al., 2015; W.-J. Guo et al., 2008; Murphy et al., 1997; Sekhar et al., 2011; Zhou & Goldsbrough, 1995).

Type I MTs contain a total of six Cys-Xaa-Cys motifs (where Xaa represents another amino acid) that are equally distributed among two domains, that are separated by an approximately 40 amino acids stretch called spacer (Fig. 2) (Cobbett & Goldsbrough, 2002; Giritch et al., 1998; Leszczyszyn et al., 2013).

The type II MTs have a similar composition to the MT1's, in which they also contain two Cys-rich domains separated by a spacer of 40 amino acids, approximately, but the first pair of Cys residues is present as a Cys-Cys motif and a motif of Cys-Gly-Gly-Cys is present at the end of the N-terminal region. However, the C-terminal domain contains three Cys-Xaa-Cys motifs (Fig. 2) (Cobbett & Goldsbrough, 2002; Leszczyszyn et al., 2013; Sekhar et al., 2011).

The third type of MT, as we can see on Fig. 2, contains four Cys residues in the N-terminal domain, in which the first three are part of the sequence Cys-Gly-Asn-Cys-Asp-Cys, while the fourth is within a highly conserved motif Gln-Cys-Xaa-Lys-Lys-Gly. Also, the Cys residues in the MT3 C-terminal are similar to those of the types I and II, and both domains are separated by a spacer of 40 amino acids, approximately, as well (Cobbett & Goldsbrough, 2002; Gu et al., 2015; Leszczyszyn et al., 2013).

Type IV MTs differ from the previous types, because they have three Cys-rich domains, with five to six Cys residues displayed in Cys-Xaa-Cys motifs. Nevertheless, these MTs have additional amino acids, eight to ten, before the first cysteine residue in the N-terminal domain (Fig. 2) (Cobbett & Goldsbrough, 2002; Gu et al., 2015; Leszczyszyn et al., 2013).

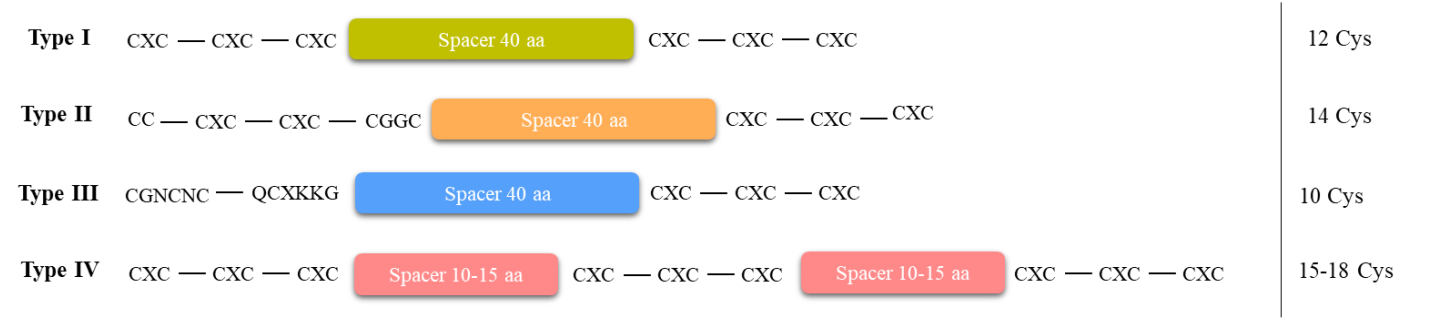


Figure 2 - Schematic representation of the four types of MT structures, according to Cobbett & Goldsbrough (2002), along with the number of Cys residues present in each type.

Previous studies have reported that the different MTs are varyingly expressed in plants: type I MTs are predominantly expressed in roots (W. Guo et al., 2003; Zhou & Goldsbrough, 1994); the type II MTs are mainly expressed in leaves (W. Guo et al., 2003; Hsieh et al., 1996); the type III are expressed in leaves and ripening fruits (W. Guo et al., 2003; Reid & Ross, 1997); and the type IV MTs were detected only in seeds (W. Guo et al., 2003).

Researchers have, for the last couple of decades, tried to identify and determine the functions and roles of the different MTs within the cell. Sekhar et al., (2011) and W.-J. Guo et al., (2008) have shown that the type I MTs increase the tolerance of the plant to Cd and are responsible for the distribution and homeostasis of Cu, respectively. Also, Dubey et al., (2021) noted that the over expression of the MT1 increased significantly the accumulation of As and Cu. According to prior studies by Lv et al., (2013) and Zhigang et al., (2006), plant type I and II MTs play an important role in the tolerance to cadmium and copper, where MT2 are induced in seedlings by copper (Murphy et al., 1997).

Lee et al., (2004) noted that the overexpression of *Arabidopsis thaliana* MT2A and MT3 enhanced the Cd resistance in plant cells. Since the type IV MTs are mainly expressed in seeds, it is more difficult to study how HMs stress would impact these MTs, but researchers noted that when MT4 was expressed in a heterologous system, it conferred tolerance to Zn, also enhancing its accumulation (W.-J. Guo et al., 2008) and that this MT could be involved in Zinc homeostasis in seeds (Imam & Blindauer, 2018).

1.2.2. Oxidative Stress

One of the first consequences caused by HM toxicity is oxidative stress, which is triggered by the increased accumulation of ROS, such as hydrogen peroxide (H_2O_2), hydroxyl radical ($\bullet\text{OH}$) and superoxide anion ($\bullet\text{O}_2^-$) (Gill & Tuteja, 2010; Noor et al., 2022). Depending on the level of toxicity of the HM, the balance of ROS and cellular scavenging activities will vary, since the metal ions trigger different reactions; for example, Fenton reaction uses H_2O_2 in a Fe^{2+} - and Cu^+ - catalysed reaction; Haber-Weiss reaction induces the conversion of H_2O_2 and $\bullet\text{O}_2^-$ to $\bullet\text{OH}$ (Mithöfer et al., 2004; Møller et al., 2007; Thakur et al., 2022). The rapid production of ROS can be induced through the exposure of the organism to determined environmental conditions, ranging from abiotic to biotic stresses, such as, HM toxicity, nutrient excess or deficiency, wounding, flooding, drought, and others. Extreme concentrations of ROS can negatively impact the organism at a molecular level through protein oxidation, lipid peroxidation and DNA damaging (Chaki et al., 2020; Hasanuzzaman et al., 2019).

Mitochondria, due to an over-reduction of the electron transport chain; chloroplasts, when the light intensity and other environmental changes can affect photosynthetic apparatus; and peroxisomes, through photorespiration, are main cell organelles responsible for the production of ROS (Mithöfer et al., 2004; Møller et al., 2007; Thakur

et al., 2022). Both mitochondria and chloroplasts produce $\bullet\text{O}_2^-$ that is then converted into H_2O_2 (Møller et al., 2007).

Plants have developed defence mechanisms to avoid oxidative stress, that are responsible for removing ROS and maintain antioxidant defence compounds. Non-enzymatic antioxidants, including glutathione reduced (GSH) and carotenoids, and enzymatic antioxidants, such as catalase, glutathione reductase, glutathione S-transferase and glutathione peroxidase, scavenge ROS to protect plants from oxidative damage (Hasanuzzaman et al., 2019; Hasanuzzaman & Fujita, 2022; Helaoui et al., 2022).

Carotenoids are located in high quantities in chloroplasts and chromoplasts. These pigments are effective antioxidants that depend on their interaction with other co-antioxidants, they also participate in several biological processes, including photosynthesis and photoprotection. But also, the nature of the ROS affects the mechanisms and rate of scavenging of free radicals (Nisar et al., 2015; Roukas, 2016).

Glutathione (GS) is considered to be a critical intracellular defence mechanism against ROS-induced oxidative damage in plants. Its reduced form is present in plant tissues and can be localized in a wide range of cell compartments, such as cytosol, endoplasmic reticulum, mitochondria, chloroplast, peroxisomes and apoplast. This metabolite has an important role in a variety of physiological pathways, for example, regulation of sulphate transport, signal transduction, conjugation of metabolites, detoxification of xenobiotics and the expression of stress-responsive genes. Also, it is the precursor for the synthesis of PCs. Besides this, GSH is also important for the function of several growth and development related events in plants, such as cell differentiation, cell death and senescence, pathogen resistance and enzymatic regulation (Gill & Tuteja, 2010; Hasanuzzaman et al., 2019).

1.3. *Arabidopsis thaliana* Heynh

Advances in molecular biology over the last few decades have led to great progresses since a reductionist approach has been established. This approach is based on the concept that complex systems are simply a sum of their parts. Even though this strategy has been successful, it requires a straightforward relationship between the gene and its function. Meaning that a simple summation of the different parts cannot adequately account for the complexity that can be observed in biological systems.

Systems biology attempts to study complex biological systems through the analysis of all components in the context of a whole. It is based on the complexity of the whole system which can only be studied with all of its components and interactions, because systems display emergent characteristics that cannot be predicted from or examined in isolated components (Ideker et al., 2001; Raikhel & Coruzzi, 2003; Van Norman & Benfey, 2009).

Therefore, there was a need to select a model organism that allowed the study of key features that would be applicable to all other plants, including crops (Leonelli, 2007; Van Norman & Benfey, 2009). *Arabidopsis thaliana* is a eudicot species from the Brassicaceae family, along with mustard and cabbages, and it became the most used model organism to study physiological and molecular aspects of plants. Due to the wide scale use of this plant and the inherent knowledge, including the complete sequencing of its small genome and the different genetic mutants available, lead to the creation of the database The Arabidopsis Information Resource (TAIR) (Huala et al., 2001).

A. thaliana is a small higher plant, with an aerial part that averages 10 cm in length; a short life cycle, 6-8 weeks; produces no fleshy fruits, but is low maintenance as it's able to grow in small and restricted spaces under fluorescent lights. As such, using *A. thaliana* as a model organism in research is convenient, because it is possible to obtain results in less time and is relatively cheap to maintain. But also, due to its genome being fully sequenced and annotated, there are many biochemical, molecular and bioinformatic tools available for *A. thaliana*, which facilitate its use as a research model organism (Leonelli, 2007; Pigliucci, 2003; Woodward & Bartel, 2018).

Several studies have used this species coupled with biotechnology approaches in order to obtain solutions to practical problems within agriculture and the environment, as well as many other areas. Researchers have also used it for studying the different stresses plants are exposed to, such as climate changes and the over-use of pesticides, fertilizers or other chemical products (Azab et al., 2018, 2020).

The genome sequencing of *A. thaliana* allowed the identification and annotation of 8 different and active MT-encoding genes (*AtMTs*), representing the four types. These MTs are: three genes for type I: MT1A (AT1G07600), MT1B (AT5G56795) and MT1C (AT1G07610); two for type II: MT2A (AT3G09390) and MT2B (AT5G02380); only one MT has been identified as type III: MT3 (AT3G15353); and two sequences have the characteristics of type IV MTs: MT4A (AT2G42000) and MT4B (AT2G23240) (Cobbett & Goldsbrough, 2002; Zhou & Goldsbrough, 1994, 1995).

The impact of high quantities of HM in the soil on plants has been studied using *A. thaliana* as a model organism, specially to try to understand how they affect the development and growth of plants and the responses of different genes and enzymes to the unfavourable conditions. In *A. thaliana*, different approaches have been implemented to study MTs since they are a mechanism of defence against HMs: some researchers, such as Lee et al. (2004), Ruta et al. (2018), Sauge-Merle et al. (2003), expressed the MT genes of *A. thaliana* in different species; others, as Dubey et al. (2021), Evans et al. (1992), Gu et al. (2014, 2015), Jin et al. (2014), Lv et al. (2013), Patankar et al. (2019), Sekhar et al. (2011), Zhigang et al. (2006) expressed heterologous MTs in *A. thaliana*. Such results pointed to a pivotal role of MTs in increasing the plants' tolerance towards high levels of HM. Other authors have directly used *A. thaliana* as an object of study and exposed it to different stresses and different HMs, mainly copper and lead, like for example: Busoms et al. (2021), García-Hernández et al. (1998), Khandekar & Leisner (2011), Kim & Kang, (2018), R. Benatti et al. (2014). These latter studies have revealed the active participation of native MTs in response to such stress conditions, but unfortunately, they were performed with small exposure periods to such stresses, such as 5-7 days, which do not reflect the real situation, which is if the plants were to grow throughout their whole life cycle under such conditions.



Figure 3- *Arabidopsis thaliana* Heynh, (Porto, 2023).

1.4. Objectives

As it has been previously mentioned, the preoccupation on soil pollution has severely intensified in the last decade. Therefore, the need to study and understand how plants react to these alterations and the consequence of these changes has become a priority.

Regarding MT roles in plant (and, in particular, *A. thaliana*) physiology and HM distribution, homeostasis and tolerance, it is possible to realise that there are many gaps in this knowledge that need to be further investigated. These include: the response of the MT gene family to a prolonged exposure to HM; the lack of knowledge regarding their redundancy; and if (and/or which) MT are, in fact, transported within the plant body, thus contributing for plant HM distribution, metabolism and tolerance.

This study had as main objective to understand the effect of Ni in the development of *A. thaliana*, in its physiology, and in the expression of the individual MT gene family members. The expression of the 8 MTs genes was analysed through RT-qPCR in different plant organs: roots, young leaves and mature leaves, in order to understand the differences between the expression of these genes in different parts of the plants, and in response to the increasing concentrations of Ni.

For this, the use wildtype and mutant plants of *A. thaliana* with different knockdown gene expressions, MT1A and MT2B, was intended, to understand how the effects of a long-term exposure to Ni would impact the expression of all *Arabidopsis* MT gene members. This would also allow to explain how the exposure to Ni affects the wildtype plants regarding its morphology and physiology.

Some parameters regarding oxidative stress of the plants in response to the increasing concentrations of Ni were also pursued, including photosynthetic pigments, hydrogen peroxide, and GSH, to determine the degree of the imposed stress and to learn more about the plant's response to this situation.

Furthermore, we propose to study the potential translocation of two MTs, *AtMT1A* and *AtMT2B*, in the vertical axis of the plant. For this the oriented cloning process to obtain chimeric *AtMT1A* and *AtMT2B* fusion proteins with the mNeonGreen reporter protein was initiated. The final constructs would be used to perform Agroinfiltration in expanded tobacco leaves and the fluorescence would be analysed/detected 3-5 days post-infiltration in other leaves with different development phases, to clarify the eventual translocation of MTs within the plant body.

2. Materials and Methods

2.1. Medium Preparation and Seed Germination

The Murashige & Skoog medium, including vitamins, (MS, 4,3 g/L), with 1% Micro Agar and increasing concentrations of Nickel (0 mg/L; 12.5 mg/L; 25 mg/L; 50 mg/L) was prepared and dispensed in petri dishes after autoclaving. Once the medium was set, the seeds of *Arabidopsis thaliana*'s WT, Columbia strain, were scattered evenly on the MS medium in a laminar flow hood.

Full strength Murashige & Skoog (MS) medium, including vitamins, (4.3 g/L; Duchefa Biochemie), was also prepared, being complemented with 2-(N-Morpholino) ethanesulfonic acid (MES) (1.0 g/L; Sigma Aldrich) and sucrose (10 g/L; Sigma Aldrich). The pH was adjusted to 5.8 using KOH before adjusting the final volume. The agar (9 g/L; Liofilchem) and increasing concentrations of Ni (0 mg/L; 2.5 mg/L; 5 mg/L; 7.5 mg/L; 10 mg/L) were added directly to the bottle in which the medium was going to be autoclaved. Afterwards, in a laminar flow hood the medium was distributed to petri dishes and the disinfected seeds were evenly placed in the plates with the help of a sterile toothpick.

In both cases, the plates were placed at 4°C for 48 h to break the seed dormancy and to synchronize the germination, and then transferred to a growth chamber with a long day photoperiod (16 h light and 8 h dark), at 21°C, with 50-60% relative humidity and light intensity at 180 $\mu\text{mole m}^{-2} \text{s}^{-1}$.

The biological samples were retrieved 21 days after germination, approximately.

To facilitate the comprehension and numbering of the WT samples, a 2-digit code was used, in which the first corresponds to the concentration of Ni and the second to the biological replicate, since for each Ni concentration at least 3 biological replicates were assembled (Table 1).

Table 1- Two-digit code used for the collected samples and biological replicates.

<i>Ni</i> Concentration	First Digit	Second Digit	Code
0 mg/L	0	1	0-1
		1	0-2
		3	0-3
2.5 mg/L	1	1	1-1
		2	1-2
		3	1-3
5 mg/L	2	1	2-1
		2	2-2
		3	2-3
7.5 mg/L	3	1	3-1
		2	3-2
		3	3-3
10 mg/L	4	1	4-1
		2	4-2
		3	4-3

2.2. Mutant Seeds

Based on the information available on TAIR, Signal (<http://signal.salk.edu/cgi-bin/tdnaexpress>) was used to find if T-DNA insertion mutants existed for the different MT-encoding genes. For this, the code of each MT was used and, based on the results, the ones that had the insertion hit in an exon were selected: MT1A, MT1B, MT2B. The seeds were ordered from NASC. All *A. thaliana* seeds used, wild type and mutants, are from the ecotype Columbia-0 (WT col-0), to eliminate interference of different genomic backgrounds on gene expression profiles.

Furthermore, *A. thaliana* MT mutants for MT1A, MT2B and MT3 were requested to researchers that had previously worked with them (Benatti et al., 2014).

The seeds were germinated in a container with greenhouse soil. Once the plant had developed enough, a leaf from each plant was collected in order to extract genomic DNA to be further used for their genotyping (see ahead).

2.2.1. Seed disinfection

With the help of a small spatula, a few seeds were placed in 1.5 mL microcentrifuge tubes with 1 mL of 70% ethanol and afterwards were gently inverted, before incubating for 5 min. In the flow cabinet, the seeds were transferred to a filter paper (Fisher Scientific), previously sterilized with 70% ethanol, and left to dry.

2.3. Mutants' Genotyping

After extracting the gDNA (see ahead) of the mutant plants, 3 PCR were performed to confirm the homozygous presence of the T-DNA insertion. These 3 PCR were prepared with the same protocol as mentioned before. For the first PCR, both forward and reverse primers of the MT of interest, flanking the T-DNA insertion site, were used; for the second the forward primer of the specific MT and the primer for the T-DNA insertion (5' cgc aag ctt gtc gac ACA GGA TAA GTG TTC ACA CG 3') were used; and lastly, in the third PCR the reverse primer for the MT and the primer for the T-DNA were used.

2.4. DNA Extraction

The genomic DNA extraction was performed using the Edwards et al., (1991) procedure. A leaf from *A. thaliana* was collected with the help of a 1.5 mL tube and using quartz sand and a small pestle the organic material was ground. After 15 seconds, 400 µL of extraction buffer (200 mM Tris-HCl pH 7.99, 250 mM NaCl, 25 mM EDTA, 0.5% (w/v) SDS) were added and the tubes vortexed for 5 sec. Then, a centrifugation at top speed followed for 1 min. The supernatant was transferred to a new 1.5 mL tube and 350 µL isopropanol were added, mixed gently, and left at RT for approximately 2 min. Another centrifugation followed for 5 min. Afterwards, the supernatant was removed, and the tube was placed in an incubator at 37°C for a couple of minutes to dry the pellet. Once the pellet was dry, 50 µL of sterilized water were added to dissolve the pellet by gently inverting the tube. The DNA was kept on ice until its quantification and then stored at -20°C until needed.

2.5. RNA Extraction

For the RNA extraction from roots (R), young (YL) and mature leaves (ML), different methods were used. The first one was the “Pure link™ RNA Mini kit” and the entire procedure was completed at room temperature. The different tissues were weighted and cut in small pieces, before being transferred to a sterile 1.5mL tube. For tissue samples around 100 mg, 1 mL of Lysis Buffer and 10 µL of 2-mercaptoethanol were added to each tube and the tissues were ground with a glass pestle until homogeneity. Then, the samples were centrifuged at 2,600 x g for 5 min, the supernatant was transferred to a new sterile 1.5 mL tube and 1.5 volumes of ethanol were added and mixed well by gently shaking the tubes. Making use of the provided spin cartridges, 700 µL of the mixture were transferred and centrifuged at 12,000 x g for 15 sec. The flowthrough was discarded and this step was repeated until the entire sample was processed. Afterwards, 700 µL of Wash Buffer I were added to the spin cartridge and centrifuged it at 12,000 x g for 15 sec. The flowthrough was discarded and 500 µL of Wash Buffer II were added to the cartridge, followed by a centrifugation at 12,000 x g for 15 sec. Again, the flowthrough was discarded and the cartridge plus collector tube centrifuged once more at 12,000 x g for 1 min to remove any remaining wash solution and to dry the cartridge. The cartridge was inserted in a sterile 1.5 mL tube and 30 µL of RNase-free water were added to the centre of the cartridge and left to incubate for 1 min, before centrifuging it at 12,000 x g for 2 min. The flowthrough was loaded again into the centre of the spin cartridge and centrifuged. The RNA was kept on ice until it was quantified and stored at -80°C until needed.

The second technique made use of TRIzol™ reagent. It started by homogenising the samples with 1 mL of TRIzol™ reagent for each 50-100 mg of tissue, with the help of a mortar and pestle. The homogenised was transferred to a 1.5 mL tube and incubated for 5 min at room temperature (RT). Then, 200 µL of chloroform for each 1 mL of TRIzol™ used were added, and the tube gently shaken and incubated for 2-3 min at RT. The samples were then centrifuged at 12,000 x g for 15 min at 4°C. Afterwards, with the aid of a micropipette, the aqueous phase was gently transferred to a new tube and 500 µL isopropanol for each 1 mL of TRIzol™ used were added. The tubes were incubated for 10 min at 4°C (ice), before being centrifuged at 12,000 x g for 10 min, at 4°C. The supernatant was discarded, and, in order to wash the pellet, 1 mL of ethanol 75% was added, gently shaking it. Next, the tube was centrifuged at 15,000 x g at 4°C, the supernatant discarded, and the pellet was left to dry for 10-15 min at RT. To resuspend the pellet, 30 µL of RNase-free water were added and the tubes were placed in a thermal

block at 58°C for 10-15 min to promote the evaporation of the remaining ethanol and to speed up the dissolution of the total RNA extracted. The RNA was kept on ice until it was quantified, and stored at -80°C until needed.

The third method used was “TripleXtractor” (GRiSP), using the same protocol as that of TRIzol™, but with the initial reagent – TripleXtractor - provided by the manufacturer.

2.6. DNA and RNA Quantification

To quantify and determine the purity of the DNA and RNA extracted, the DS-11+Spectrophotometer (DeNovix) and the respective programmes included in the device were used. The blank was measured using 1 µL of the same solution used to dissolve the nucleic acids, followed by 1 µL of the sample. The results obtained were in ng/µL, absorbance (Abs) at 260 nm, Abs ratio of 260 nm and 230 nm, and the Abs ratio between 260 nm and 280 nm. For readings with more than one sample, the sensor was cleaned with water and tissue paper between each sample.

To verify the quality of the RNA extraction, an electrophoresis was performed in a 0.8% (w/v) agarose gel, as previously described. Before loading the gel, 300 ng of total RNA; 1.5 µL of RNA buffer 10x; and H₂O to a final volume of 15 µL were mixed in small droplets on a piece of parafilm, over ice.

2.7. Metallothionein Sequences retrieval

For obtaining the *A. thaliana* metallothioneins' nucleotide sequences, TAIR (<https://www.arabidopsis.org/>) was accessed to find out how many MTs were annotated for this biological model. After discovering the existence of 8 different genes, and using the “genes” data type, it was possible to obtain the corresponding cDNA sequences. Next, the NCBI database (<https://www.ncbi.nlm.nih.gov/>) and its different tools: “Nucleotides”, “Proteins” and “Gene”, were accessed to obtain the mRNA, protein, and genomic DNA sequences, respectively (Sayers et al., 2021). The Ensembl Plants webtool (https://plants.ensembl.org/Arabidopsis_thaliana/Info/Index) was also used to identify and locate the exons in the retrieved genomic DNA sequences (The International Arabidopsis Informatics Consortium, 2012).

2.7.1. Primer Design

To study the expression of the eight different MTs, specific primer pairs were designed for each gene. For this, an initial aligning of the sequences of all mRNAs was done using Clustal Omega (<https://www.ebi.ac.uk/Tools/msa/clustalo/>); the results were obtained in the format Pearson/FASTA (Sievers et al., 2011). With this outcome and making use of PrimerIdent (http://primerident.up.pt/primerident_1.htm) online tool, the primers with the strongest complementarity to the sequence of interest and with the weakest complementarity to the other sequences were chosen (Pessoa et al., 2010). The following settings were considered: primer size, minimum 18 bp and maximum 24 bp; primer T_m, minimum of 50°C and maximum of 60°C; primer GC%, minimum of 45 and maximum of 55; no CG clamp; Max Complementarity, self: 5.00, 3'self: 3.00.

With the output of PrimerIdent, the selected primers were tested in Primer3, where the settings used were the default, except “50-150” to the product sizes range was included.

Before ordering the selected primers, their binding sites in the sequence of interest were checked through an alignment of the primers, genomic DNA sequence of the MT in question and the sequence of the exons. This allowed to verify if the primers would align in a different or in the same exon of the corresponding gene.

The primers used for the cloning experiments were designed making use of the program SnapGene Viewer. For the forward primers, the first 20 amino acids were selected, to which 6 base pairs of the restriction site *Xba*I were insert at their 5' end. This process was repeated for the reverse primer, and also for its 5' end, selecting the last 20 amino acids and adding 6 base pairs of the restriction site *Sal*I. In addition, an assortment of base pairs was also added to the 5' end of each primer to allow the restriction enzymes to better anchor to the DNA molecule.

The primers were manufactured by STAB VIDA, lda. Because all these primers are the outcome of a part of this work, their sequences and other related features will be presented in the results section.

2.7.1.1. Primer Resuspension

The ordered primers were resuspended in a 2 mM stock solution. In order to do this, the microtube containing the lyophilised primers was shortly centrifuged, so that the powder would collect at the bottom, and the necessary quantity of sterile and deionized

H₂O was added. The primers were allowed to hydrate for a minute at room temperature. Next, making use of the vortex a 10-seconds stir was done to dissolve the primers, before spinning in the centrifuge and keeping on ice. From the stock solutions, the working solutions were prepared. This consisted of using sterile and deionized H₂O to prepare a final solution with a concentration of 10 µM. Afterwards, both solutions were stored at -20°C and thawed in ice when needed.

2.8. PCR Optimization for the designed primer pairs

The determination of the optimum T_a for each primer pair was performed using gDNA of *A. thaliana* WT and by starting 2 to 3 degrees Celsius below the T_m indicated by the manufacturer and then increasing the T_a until a single and a well-defined band would appear after an agarose gel electrophoresis.

The PCR mix was composed of: 1x Taq Master Mix, 50 ng DNA; 0.5 µM of each primer in a final volume of 10 µl. The PCR was carried out in an Analytik Jena Biometra TAdvanced Thermo Cycler with the program: 95°C for 2 min; 35 cycles of 95°C for 30 sec, T_a for 30 sec, 72°C for 1 min; and 72°C for 5 min. The 10 µL PCR volume was mixed with 1 µL of Fermentas 6x DNA Loading Dye and loaded in a 2.5% (w/v) Agarose gel prepared with SB 1x and dyed it with NZYTech™ GreenSafe Premium. The electrophoresis ran for 10 min, at 200 V, 400 mA using SB 1x as buffer. The amplicons' sizes were compared with Thermo Scientific™ GeneRuler 100 bp Plus DNA Ladder or Thermo Scientific™ MassRuler DNA Ladder Mix.

2.8.1. Amplification of Metallothioneins' cDNAs for Cloning

The MTs amplified through PCR to be used as insertion fragments in the cloning process *a posteriori* were the *AtMT1A* and *AtMT2B*. The same strategy previously mentioned to optimize the primers' T_a was also used for these primers, containing the restriction sites, that were used to amplify the full cDNA sequence corresponding to the complete ORF of these MTs. To obtain the inserts, a cDNA pool was used as template, following the same PCR conditions as described above. Afterwards, the band was purified (see ahead).

2.9. Reverse Transcription (cDNA Synthesis)

For the cDNA synthesis the SensiFAST™ cDNA Synthesis Kit Protocol (Meridian Bioscience) was used and everything was prepared over ice. The reaction consisted in mixing in a sterile tube 1 µg of total RNA; 4 µL of 5x TransAmp Buffer; 1 µL of Reverse Transcriptase; the final volume being completed to 20 µL with sterile H₂O and mixed gently. In a thermocycler, the following program was used: 15 min at 25°C; 30 min at 48°C; 5 min at 85°C; and hold at 4°C. The reactions were stored at 4°C until needed.

The Xpert cDNA Synthesis Supermix (Grisp Research Solutions) was also used for cDNA synthesis according to the instructions of the manufacturer. For this, a mixture was prepared with 1 µg of total RNA, 4 µL of the Master Mix and the total volume was adjusted to 20 µL with sterile and deionized H₂O. Afterwards, making use of the thermocycler, the reaction was heated at 37°C for 30 min, for the cDNA synthesis to occur; followed by 10 min at 60°C and 3 min at 95°C, to inactivate the reverse transcriptase. The cDNAs were placed at -20°C and thawed over ice when needed.

For regular PCRs the obtained cDNA was diluted 1:5.

2.10. RT-qPCR

The master Mixes used for the RT-qPCR assays were “iTaq Universal SYBR Green Supermix” (Bio-Rad) for the YL and R, and the “GreenHot Master Mix” (BIORON, Romemberg, Germany) for the ML. For the study of the gene expression of each MT, 3 biological replicates were made, and, for each biological replicate, 3 technical replicates were made. For each reaction, 4 µL of diluted cDNA, and 6 µL of a mixture containing 5 µL of the 2x Master Mix, 0.3 µL of each 10 µM primer and 0.4 µL of sterile and deionized H₂O were mixed.

Before starting RT-qPCR analysis and because the ordered primers were not tested before, it was important to determine the primer efficiency and the most suitable concentration of cDNA to be used *a posteriori*. For this, a master mix, as mentioned above, was prepared for each of a 3 tenfold dilution series of the cDNA template - 1:10; 1:100; 1:1000. For that, 4 µL of each dilution were added to the respective mix. For this assay *A. thaliana* WT cDNA from ML was used and for each dilution 3 technical replicates were assembled. The 96 well plate was sealed and spined to ensure that the reaction mixtures would settle to the bottom of the wells. The plate was placed in the Bio-Rad CFX96 thermocycler and the protocol used was the one recommended by the respective

manufacturer. The program used for each analysis was according to the instructions of the master mix used: for YL and R, 30 sec at 95°C; 40 cycles of 5 min at 95°C, 30 min at 60°C; and for the ML, 93°C for 3 min, 40 cycles of 95°C for 10 sec, Ta for 10 sec and 72°C for 45 sec. To determine the melting curve, a cycle of 5 sec at 65°C and the increase of temperature until 95°C, increasing 0.5°C every 5 sec, was added to the end of the reaction.

The reference genes for the YL and ML were: SAND family (*SAND*), actin 2 (*ACT2*) and the yellow-leaf-specific gene 8 (*YLS8*); for the R the last two were used.

Table 2 - Reference genes used for the RT-qPCR, with the respective 5' and 3' primer sequences, the size of the amplicon (bp) and the reference from where the primers were obtained.

Locus	Gene	Primer	Sequence (5'→3')	Amplicon size (bp)	Reference
AT5G08290	YLS8	Forward	AAGATCAACTGGGCTCTCAAGG	141	Ferreira et al. (2023)
		Reverse	TGGGAAGCTCGATTAGTAACGG		
AT3G18780	ACT2	Forward	CTTGCACCAAGCAGCATGAA	68	Takeuchi & Higashiyama (2012)
		Reverse	CCGATCCAGACACTGTACTTCCTT		
AT2G28390	SAND	Forward	AACTCTATGCAGCATTTGATCCACT	61	Remans et al. (2008)
		Reverse	TGATTGCATATCTTTATCGCCATC		

The annealing temperatures of the results were also analysed to ensure they would not differ from the expected.

2.11. Cloning of the full-length cDNAs of *AtMT1* and *AtMT2B*

Not much is known regarding the protein domains of the MTs and their functionality, so the placement of a reporter protein in the sequence of the protein of interest could potentially hinder their normal functions or completely inactivate the protein. Therefore, to obtain chimeric *AtMT1A* and *AtMT2B* fusion proteins with the mNeonGreen reporter protein, the reporter protein was added to the 3' end of the sequence of the MTs, so that the protein would remain active and functional.

2.11.1. DNA Purification from the gel

The kit used was the GFX PCR DNA and Gel Band Purification (Illustra, Cytivia) and the protocol was followed according to the instructions provided by the manufacturer. In a 2.5% (w/v) agarose gel, the DNA was separated, and the band of interest cut with a sterile surgical blade and placed in a 1.5 mL sterile microtube and its mass was determined. For each 10 mg of agarose gel 10 µL of buffer type 3 were then added (if the gel was lighter than 300 mg, the minimum recommended of buffer should be 300 µL). The tube was incubated at 60°C for 15-30 min, or until the agarose dissolved, with small inversions every 3 min. The mixture was centrifuged and transferred to the assembled GFX MicroSpin column and Collector tube, which were part of the kit. Subsequently, it was incubated for 1 min at RT before being centrifuged for 30 sec at 16,000 x g, the flowthrough was discarded. Then, 500 µL of the type 1 buffer was added to the column and centrifuged again at 16,000 x g for 30 sec, discarding the collector tube afterwards, and transferring the GFX MicroSpin column to a new sterile micro tube. Next, 20 µL of buffer type 6 were added to the column, followed by a 1 min incubation at RT before centrifuging it for 1 min at 16,000 x g, to collect the purified DNA, which was later quantified, as mentioned before. The DNA was stored and conserved at -20°C until necessary.

2.11.2. Restriction and Ligation of the insert and vector

Both MT1A and MT2B have small sequences of DNA -between 100 and 200 bps-, which could complicate its cloning directly into the final vector, since pMDC83 (which already possesses the DNA sequence encoding the mNeonGreen reporter protein) is significantly bigger than the inserts and pSK- 10779 bps. The cloning was performed using a variety of plasmid vectors, such as, pSK, pMDC83 and pSMART, the last one belongs to a cloning kit of PCR products. Different strategies for the cloning process were also used, for instance, blunt end ligation using *EcoR321* and *HincII* as restriction enzymes (RE); but also, sticky ends, for which *XbaI* and *SaI* were used as RE.

In order to obtain the final constructs of DNA-pSK-*AtMT1A*, pSK-*AtMT2B*, pMDC83-*AtMT1A*, pMDC83-*AtMT2B*- restrictions of the vectors pSK and pMDC83 and of the amplicons *AtMT1A* and *AtMT2B* were performed. As such, 1 µL of each corresponding enzyme (*XbaI* and *SaI* or *EcoR321* or *HincII*), 16 µL of DNA of interest and 2 µL of 10x Fast Digest Buffer (NZYTech) or 10x Anza Buffer (Thermo Fisher Scientific), depending on the enzyme used, were mixed and the final volume was adjusted to 20 µL with sterile

and deionized H₂O, when needed. The 1.5 µL microtubes were placed in a water bath at 37°C for 30 min, to allow the RE to cut the DNA.

A treatment with alkaline phosphatase (CIAP (Calf Intestinal Alkaline Phosphatase), 20 U/µL, Invitrogen) was done after the vectors' restrictions. Therefore, 2 µL of the alkaline phosphatase were added to the microtube after its first incubation, further incubating it for 15 min at 50°C, if the restriction resulted in blunt ends (*Eco*R321 or *Hinc*II), or 15 min at 37°C, if the restriction resulted in sticky ends (*Xba*I and *Sal*I).

Another strategy consisted in the gel purification of the restriction fragments and vectors, after the restriction and the CIAP treatment, and their quantification, as described previously, before proceeding to their ligation.

The ligation of the insertion fragments into the vectors was performed with the T4 DNA ligase (NZYTech) and the protocol of the manufacturer was followed. For this several quantities of plasmid were used, from 20 to 50 ng in different trials, as well as the amount of the insertion fragments were altered, from 10 to 1000 times, considering the concentration calculated using the formula $insert\ (ng) = \frac{vector\ (ng) * insert\ (bp)}{vector\ (bp)}$. The reaction consisted of 4 µL of T4 DNA ligase Buffer and 1 µL of ligase, X µL of vector and Y µL of insert; the final volume of 20 µL was completed with sterile and deionized H₂O. The reaction was placed in a refrigerated bath at 22°C overnight.

Complementary to this, the PCR pSmart (CloneSmart Blunt Cloning Kits, Lucigen) cloning kit was also used with the intention to create the constructs pSMART-*AtMT1A* e pSMART-*AtMT2B* using the corresponding PCR products. During the use of this kit, the manufacturer's instructions were followed, but regarding the concentrations of insert and vector, several trials with the alterations mentioned above were also performed.

2.11.3. *Escherichia coli* DH5α Transformation

Competent cells of the *E. coli* DH5α strain were used. One hundred µL of the cell suspension were placed in a previously chilled 1.5 mL sterile tube, along with 10 µL of the ligation reaction before gently mixing and incubating in ice for 30 min. After the incubation, and to ensure the entry of DNA into the cells it was necessary to apply a thermal shock, which was done by placing the tube in a water bath at 42°C for 45 sec, followed by 2 min in ice to stabilize the bacterial membranes. Subsequently, near an open flame, 900 µL of LB medium (Luria Broth Base, Miller, Duchefa Biochemie) were added to the cells which were placed under agitation for 60 min at 37°C. Afterwards, the

tube was centrifuged for 1 min at 1,800 x g, 900 µL of supernatant were removed and the remaining 100 µL of the mix were plated in the corresponding media (see below). The plates were placed in the incubator at 37°C to dry, and then they were inverted and kept at the same temperature overnight.

2.11.4. Culture Medium for plating bacteria

In 9 cmø petri dishes, sterile LB Medium (Luria Broth Base, Miller, Duchefa Biochemie), containing 1.5% (w/v) agar (Liofilmchem) and the respective antibiotic as selective agent was poured. For pSK, ampicillin at 50 µg/mL and X-GAL at 25 µg/mL were used, since this plasmid allows the recognition of colonies carrying the recombinant plasmid through the loss of blue colour in the presence of X-GAL. For both pSmart and pMDC83, kanamycin at 50 µg/mL was used.

2.11.5. Culture of Bacteria

In sterile conditions, 5 mL of sterile liquid LB medium (Luria Broth Base, Miller, Duchefa Biochemie), with the respective antibiotic for each plasmid in the corresponding concentrations, were transferred to a 15 mL sterile tube. Making use of a sterile toothpick, a single colony was picked and inoculated into the LB medium and let to grown overnight at 37°C at 200 rpm (GFL 3031, GFL).

2.11.6. Transformed *E. coli* Stocks

To prepare the bacteria stocks, in sterile conditions, 500 µL of sterile 50% glycerol and 500 µL of the liquid cell culture with the appropriate antibiotic were mixed in a 1.5 mL sterile microtube and kept at -80°C until necessary.

2.11.7. Plasmid Purification

In order to quickly obtain a high purity plasmid DNA from the transformed *E. coli* DH5α liquid cultures, the NZYMiniprep kit (NZYtech) was used. The protocol followed was according to the instructions provided by the manufacturer. One mL of the liquid cell culture was centrifuged during 30 sec at 13,000 rpm (Centrifuge 5418/5418R, Eppendorf) and the supernatant was rejected. The sediment was resuspended in 250 µL of the A1 Buffer, before adding 250 µL of the A2 Buffer to the mixture and incubating

at RT for 4 min. Afterwards, 300 μ L of the A3 Buffer were added, mixed through inversion and centrifuged for 3 min at 13,000 rpm. The supernatant was transferred to a column placed inside a 2 mL collector tube, both provided by the manufacturer, and centrifuged for 30 sec at 13,000 rpm. The flow-through was dismissed and, to the column, 600 μ L of the AS Buffer were added and centrifuged twice for 1 min, always discarding the flow-through. Then, the column was transferred to a new 1.5 mL tube, and 30 μ L of AE Buffer were added and the tube was incubated at RT for 1 min, before being centrifuged for 1 min at 13,000rpm to obtain the purified plasmids. The tube was kept at -20°C until needed.

2.12. Biochemical Parameters

2.12.1. Quantification of Hydrogen Peroxide (H₂O₂)

Two hundred mg of the Aerial Part (AP) and R were collected. The samples were homogenized with 1.2 mL of phosphate buffer 50 mM pH 6.5, with the help of quartz sand over ice. The homogenised was transferred to a 2 mL tube and centrifuged at 6,500 x g for 20 min at 6°C. Afterwards, 500 μ L of the supernatant was later transferred to a new 2 mL tube, to which were added 500 μ L of titanium sulphate 0.1% (w/v) in sulphuric acid 20% (w/v), to obtain a 1:1 mix with a final volume of 1 mL. This was later centrifuged at 6,000 x g for 15 min at 6°C and the supernatant was saved for the Abs readings at 410 nm, using a glass cuvette in the DS-C Cuvette Spectrophotometer (DeNovix). The readings were done over a blank composed of 1:1 phosphate buffer and titanium sulphate. The H₂O₂ quantity was expressed in nmole/g of fresh weight (FW), making use of a molar extinction coefficient of 0.28 mM⁻¹cm⁻¹ (Alves et al., 2023).

2.12.2. Extraction and Quantification of Photosynthetic Pigments

For this, YL and ML were used, 50 mg of biological material were homogenised with 80% acetone in a final volume of 5 mL (2 mL + 2 mL + 1 mL), with the help of quartz sand. Between each step of the homogenisation, the sample was transferred to a 15mL tube and protected from light, so the photodegradation of the pigments could be avoided. Then, the tubes were centrifuged at 10,000 x g for 10 min, from which the supernatant was transferred to a new 15 mL tube, also protected from the light. The final volume was adjusted to 10 mL with 80% acetone and mixed by inversion. In the DS-C Cuvette Spectrophotometer (DeNovix) and using a glass cuvette, the Abs was obtained for the following wave lengths: 480 nm; 495 nm; 645 nm; 655 nm.

The equations (1) and (2) were used to correct the influence of the chlorophylls and of other photosynthetic pigments could have in the Abs of the samples. In this equations Abs^o (480 nm) and Abs^o (495 nm) represent the values obtained from the measurement of the samples.

$$(1) \text{ Abs (480 nm) corrected} = \text{Abs}^o (480 \text{ nm}) - 0.566 \times \text{Abs (645 nm)} + 0.121 \times \text{Abs (655 nm)}$$

$$(2) \text{ Abs (495 nm) corrected} = \text{Abs}^o (495 \text{ nm}) - 0.112 \times \text{Abs (645 nm)} - 0.0036 \times \text{Abs (655 nm)}$$

The contents of the different photosynthetic pigments were expressed in mg/L-chlorophyll A (Chla) (3), chlorophylls B (Chlb) (4), β -carotenes (β -car) (5) e lutein (Lut) (6).

$$(3) [\text{Chla}] (\text{mg/L}) = 19.00 \times \text{Abs (655 nm)} - 7.61 \times \text{Abs (645 nm)}$$

$$(4) [\text{Chlb}] (\text{mg/L}) = 21.45 \times \text{Abs (645 nm)} - 5.92 \times \text{Abs (655 nm)}$$

$$(5) [\beta\text{-car}] (\text{mg/L}) = 17.16 \times \text{Abs (495 nm)} - 3.96 \times \text{Abs (480 nm)}$$

$$(6) [\text{Lut}] (\text{mg/L}) = 11.51 \times \text{Abs (480 nm)} - 20.61 \times \text{Abs (495 nm)}$$

From these values, the final calculations for each pigment were determined and expressed in mg/g FW (Tosin et al., 2021).

2.12.3. Quantification of Reduce Glutathione (GSH)

The biological material selected, AP and R, was homogenised using quartz sand and 1 mL of 3% sulfosalicylic acid (w/v) was used for each 100 mg of tissue. The final homogenised was transferred to a 2 mL tube and incubated in ice for 10 min. Afterwards, it was centrifuged at 10,000xg for 10 min at 6°C and the supernatant was transferred to a new tube and kept in ice.

The GSH standards were prepared for a final volume of 100 μ L from a concentrated solution of 1 mM of GSH in 3% sulfosalicylic acid (w/v), through its dilution to increasing concentrations- 0, 40, 60, 80, 100 μ M.

Seven hundred and fifty μ L of the working solution, that consisted of 14 mL of 1 mM ethylenediaminetetraacetic acid (EDTA) in 100 mM phosphate buffer (pH 7.0), were mixed with 400 μ L of 1.5 mg/mL Ellman's Reagent (5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB) in DMSO, 200 μ L of H₂O and 50 μ L of the sample's supernatant or the GSH

standard. These solutions were mixed by inversion and placed in obscurity for 10 min at RT; then the readings were done at 412 nm. From the standard solutions, a standard curve was obtained from which it was possible to calculate the GSH concentrations in nmol/g of FW (Martins et al., 2021).

2.13. Biometric Parameters

MS culture medium was prepared, with and without the different concentrations of Ni previously mentioned and poured in a square petri dish. The surface-disinfected seeds were placed in two horizontal lines and evenly spread. The plates were placed for 48 hours at 4°C before being transferred to the growth chamber. In the growth chamber, the plates were kept at a 70° angle, approximately, so the R could grow vertically.

Pictures of the plates were captured weekly, and 21 days post-germination the leaf area of ML and YL along with the R length were measured. Pictures of the leaves were captured using a magnifying glass and the programme Motic Image Plus 2.0 (Motic China Group Co, Ltd. 2007), the final images of the leaves, along with the images of the R, were analysed using the programme Image J (Schneider et al., 2012).

2.14. Statistical Analysis

For all assays a minimum of 3 biological replicates ($n \geq 3$) were prepared. Moreover, for the RT-qPCR assays 3 technical replicates over every biological replicate were also performed.

For the statistical analysis the program GraphPad Prism 8.0 (GraphPad Software Inc., USA), in which the differences between the control situation and the results from the Ni treatments were compared through a one-way analysis of variance (One-way ANOVA) and to be considered significant the p-value had to be lower than 0.05. The RT-qPCR results were first analysed in the program Bio-Rad CFX Maestro 2.0 before being analysed by the GraphPad Prism 8.0 software.

3. Results

3.1. Plant Growth

One month after the exposure to increasing Ni concentrations (0 mg/L; 12.5 mg/L; 25 mg/L; 50 mg/L of Ni), the germination rate was determined, as shown in table 6. Overall, a percentage above 95% of seeds germinated under all situations, but the seedlings did not develop further than the R apex.

Table 3 - Germination rate of A. thaliana WT when exposed to increasing concentration of Ni - 0 mg/L; 12.5 mg/L; 25 mg/L; 50 mg/L.

<i>Ni concentration</i>	<i>Germinated seeds</i>	<i>Total Seeds</i>	<i>Germination Rate (%)</i>
0 mg/L	84	84	100
12.5 mg/L	123	125	98.4
25 mg/L	101	104	97.1
50 mg/L	87	91	95.6

After one month of exposure, the seedlings in the media without Ni, control, were smaller than expected, as seen in Figure 4 (Fig 4- (A, B)), the stems did not develop at this point. With the lowest concentration of Ni, 12.5 mg/L, the seedlings started to develop but eventually died. The few seedlings that survived displayed some leaf discoloration (Fig.4- D, E). With the highest concentrations of Ni, 25 and 50 mg/L, the germination rate was high, 97.1% and 95.6%, respectively, but the seedlings did not develop further than the R apex (Fig. 4- (G, H, J, K)).

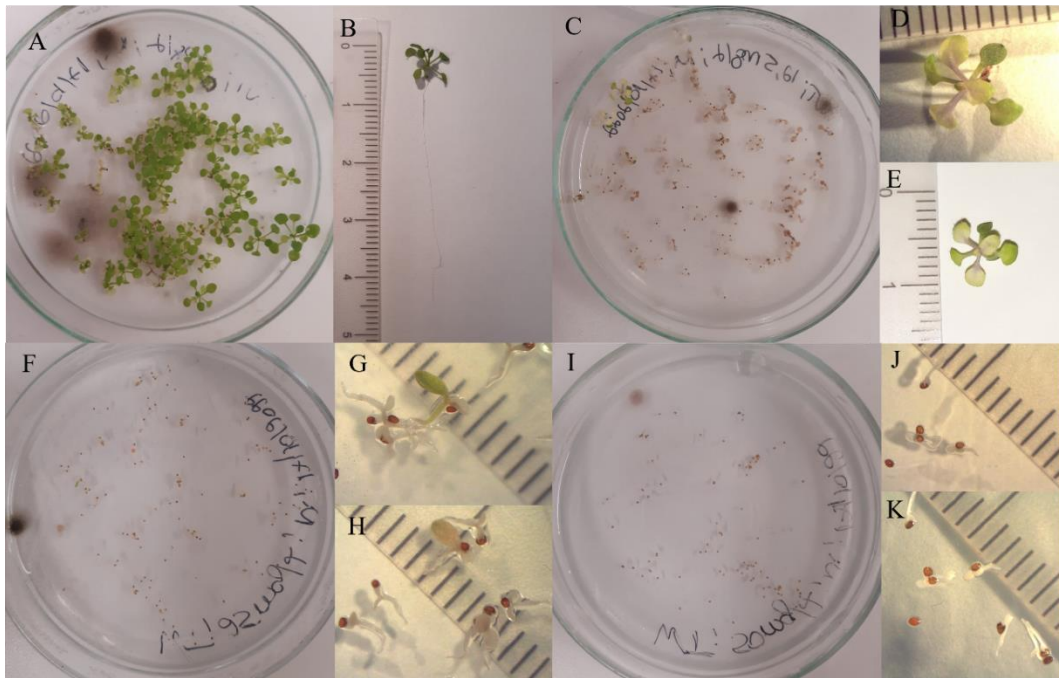


Figure 4-A. *thaliana* WT grown in MS medium with increasing concentrations of Ni 0 mg/L (A, B); 12.5 mg/L (C, D, E); 25 mg/L (F, G, H); 50 mg/L (I, J, K), one month after exposure.

Because of the growth impairment obtained under such Ni concentrations, a decrease of its concentration in the Media was done to 2.5, 5, 7.5 and 10 mg/L. Additionally, a more complete media, with sucrose and MES, was used to ensure the stabilization of the pH and to improve plant development. The germination rate for each stress condition was determined 7 days after germination (Table 4). Twenty-one days post-germination, approximately, the seedlings were bigger and, in some cases, almost fully developed (Fig. 7).

Table 4 - Germination rate of *A. thaliana* WT when exposed to increasing concentration of Ni - 0 mg/L; 2.5 mg/L; 5 mg/L; 7.5 mg/L; 10 mg/L.

<i>Ni</i> concentration	Germinated seeds	Total Seeds	Germination Rate (%)
0 mg/L	34	35	97
2.5 mg/L	24	26	92
5 mg/L	14	18	78
7.5 mg/L	19	22	86
10 mg/L	22	27	81

As it can be seen in Fig. 6, the 7-days old seedlings exposed to the highest concentrations of Ni had their growth and development impaired. However, this wasn't noticeable in plants with 21 days (Fig.7).

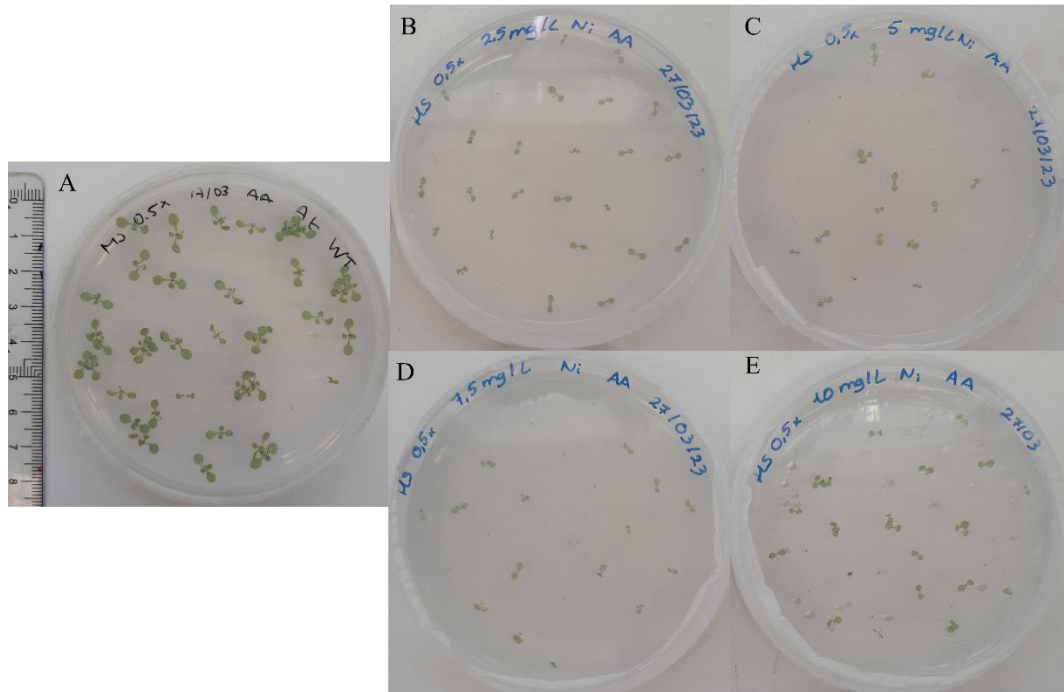


Figure 5 - *A. thaliana* WT grown in 1x MS medium with increasing concentrations of Ni - 0 mg/L (A); 2.5 mg/L (B); 5 mg/L (C); 7.5 mg/L (D); 10 mg/L (E), 7 days post-germination, approximately.

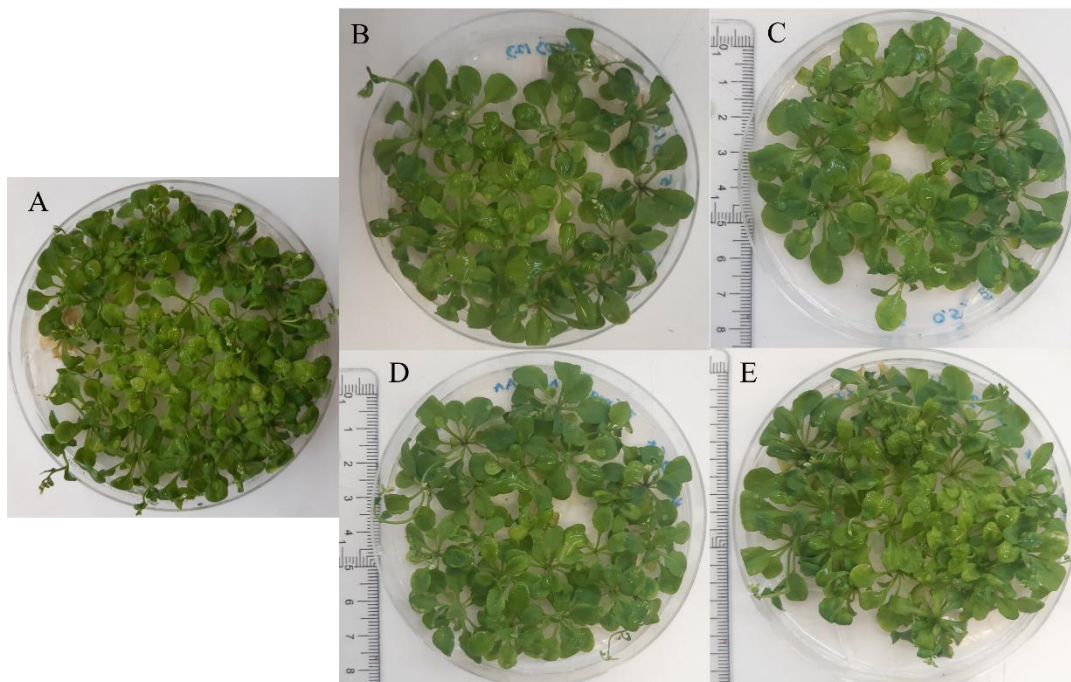


Figure 7 - *A. thaliana* WT grown in 1x MS medium with increasing concentrations of Ni - 0 mg/L (A); 2.5 mg/L (B); 5 mg/L (C); 7.5 mg/L (D); 10 mg/L (E), 21 days post-germination, approximately.

3.2. Mutants and Genotypes

To study the importance of MTs and the roles they could play in the homeostasis of Ni and, afterwards, how its absence would affect *A. thaliana*, mutants with the knockout expression for MT1A, MT2B, MT3 were ordered to see how the absence of these MTs would affect the plant and to compare to the results obtained with the WT.

Upon the seeds arrival, their germination rate was accessed to understand if they would vary from the WT. As shown in table 5 and Fig. 8, the germination rate was very low when compared to the WT, MT3 was the mutant with the lowest rate. Also, the “mutant” seedlings were smaller than the WT’s.

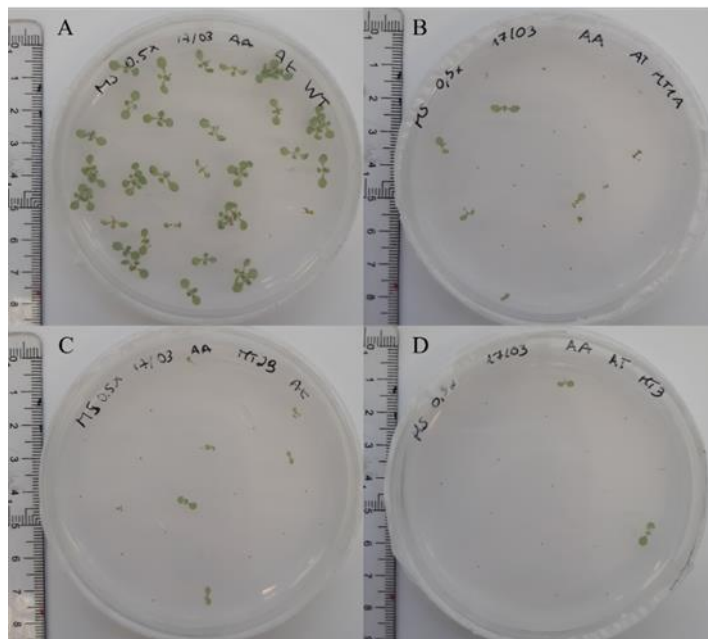


Figure 6 - *A. thaliana* WT (A) and knockout mutants (B, C, D) in 1x MS medium, 7 days post-germination. A- WT, B – MT1A, C-MT2B, D-MT3

Table 5 - Germination rate of *A. thaliana* WT and knockout “mutants”.

	Germinated seeds	Total Seeds	Germination Rate (%)
WT	34	35	97.1
MT1A	10	26	38.5
MT2B	7	22	31.8
MT3	2	18	11.1

It was necessary to verify the genotype of the plants to ensure the homozygosis of the mutants before continuing the study. So, for each genome extracted 3 PCRs sets were prepared and analysed by gel electrophoresis (Fig. 9) afterwards. All the plants genotyped proved to be heterozygotes, since they all amplified with the 5' and 3' primers of the respective MT (lane 1), as well as with the primer do the T-DNA (lane 3). Since all of the plants genotyped were heterozygotes, the MT expression study in mutants was not further pursued due to time constrictions, as these plants seeds would have to be germinated and the F1 seedlings tested again, which would take more time and, therefore, the remainder of the study was done using *A. thaliana* WT exclusively.

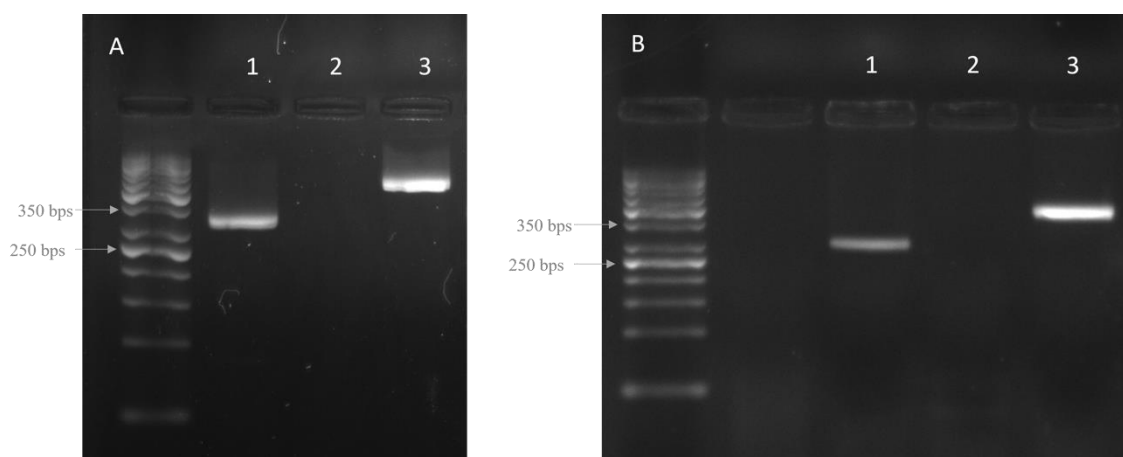


Figure 7 - Example of the result of the genotyping of the mutants with the knockdown gene MT1A (A) and MT2B (B). Lane 1 corresponds to the PCR using the forward and reverse primers for the MT in question; lane 2 corresponds to the PCR using the primers forward of the MT and the primer for the T-DNA; and lane 3 corresponds to the results of the PCR using the primer reverse of the MT and the primer for the T-DNA. The PCRs were performed using gDNA extracted from the leaves of mutant plants. The gene ruler used- GRS Ladder 50bp, grisp.

3.3. Primer design and PCR optimization

After the sequence alignment of the mRNA (see supplements, S1) was analysed, the initial primers pairs designed with the objective of being used for RT-qPCR analysis are displayed in table 3. Since it was difficult to obtain a pair of primers that specifically would amplify MT1A, a primer pair that amplified both MT1A and MT1B (MT1A_B) was designed.

Because these primers were not used previously, and the MTs have small and similar DNA sequences, their respective T_a were optimised by a normal PCR, using WT *A. thaliana* gDNA, to ensure the size of the amplicons was within the correct range (50-200 bp) and to ascertain the T_a for each pair. Such optimization started by referring to the T_m depicted by the manufacturer, but afterwards it was decided to calculate their T_m making

use of the equation: $T_m = 2 * (A + T) + 4 * (G + C)$, and decreasing the T_a a couple of degrees, as it can be seen on the table 3.

Table 6- Primers designed to be used in RT-qPCR, Forward and Reverse sequences (5'→3'), the size of the amplicon (bp) when using gDNA as template and the starting T_a tested.

Gene	Primer	Sequence (5'→3')	Amplicon size (bp)	Initial T_a (°C)
MT1A_B	<i>Forward</i>	CCTCCTGCAAATGTGGTGAC	341	53
	<i>Reverse</i>	GTTGTCGCACTCCTTGTTGT		
MT1B	<i>Forward</i>	AGAGATGTGTGTGTTGTTGG	152	50
	<i>Reverse</i>	TTGGTGAGAGTGGGACTTG		
MT1C	<i>Forward</i>	GCTTGCTTGACTTGTTCC	114	47
	<i>Reverse</i>	TGGTTTTTCGTTAGAGAGGAG		
MT2A	<i>Forward</i>	GGTTGCAAAATGTACCCTGAC	127	21
	<i>Reverse</i>	TCTCAGCGTTGTTACTCTCC		
MT3	<i>Forward</i>	ACAAGACCCAGTGCGTAAAG	370	52
	<i>Reverse</i>	ATGGCCTCCTTGTAAGCTCTC		
MT4A	<i>Forward</i>	CCGATCATGTCTGTTCACTCTC	632	50
	<i>Reverse</i>	TGCCTGTATCTGCCATTTTC		
MT4B	<i>Forward</i>	AGACACAGGCAAAGGAAG	275	49
	<i>Reverse</i>	AGCAGAGGTTTGAGTTTGAG		

For the first PCR optimization round the T_a mentioned in table 6 was used. The results were analysed via gel electrophoresis and, as seen in fig. 10, such temperatures were not ideal for most of the amplicons pursued, since no bands were obtained, and only MT4B had its sequence amplified.

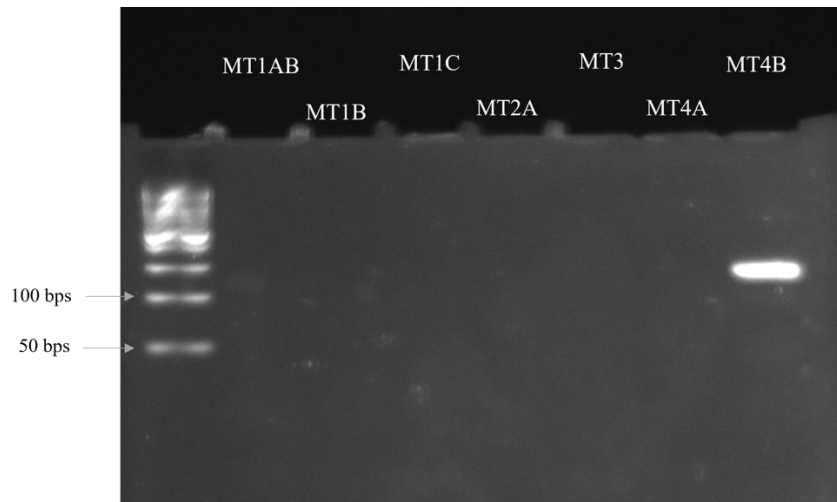


Figure 8 – Agarose gel electrophoresis with the results from the first optimization PCR using gDNA from WT *A. thaliana* and testing the primers MT1AB, MT1B, MT1C, MT2A, MT3, MT4A and MT4B (left to right, mentioned above the corresponding lanes). The gene ruler- GRS Ladder 50bp, grisp.

According to the results of the first PCR, some adjustments were made: first, 30 seconds to the elongation time were added, and then a 1°C-increase of the T_a at each PCR optimization round was performed until the amplification resulted in a clear and prominent band after electrophoresis, also corresponding to the right size according to the alignment made using Blast Nucleotide (NCBI) (Sayers et al., 2021).

Some of the primers proved not to be good for RT-qPCR. As shown in fig. 11, the primers for MT1A_B never showed just two bands, as it was expected, so it was decided to try and find new primers that would specifically amplify MT1A. In the case of the primers for MT1C, it was tricky to obtain a clean and prominent band in the gel (Fig. 11). Therefore, it was decided to use the primers depicted in Kim & Kang, (2018) that had design a set of primers for these MTs along with the primers for MT2B (Table 7), to use them in RT-qPCR.

Table 7 -Sequence of the forward and reverse primers used in RT-qPCR, the final T_a for optimal results and reference of the primers found in the literature.

Gene	Primer	Sequence (5'→3')	Amplicon size (bp)	T_a (°C)	Reference
MT1A	Forward	CCTGCAAATGTGGTGACTCT	370	57	Kim & Kang (2018)
	Reverse	ACCCACAGCTGCAGTTTGAT			
MT1B	Forward	AGAGATGTGTGTGTTGTTGG	152	53	
	Reverse	TTGGTGAGAGTGGGACTTG			
MT1C	Forward	CCTGCAAATGTGGTGATTCGT	229	59	Kim & Kang (2018)
	Reverse	ACAGTTACAGCTTGACCCGCA			
MT2A	Forward	GGTTGCAAAATGTACCCTGAC	127	51	
	Reverse	TCTCAGCGTTGTTACTCTCC			
MT2B	Forward	GTGGAAGCTGTGGTTGTGG	334	58	Kim & Kang (2018)
	Reverse	AACGAAAGTCTCGCCGGAAG			
MT3	Forward	ACAAGACCCAGTGCGTAAAG	370	55	
	Reverse	ATGGCCTCCTTGCTAGCTCTC			
MT4A	Forward	CCGATCATGTCTGTTCACCTCTC	632	55	
	Reverse	TGCCTGTATCTGCCATTTTC			
MT4B	Forward	AGACACAGGCAAAGGAAG	275	54	
	Reverse	AGCAGAGGTTTGAGTTTGAG			

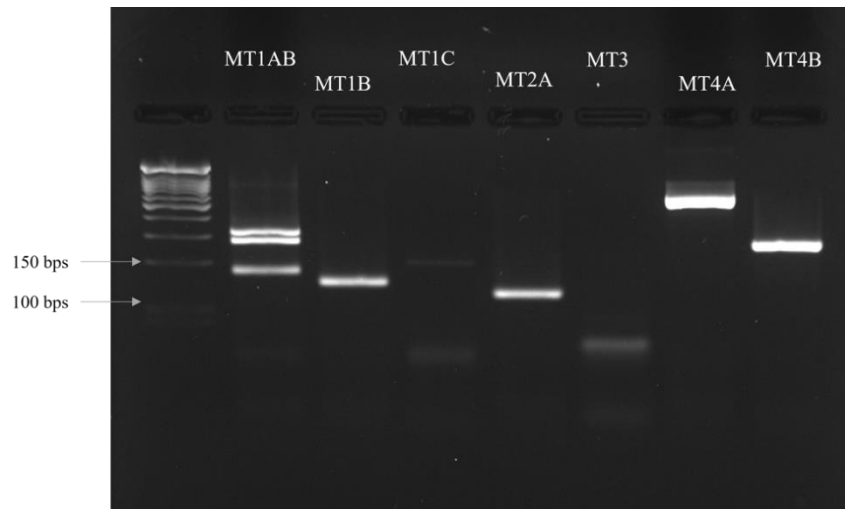


Figure 9 - Agarose gel electrophoresis with the results of the optimized PCR using gDNA of WT *A. thaliana* for each set of primer pairs designed. The MT for each amplicon is mentioned above the corresponding lane (MT1AB, MT1B, MT1C, MT2A, MT3, MT4A and MT4B, - left to right). The gene ruler used- GRS Ladder 50bp, Grisp.

The same procedure was used to determine the correct T_a for these new primer pairs, as previously described for the primer pairs designed in this work. The optimal T_a for each primer pair is depicted in Table 7, as it led to a clear and distinct band for each MT analysed (Fig. 11 and 12).

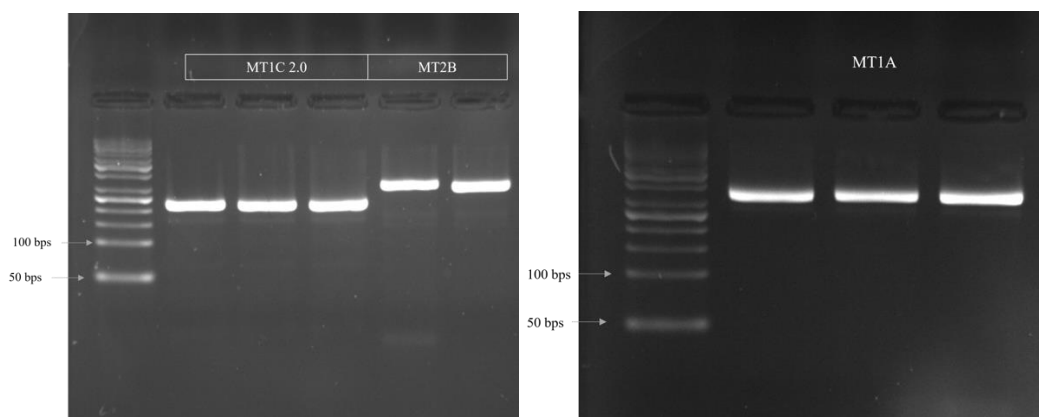


Figure 10 - Agarose gel electrophoresis gel with the results of the optimized PCR using gDNA from WT *A. thaliana* for the new pairs of primers for MT1C, MT2B (left) and MT1A (right). The gene ruler used- GRS Ladder 50bp, grisp.

3.4. Primers and PCR optimization for the amplification of insert fragments for posterior cloning

The primers designed for the amplification of the MTs to be used as inserts in future cloning assays are displayed in table 8. These primers were tested the same way the primers mentioned above, but the template used for these PCRs was the cDNA of WT *A. thaliana* leaves. In both cases, the optimal T_a was 60°C and the resulting band in the electrophoresis gel was clear and prominent (Fig. 13).

Table 8 - Sequence of the forward and reverse primers used to amplify the insets to be used for cloning and the optimal annealing temperature to be used in PCR. The added restriction sites for *Xba*I in the forward- and *Sa*II in the reverse primers are underlined.

Gene	Primer	Sequence (5'→3')	Amplicon size (bp)	$T_a(^{\circ}\text{C})$
MT1A+RE	Forward	AAT' <u>CTAGA</u> AATGGCAGATTCTAACTGTGG	152	60
	Reverse	AAAG' <u>TCGAC</u> ACAGTTACAGTTTGACCCAC		
MT2B+RE	Forward	AAT' <u>CTAGA</u> AATGTCTTGCTGTGGTGGAAG	248	60
	Reverse	AAAG' <u>TCGAC</u> TTTGCAGGTACAAGGGTTGC		

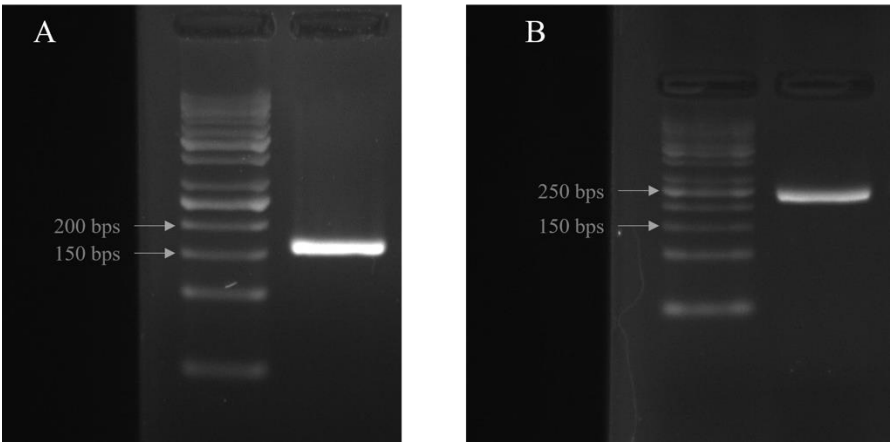


Figure 11 - Agarose gel electrophoresis with the results of the optimized PCR using cDNA from WT *A. thaliana* as template for the primer pairs MT1A+RE (A) and MT2B+RE (B) using the optimal annealing temperature for each pair. The gene ruler used- GRS Ladder 50bp, grisp.

3.5. RNA Quality

The spectrophotometry quantification allowed for the determination of the concentration of the RNA as well as the ratio between the 260 nm and 280 nm readings, which indicates the RNA purity and should fall between 1.50 and 2.00 for a satisfactory purity degree (Table 9).

Table 9 - RNA quantification and purity results. YL – Young leaves; ML – Mature leaves; R – Roots. 0-1 to 0-3 – control; 1-1 to 1-3 – 2.5 mg/L Ni; 2-1 to 2-3 – 5 mg/L Ni; 3-1 to 3-3 – 7.5 mg/L Ni; and 4-1 to 4-3 – 10 mg/L Ni.

	YL		ML		R	
	<i>Concentration</i>		<i>Concentration</i>		<i>Concentration</i>	
	<i>(ng/μl)</i>	<i>A(260)/A(280)</i>	<i>(ng/μl)</i>	<i>A(260)/A(280)</i>	<i>(ng/μl)</i>	<i>A(260)/A(280)</i>
0-1	3298.4	2.04	2168.5	1.96	998.53	1.59
0-2	2738.1	1.99	2232.3	1.98	831.05	1.85
0-3	2514.8	2.00	3153.9	2.05	647.42	1.65
1-1	4092.9	2.09	2165.1	2.01	1584.5	1.81
1-2	2485.9	2.05	2810.1	2.01	1505.6	1.86
1-3	1318.4	1.99	2179.7	2.00	1508.7	1.79
2-1	2990.1	2.03	2565.9	1.97	1358.5	1.75
2-2	3154.0	2.03	1837.4	1.97	1158.2	1.92
2-3	3715.0	2.09	3046.2	2.06	1436.0	1.83
3-1	2554.8	1.96	2833.6	2.06	2019.1	1.66
3-2	1109.5	2.02	2026.3	1.95	1532.0	1.86
3-3	3308.1	2.00	2411.4	2.00	858.33	1.88
4-1	3831.3	2.08	2232.4	1.98	1511.8	1.88
4-2	2237.3	2.00	2048.9	1.94	1264.0	1.76
4-3	2146.2	2.03	2416.4	2.01	1324.8	1.80

The integrity and quality of each RNA was analysed via gel electrophoresis. As shown in Fig. 13, every RNA possesses 2 bands corresponding to the subunits of the eukaryotic ribosomal RNA, 28S and 18S, indicating that the RNA did not suffer degradation. Other bands are also present, which correspond to procaryote rRNA, existent in mitochondria and plastids, and tRNAs.

The rRNA bands were masked by the dye stain present in the gel (Fig. 13-C), but since we were able to see the top band of the pair and it possesses the remnant bands, the RNA was considered pure.

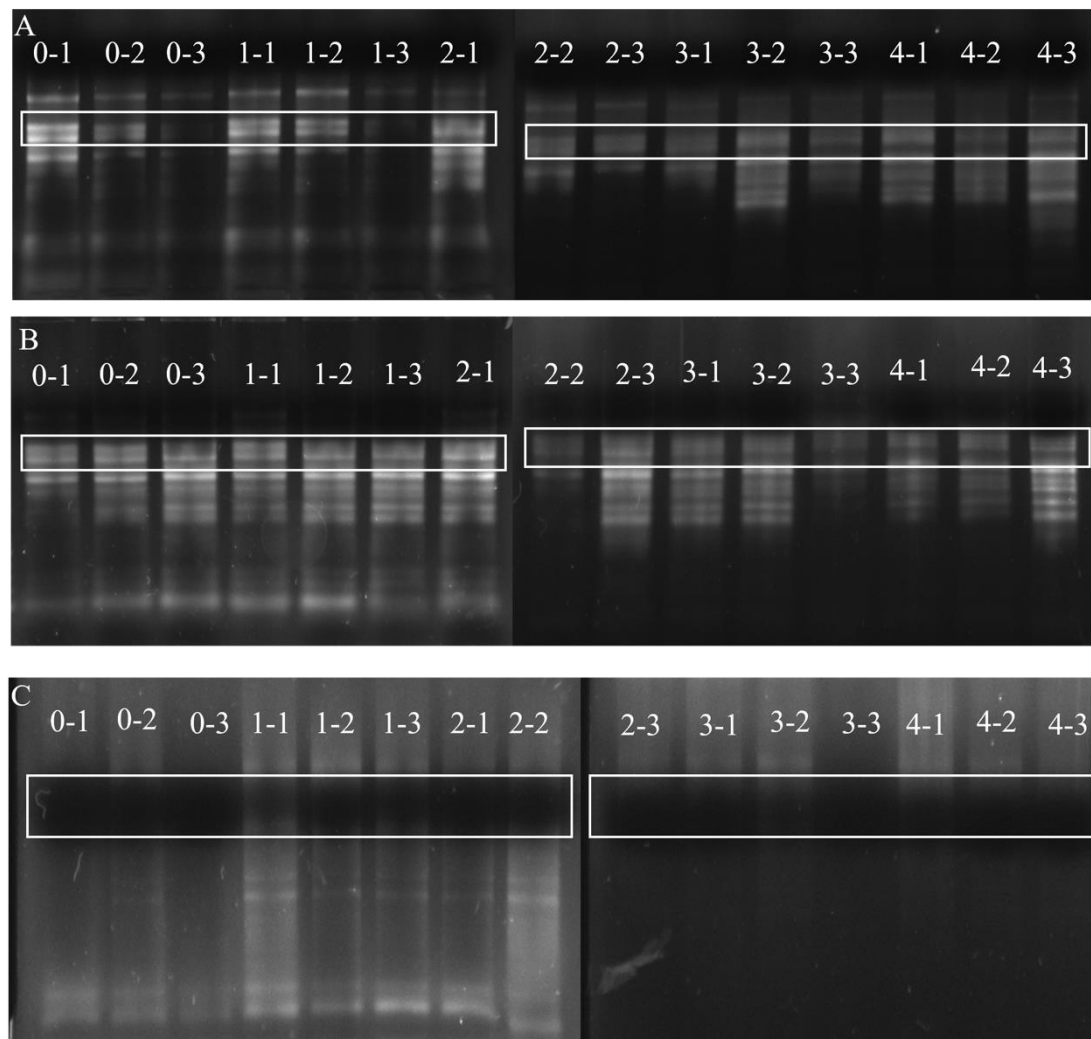


Figure 12 - RNA quality assessed by a 0.8% (w/v) gel electrophoresis of 300 ng of the total RNA extracted. A-Young leaves RNA. B- Mature leaves RNA. C- Roots RNA.

3.6. Confirmation of the expression of the MT genes in the different organs of *A. thaliana* WT by qualitative RT-PCR

Comparing the expression of the different MT genes in R and in shoots some differences were noticed, such as, the Type 4 MTA was not expressed in R (Fig.14-A), while faint bands were noticeable in the shoots (Fig. 14-B). The original pairs of primers for MT1AB and MT1C revealed not to be appropriate for RT-qPCR, since their results were variable, as sometimes a band could be obtained (Fig. 15-B) while others it was not present for the same organ (Fig. 14-B). The remaining MTs (MT1B, MT2A and MT3) were expressed in both organs.

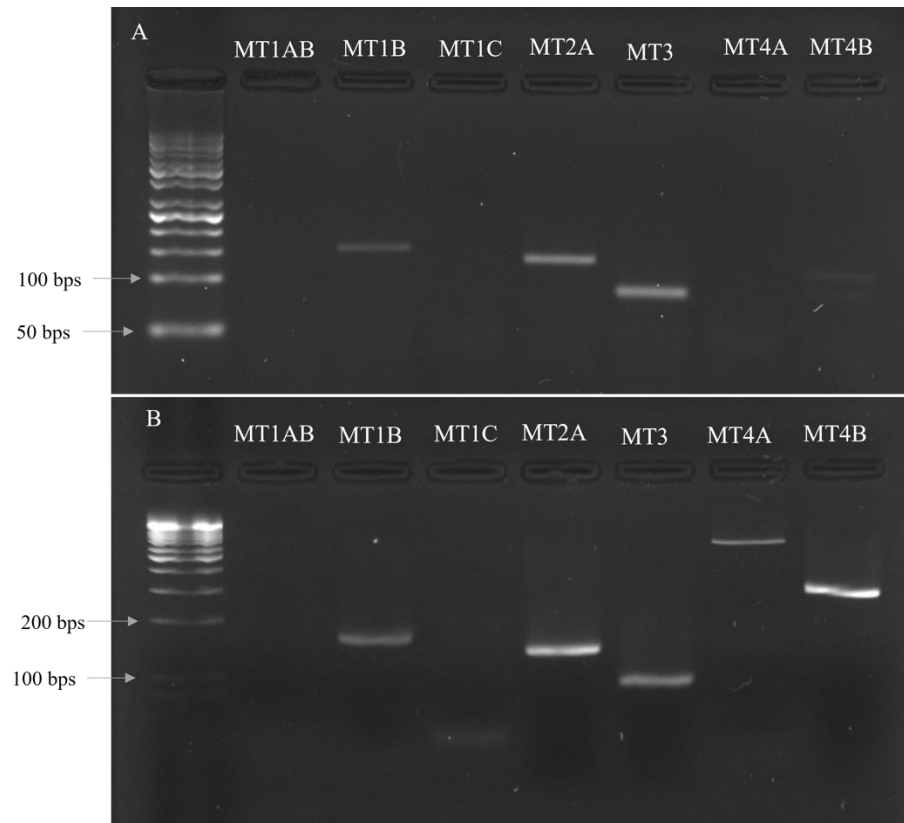


Figure 13 - Typical agarose gel electrophoresis with the results of the PCR for each set the original primers pairs (MT1AB, MT1B, MT1C, MT2A, MT3, MT4A and MT4B, -left to right) using cDNA of roots (A) and shoots (B) from WT *A. thaliana*. The gene ruler used were A – GRS Ladder 50 bp, grisp; B -MassRuler DNA Ladder Mix, ready-to-use, Thermo Scientific.

When the leaves were separated into YL and ML and this analysis was performed again, some differences were observed (Fig 15): the MT4A and MT4B, as well as MT3, presented more intense bands in ML. Once again, the original MT1AB and MT1C primer pairs were unreliable, as the results were not constant between PCRs.

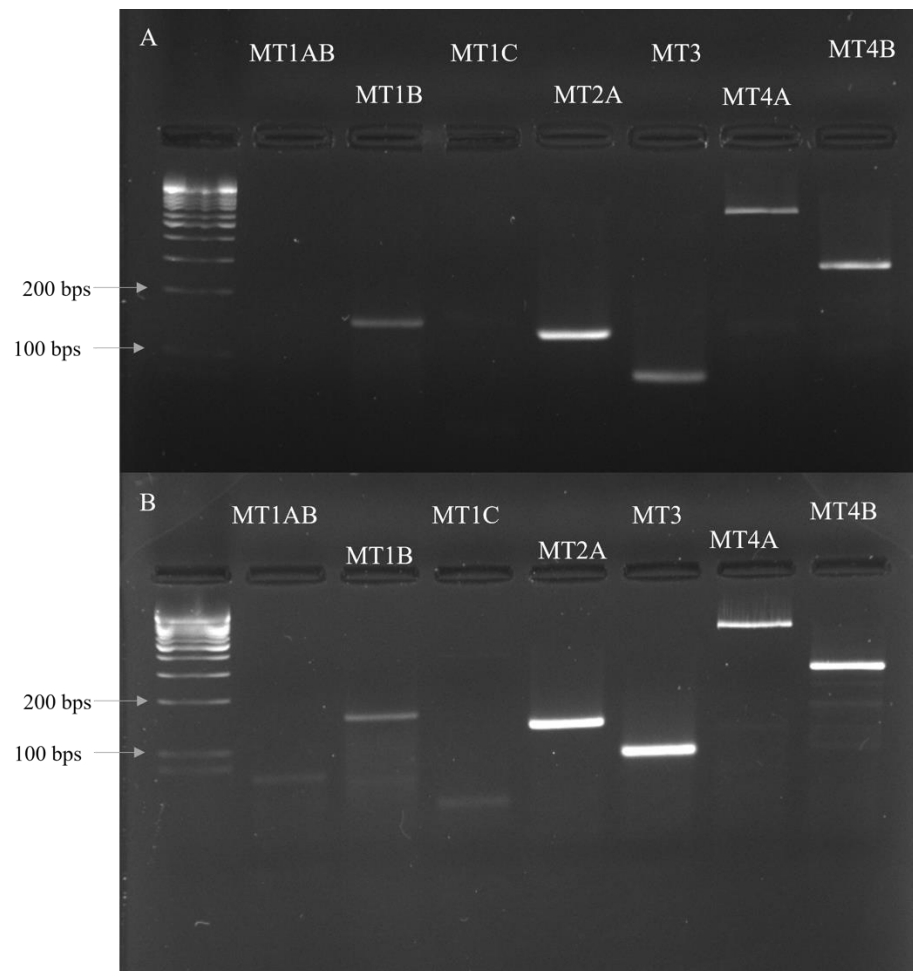


Figure 14 – Typical agarose gel electrophoresis with the results of the PCR for each set the original pairs of primers (MT1AB, MT1B, MT1C, MT2A, MT3, MT4A and MT4B, -left to right) using cDNA of young leaves (A) and mature leaves (B) from WT *A. thaliana*. The gene ruler used MassRuler DNA Ladder Mix, ready-to-use, Thermo Scientific.

Using the new primer pairs for MT1A, MT1C and MT2B it was possible to determine that MT1A had a more intense band in ML than in YL and R. However, MT1C and MT2B had similar intensities in their bands in ML and YL, but the corresponding bands obtained with the cDNA from R seem to be less intense. MT1B also seems to be a little unstable in R because in Fig.14 there is a band whereas in Fig 16, there is no band in the lane corresponding to the root cDNA. Interestingly, in the lanes corresponding to the cDNA from YL and ML, MT1C appears contaminated, as there are two bands, however, the PCR results from the root it does not happen.

Overall, the intensity of the bands obtained with the R' cDNAs appears to be lighter than those obtained with the leaves'.

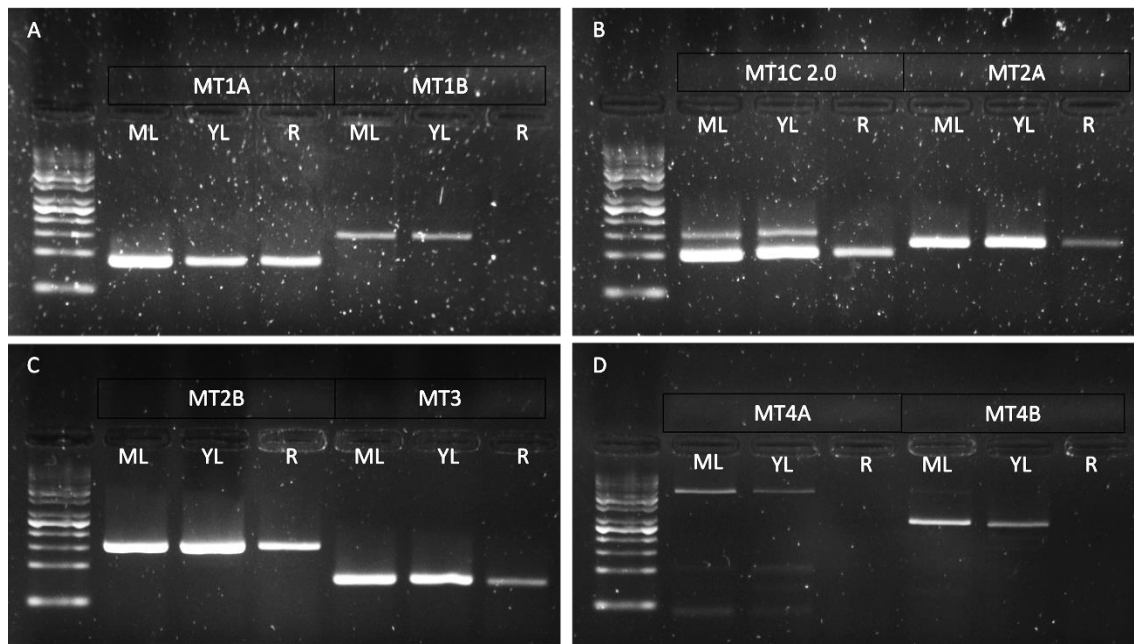


Figure 15 - Typical agarose gel electrophoresis with PCR products using cDNA from WT *A. thaliana* from the different organs and the MTs primers at the corresponding annealing temperature. A- MT1A and MT1B. B- MT1C and MT2A. C- MT2B and MT3. D- MT4A and MT4B. ML-Mature leaves; YL- Young leaves; R-Roots. The gene ruler used- GRS Ladder 50 bp, grisp.

3.7. RT-qPCR

3.7.1. Primer Validation and qPCR optimization

Before starting the study on gene expression by qPCR, it was necessary to validate the primers according to their efficiency and to optimize the reactions. For this, 3 dilutions of the cDNA (1:10; 1:100, 1:1000) were tested to be used as template for the reactions.

Because the optimal T_a for the primers was previously optimised, it was only needed to test the efficiency of the primers and to determinate the best concentration of cDNA to be used. For this the number of Cq ideal should be between 20 and 25. To test the primer efficiency the cDNA from mature leaves for the Type I, II and III MTs was used, while the cDNA from siliques was used for the Type IV MTs. As reference genes, *SAND* and *YLS8* were selected from previous publications that mentioned their efficiency and, because *SAND* was previously tested in stress conditions imposed by high concentrations of HM, the efficiency provided by the authors was used in this study. Contrary to this, the primers selected for *ACT2* were not previously used in a similar study and their efficiency was not available, and therefore their efficiency was tested as well in this study (Table 10).

Table 10 - List of genes and primer sequences used in the qPCR analysis, and the corresponding efficiency (E), correlation coefficients (R^2); slope, Melting Temperature and the tissue used.

<i>Primers</i>	<i>E</i>	<i>R²</i>	<i>Slope</i>	<i>Melting Temperature</i>	<i>Tissue Used</i>
<i>MT1A</i>	100.1%	0.975	-3.320	78.5°C	Mature Leaves
<i>MT1B</i>	109.1%	0.98	-3.121	78°C	Mature Leaves
<i>MT1C</i>	105.3%	0.992	-3.200	79°C	Mature Leaves
<i>MT2A</i>	96.1%	0.998	-3.419	81°C	Mature Leaves
<i>MT2B</i>	105.9%	0.998	-3.188	83.5°C	Mature Leaves
<i>MT3</i>	100.0%	0.999	-3.322	79°C	Mature Leaves
<i>MT4A</i>	97.5%	0.964	0.625	78.5°C	Silique
<i>MT4B</i>	100.0%	0	0.000	83.5°C	Silique
<i>ACT2</i>	103.8%	0.999	-3.233	77°C	Young Leaves

Regarding the efficiency of the primers to be used in RT-qPCR, there is the need to take into consideration the efficiency (E), the correlation coefficients (R^2) and the slope. For optimal results, the E should be withing 90 and 110%, the R^2 needs to be superior to 0.980 and the slope should be between -3.6 and -3.1. As showed on table 10, all primers analysed were within optimal range to be used, except for MT4B. Since in the end of the RT-qPCR a melting curve was performed, it was used to verify if the PCR assays produced single products and the lack of contamination of the RT-qPCR products.

3.7.2. Gene Expression study of the MTs

To study the MT genes' expressions in response to the increasing concentrations of Ni, a dilution 1:10 of the cDNAs from the different organs of the plants was used, along with the primers mentioned in table 8.

As depicted in Fig. 17, which refers to YL, MT1A showed a significant decrease in its expression with the increase of Ni concentration of 22.04%, 46.1%, 71.34%, 79.13%, respectively (A). MT1B (B) and MT1C (C) displayed an increase in their expression with high Ni levels, while MT1B had a significant increase in plants exposed to the two highest concentration of Ni, 7.5 mg/L and 10 mg/L of 117.4% and 84.62%, respectively, MT1C had a significant intensification in plants exposed to 2.5 mg/L Ni of 169.6% and in 10 mg/L Ni of 173.4%. Both MT Type II showed a significant decrease in their expression in the two highest concentrations, but only MT2A did display the same significant result in

5 mg/L. The reductions in the MT2A were of 43.85%, 59.19% and 52.12%, in 5 mg/L to 10 mg/L of Ni, respectively. The second MT type II, MT2B, showed a decrease in both situations: 49.25% and 44.91%, respectively. MT3 had a similar response as MT2B, in which they both had a significant reduction in expression in the two highest concentrations. However, the decrease in the Type III MT was of 29.30% in 7.5 mg/L and 50.08% in 10 mg/L. The MTs Type IV showed a variable expression, but the software didn't consider it significant.

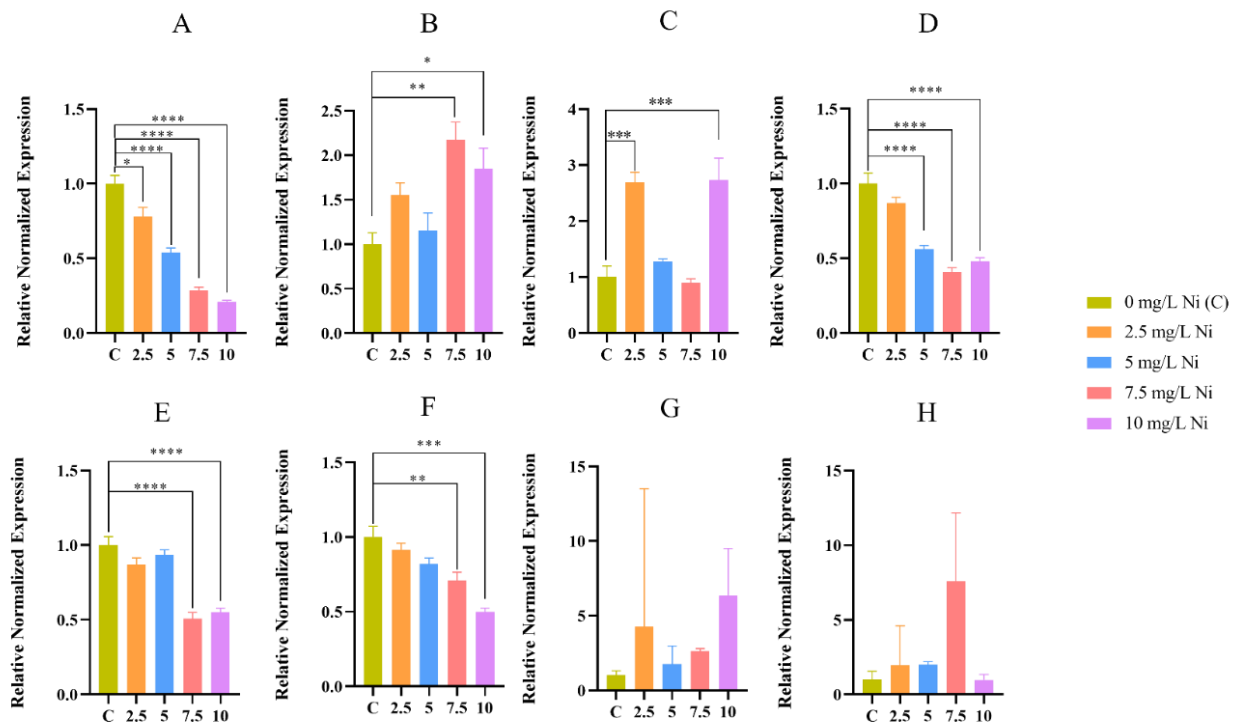


Figure 16 - Relative Normalized expression of the MT1A (A), MT1B (B), MT1C (C), MT2A (D), MT2B (E), MT3 (F), MT4A (G) AND MT4B (H) in young leaves of plants exposed to increasing concentrations of Ni- 2.5, 5, 7.5, 10 mg/L. Asterisks represent significant differences at $p < 0.05$ between the control and treatments.

Overall, in ML, the expression of the different MT seemed to decrease with the highest concentrations (Fig 18), especially the types I, II and III. Regarding type I, only MT1A showed a significant reduction in its expression in the two highest concentrations of 75.28% and 71.77%, respectively. On the other hand, both MT2A and MT2B had a decrease in their expression in plants exposed to 7.5 mg/L, 82.81% and 81.26% respectively, but only MT2A had a reduction in the highest concentration of Ni of 65.29%. While there seems to have a reduction in the gene expression in MT3, due to the high standard deviation the programme didn't consider the reduction significant. Like it happened in YL, the Type IV MTs showed variable expression but the values weren't considered significant.

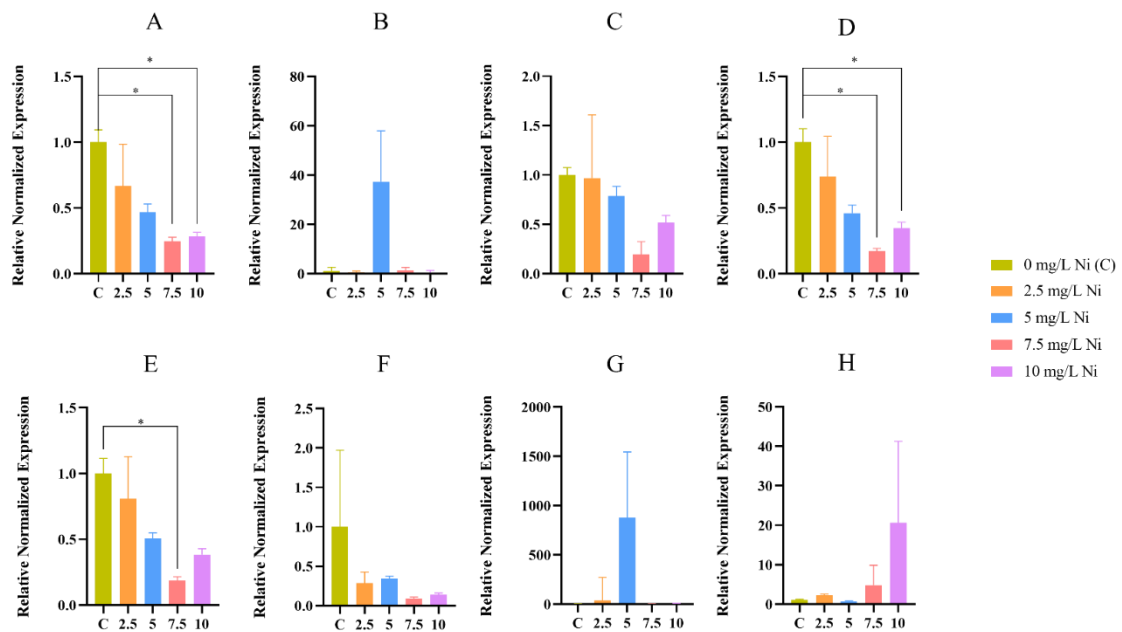


Figure 17 - Relative Normalized expression of the MT1A (A), MT1B (B), MT1C (C), MT2A (D), MT2B (E), MT3 (F), MT4A (G) AND MT4B (H) in mature leaves of plants exposed to increasing concentrations of Ni- 2.5, 5, 7.5, 10 mg/L. Asterisks represent significant differences at $p < 0.05$ between the control and treatments.

In the R, an overall increase in the expression of the MT genes was noticed, except for the type IV (Fig 19).

Within the Type I MTs, only MT1A and MT1C showed significant increases in their expression in plants exposed to the different concentrations of Ni. The expression of the MT1A gene seems to be almost linear with the increase of the Ni concentration, but in 5 mg/L of Ni there was a slight decrease when compared to the previous concentration. Nevertheless, this gene showed an increase in expression of 264.3%, 206.9%, 378.2%, 421.4%, from the lowest to the highest concentration of Ni. The increase in expression of the MT1C gene was significant in every concentration of Ni and a pattern was visible in the graph, in which the increase in expression in the concentrations of 2.5 mg/L and 7.5 mg/L of Ni are followed by a slight decrease, when compared to the prior concentration, in the concentrations of 5 mg/L and 10 mg/L. This gene also had the highest increase in expression in all concentrations when compared to the rest of the MTs in this part of the plant, of 673%, 419.7%, 700.4% and 342.5%, respectively. Interestingly, the Type II MTs did not suffer any significant alterations in the R, even though they seemed to have an increase in expression. On the other hand, MT3 only had a significant increase in the lowest concentration of Ni, 2.5 mg/L. Contrary to what happened with the first three types of MTs, the Type IVs appeared to have a decrease

in their expression in plants exposed to every concentration of Ni. MT4A had a reduction in expression of 100% in plants exposed to 2.5 mg/L and 7.5 mg/L, 77.85% and 80.96% in 5 mg/L and 10 mg/L, respectively, while the MT4B had a decrease in expression of 96.71%, 99.35%, 99.89% and 99.27%, from the lowest to the highest concentration of Ni.

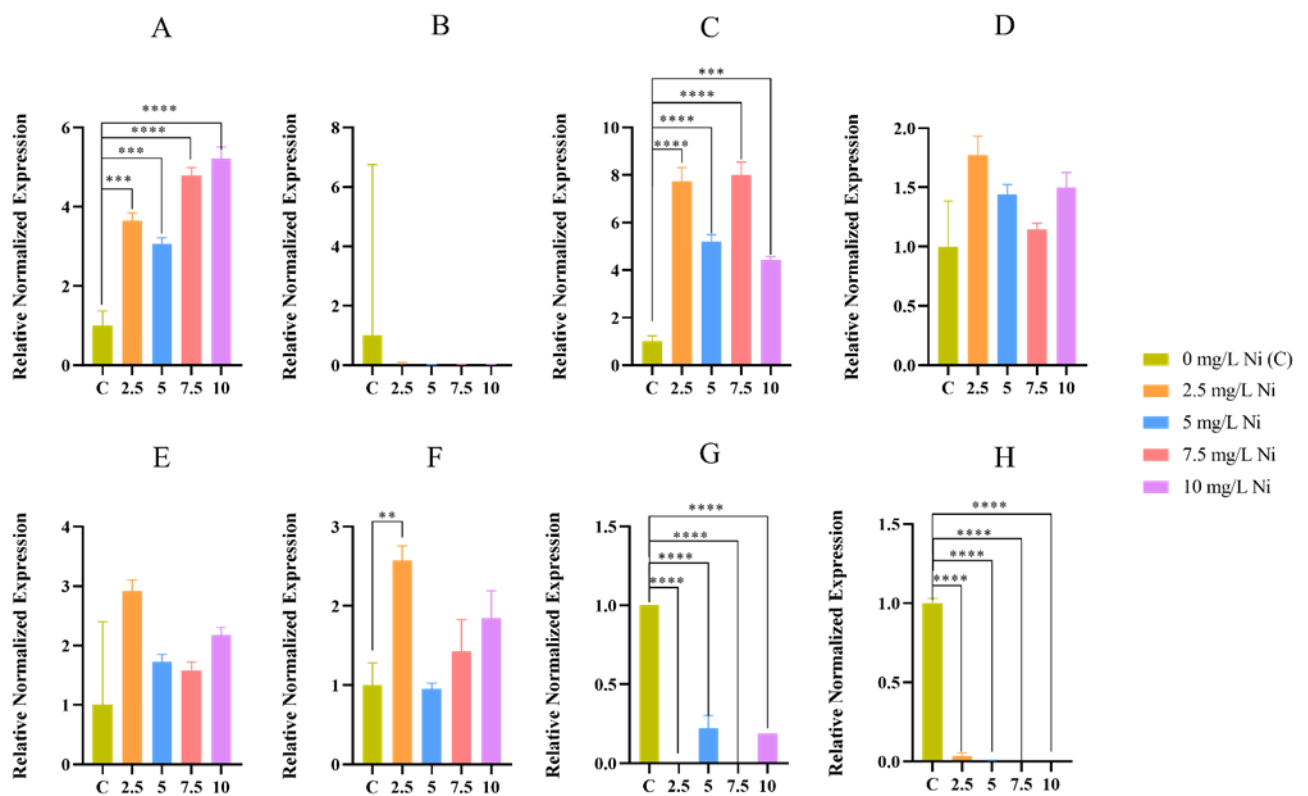


Figure 18 - Relative Normalized expression of the MT1A (A), MT1B (B), MT1C (C), MT2A (D), MT2B (E), MT3 (F), MT4A (G) AND MT4B (H) in roots of plants exposed to increasing concentrations of Ni- 2.5, 5, 7.5, 10 mg/L. Asterisks represent significant differences at $p < 0.05$ between the control and treatments.

To validate these results, one sample of the triplicates from each biological sample was analysed through agarose gel electrophoresis, to ensure the lack of contamination and that the amplicon had the correct size. The NTC was also analysed to verify that the mixtures used were not contaminated (Fig 20). This coupled with the analysis of the melting temperature, ensuring that is equal to that of showed in table 10, further validated the results.

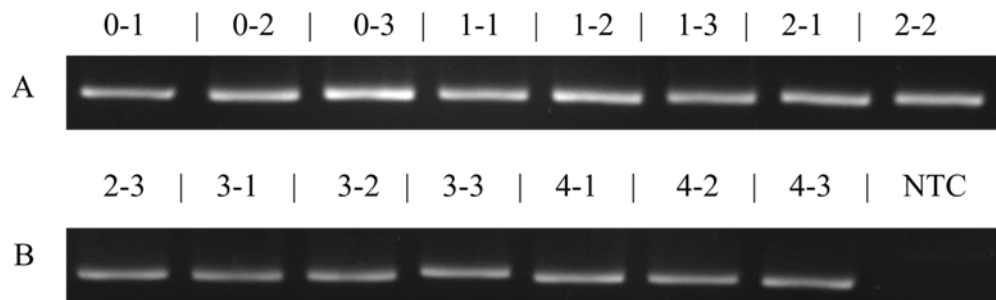


Figure 19 - Example of a gel electrophoresis analysis of the RT-qPCR for MT2A, using the cDNA of young leaves (A, B) from one of the technical replicates from each of the biological samples and NTC. 0-1 to 0-3 – control; 1-1 to 1-3 – 2.5 mg/L Ni; 2-1 to 2-3 – 5 mg/L Ni; 3-1 to 3-3 – 7.5 mg/L Ni; and 4-1 to 4-3 – 10 mg/L Ni.

3.8. Biochemical parameters

3.8.1. Effect of the increasing concentrations of Ni in the H₂O₂ levels

The levels of Hydrogen Peroxide were measured in the AP of the plant, and in the R, as this latter organ is the first organ of the plant that is in contact with the imposed stress.

The results obtained were not considered significant, but a tendency for the AP H₂O₂ levels to decrease, and in the R to increase with the increasing Ni concentration was noticeable (Fig 21-A and B, respectively).

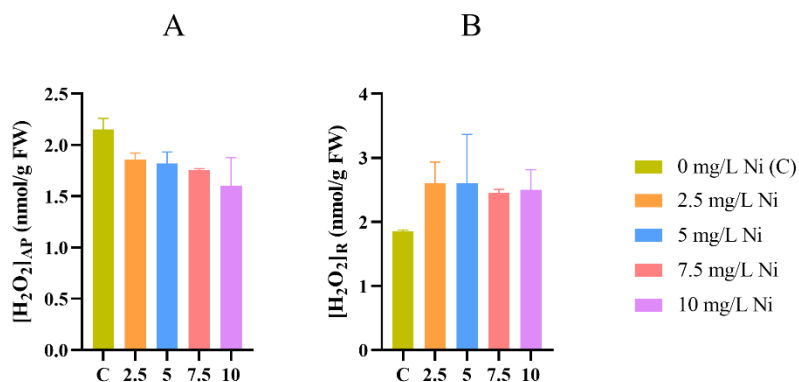


Figure 20 - Average + SD levels of Hydrogen Peroxide, expressed as nmole/g FW in the aerial part (AP) (A) and in the roots (R) (B) of plants exposed to increasing concentrations of Ni – 0, 2.5, 5, 7.5, 10 mg/L.

3.8.2. Effect of the increasing concentrations of Ni in the Photosynthetic pigments levels

The levels of the photosynthetic pigments were analysed for YL and ML, and the results obtained are displayed in Fig. 22.

In YL, the variations of the average levels of the chlorophyll a, chlorophyll b, β -carotenes and lutein weren't considered significant.

Interestingly, the results obtained for ML, the variation of the concentrations of the pigments weren't considered significant. However, we were able to notice that the levels of chlorophyll a were affected by the three highest concentrations of Ni (27.3% - 7.5 mg/L; 17.6% - 10 mg/L), and especially by the 5 mg/L, since this concentration appear to cause a bigger reduction in its quantity (32.6%) (Fig. 22-E). Also, chlorophyll b seems to be negatively affected by all the concentrations of Ni (36.1% - 2.5 mg/L; 26.6% - 7.5 mg/L; 16.8% - 10 mg/L), and, once again, 5 mg/L showed a greater impact in its quantity (47%) (Fig. 22-F). Regarding lutein, the concentrations of Ni that seemed to impact the concentration of this pigment were the lowest, 2.5 mg/L of Ni (28.3%), followed by the 5 mg/L (28.1%) (Fig. 22-H).

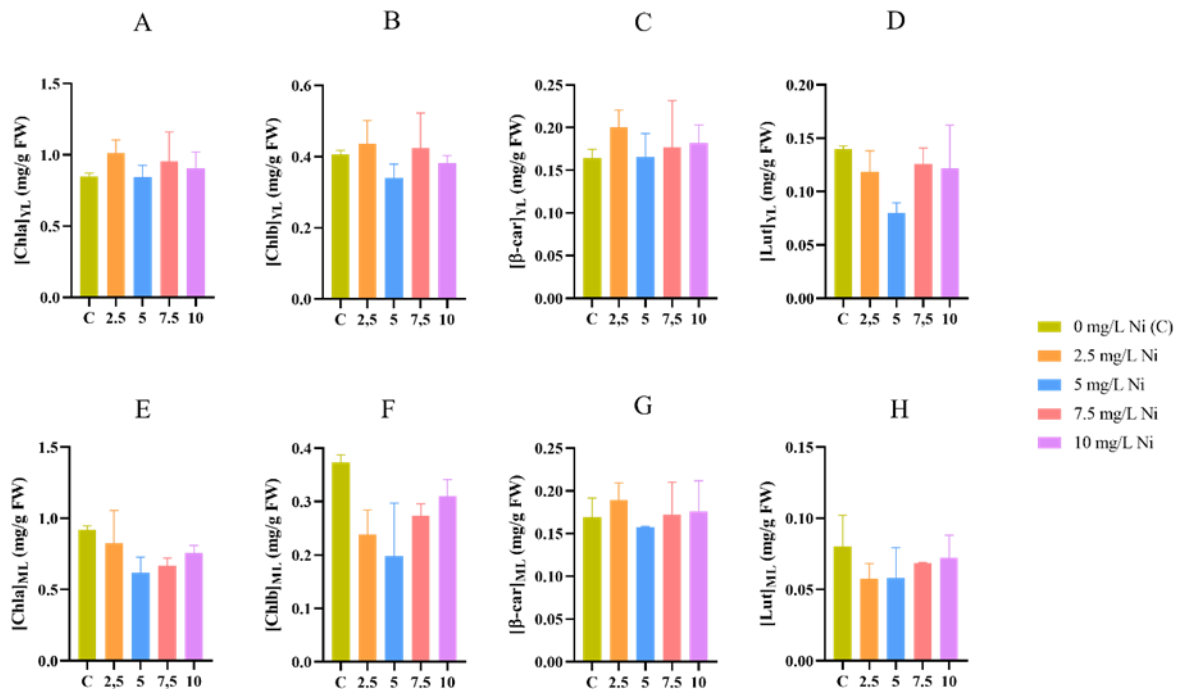


Figure 21 - Average + SD levels of Chlorophyll a in young leaves (A) and mature leaves (E); Chlorophyll b in young leaves (B) and mature leaves (F); β -Carotenes in young leaves (C) and mature leaves (G); and Lutein in young leaves (D) and mature leaves (H), expressed as mg/g FW in plants exposed to the different concentrations of Ni - 0, 2.5, 5, 7.5, 10 mg/L.

3.8.3. Effect of the increasing concentrations of Ni in the GSH levels

The concentration of GSH was measured in the AP and R. The results obtained revealed that no significant changes were detected upon the exposure of the plants to the different Ni concentrations. Even with no significant variations, it was possible to visualise a similar pattern to that of the concentration of H_2O_2 : with the increase of Ni concentration, the GSH quantity decreases in AP and increases in R (Fig 23).

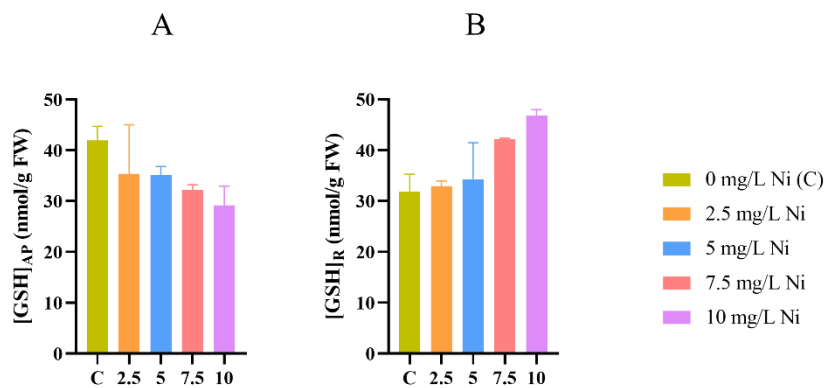


Figure 22 - Average + SD levels of GSH in aerial part of the plant (AP) (A) and in the roots (R) (B) of plants exposed to increasing concentrations of Ni – 0, 2.5, 5, 7.5, 10 mg/L.

3.9. Effect of the increasing concentrations of Ni in biometric parameters

There were no significant alterations in the biometrical parameters analysed, especially in the YL area (Fig. 24), but a tendency for the ML area to increase with the increasing Ni concentrations could be noticed.

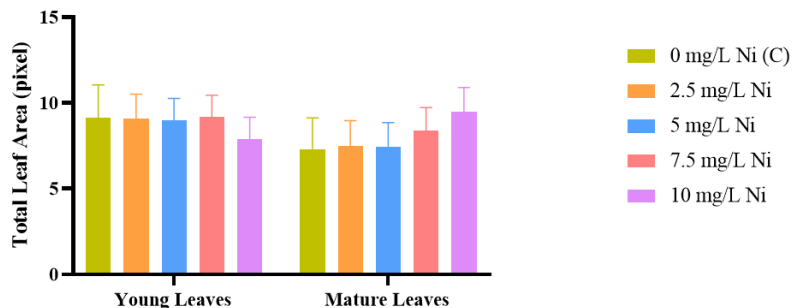


Figure 23 - Average + SD total leaf area, expressed in pixels, of young leaves and mature leaves of plants exposed to increasing concentrations of Ni – 0, 2.5, 5, 7.5, 10 mg/L.

Similarly, to the foliar area, the root length did not suffer significant alterations, even though a slight increase in the R length of plants exposed to 5 mg/L of Ni (Fig. 25) could be observed.

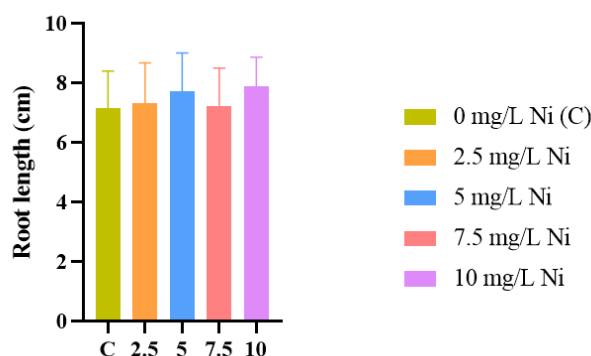


Figure 24 – Average + SD root length in cm of plants exposed to increasing concentrations of Ni – 0, 2.5, 5, 7.5, 10 mg/L.

3.10. Genetic transformations (*E. coli* and higher plants)

The oriented cloning processes to obtain fusion protein of MT1A or MT2B with a fluorescence green protein reporter was initiated.

When pSK was used as the first vector for cloning MT1A and MT2B fragments, even though it was attempted several times by altering the protocol using different strategies: increasing the ratio between the insert and the vector, to enhance the probability of ligation between the vector and the insertion fragment; purifying the vectors and the insertion fragments after the restriction reactions; using alkaline phosphatase to prevent the two ends of the vector to re-ligate, there was no success in their cloning, as only colonies with the re-ligated vector grew (blue colonies = non recombinant; fig.26). Even so, and because the insets are very small, some colonies were picked and propagated for plasmid extraction, followed by their restriction to discard the possibility of the small insert could have been cloned in frame with the *LacZ'* gene, but they just confirmed to be non-recombinant.

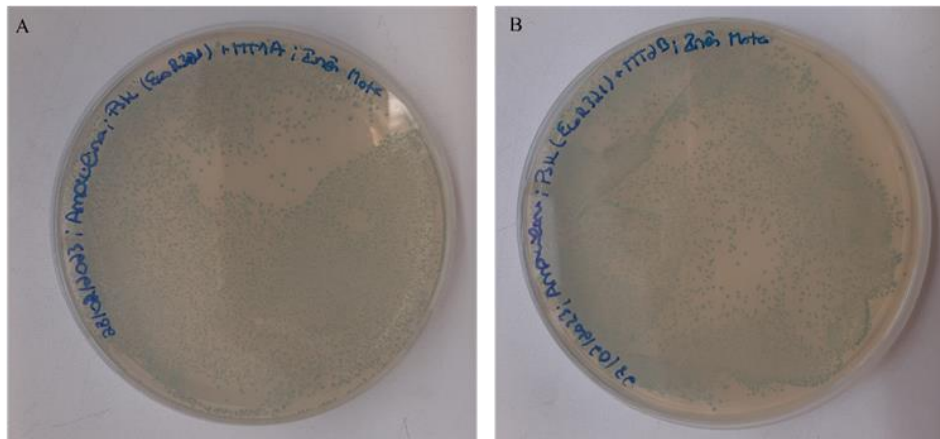


Figure 25 - Petri dishes with LB medium with ampicillin and X-GAL. A-Transformed cells with the ligation of MT1A and pSK with blunt ends, created by *EcoR321*; B- Transformed cells with the ligation of MT2B and pSK with blunt ends, created by *EcoR321*.

When using the pSMART vector, all the instructions provided by the manufacturer were thoroughly followed. Nevertheless, higher quantities of insertion fragments were also tested. However, no results were obtained after *E. coli* transformation, i.e., no colonies were present at the end of the procedure.

Regarding the use of pMDC83, no results were obtained, even when the same approaches used for cloning in pSK were used. Similarly, some potential colonies appeared in the culture medium, and a *XbaI* and *SaI* restriction digestion was performed to excise the inserts. There were no bands corresponding to the insert, but band with a bigger size than what was expected for the plasmid was visible, indicating the ligation of several plasmids in tandem (Fig.27).

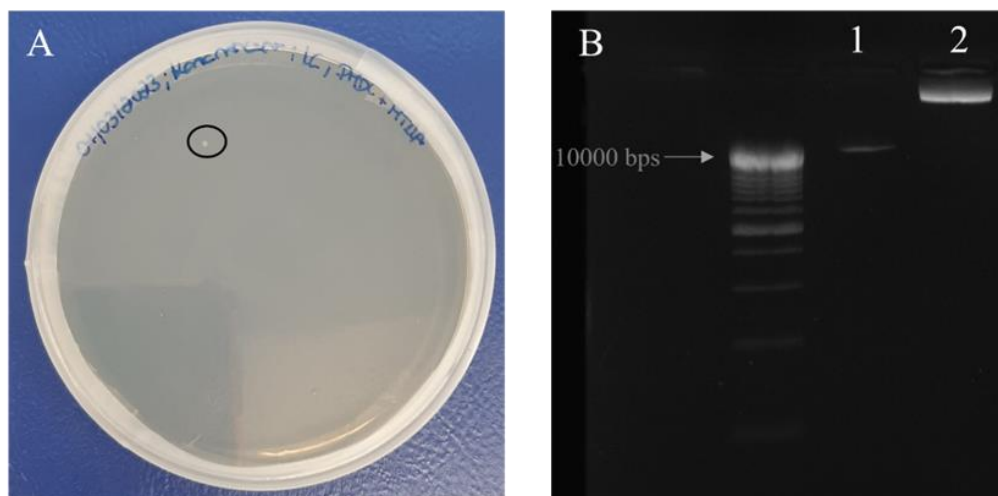


Figure 26 - (A) *E. coli*. colony resulting from the transformation with the sticky end ligation between pMDC83 and MT1A, using the restriction enzymes *XbaI* and *SaI*, in culture medium with kanamycin. (B) Electrophoresis of the mini-prep from the colony in (A); the gene ladder used is GeneRuler 1 kb DNA Ladder, thermoscientific; 1- pMDC83; 2- miniprep pMDC83 + MT1A digested with *XbaI* and *SaI*.

4. Discussion

Ni is an essential micronutrient that in high quantities can become toxic and due to anthropogenic activities, the presence of this HM has increased in the environment. Therefore, the need to understand how Ni toxicity affects living organisms has increased throughout the years.

Most investigators opt to study Cd (W.-J. Guo et al., 2008; Lv et al., 2013; Roosens et al., 2005), Cu (R. Benatti et al., 2014; Sekhar et al., 2011; Zhigang et al., 2006), Cr and As (Dubey et al., 2021), since these HMs are responsible for more critical consequences in plants and humans. So far, most of the research inherent to the impact of Ni in plants has been focused on the oxidative response of the organism (Helaoui et al., 2022; Maheshwari & Dubey, 2009). However, one of the defence mechanisms against HMs has been overlooked, MTs, and so, in this study, the understanding of how Ni influences the expression of the MT-encoding genes in a dosage- and organ-specific way was pursued.

To determine the appropriate concentrations range in the culture medium for *A. thaliana* to be used in the study, increasing Ni concentrations were used: 0 mg/L; 12.5 mg/L; 25 mg/L; 50 mg/L. Even though a high percentage of seed germinated, in the two highest concentrations of Ni the seedlings did not develop further. This could have happened because the concentration of Ni caused a high level of toxicity. In fact, previous researchers have described that one of the toxic effects of Ni in different species is the inhibition of seed germination (Hassan et al., 2019). Even though not many studies used *A. thaliana* as a model organism, some studies used species from the Brassicaceae family to analyse Ni and its impact in plants. Sharma et al. (2008) used *Brassica juncea* and noticed that seed germination and seedling growth were reduced due to Ni toxicity when exposed to 0, 25, 50 and 100 mg/L Ni. Other reports, such as Masidur Alam et al. (2007) and Pandey & Sharma (2002), where *Brassica oleracea* and *Brassica juncea* seedlings, respectively, with 10 and 42 days after sowing were exposed to excessive concentrations of Ni, 50 or 100 μ M and 500 μ M, suffered an inhibition in their growth. Also, different researchers have noted that for different species different concentrations of Ni caused negative impacts in their objects of study. Bazihizina et al. (2015) noted a decline in plant growth when *Psidium guajava* was exposed to 1000 μ M Ni; Gajewska et al. (2006) exposed *Triticum aestivum* seedlings to 100 μ M and noted a decrease in root fresh weight; and Shukla & Gopal (2009) recorded a retarded plant growth in *Solanum tuberosum* after exposure to 300 μ M Ni. So, since *A. thaliana* is a much smaller plant

than the models just described, the LD₅₀ (Median Lethal Dose) concentration of Ni could be lower for this model organism and, facing these results, it was decided to use lower Ni concentrations in the following assays: 2.5, 5, 7.5 and 10 mg/L, which allowed to obtain enough plant biomass needed for the experiments planned and discussed ahead.

Regarding the mutants ordered, after their genotype was accessed to ensure the insertion of the T-DNA in the correct place and that they were homozygotic. The size of the amplicon of the first PCR with the primers for the respective MT (Fig. 9- lane 1) is within the expected size and smaller than the size of the amplicon of the third PCR with the primers for reverse of the MT and the primer for the T-DNA. (Fig.9 -lane 3). Which means that each genome has the gene intact, without the T-DNA insertion, in one of the chromosomes, and the T-DNA insertion in the other chromosome. All mutants genotyped presented the same PCR results as seen on Fig. 9, therefore all mutants were classified as heterozygotes. This line of research was not continued because there was no time available to obtain these seeds' F1 homozygotes and perform the same procedures as those done with the wildtype plants. Nevertheless, a delay in mutant plant growth, when compared to the WT, was detected in all heterozygous seedlings. R. Benatti et al., (2014) stated that the mutants did not show any phenotypical differences from the WT. So, the delayed development observed may be possibly due to the seeds sent being old and, therefore, were not performing as expected.

In this work, the seedlings were grown in contact with varying concentrations of Ni in order to mimic the conditions plants would be exposed to if grown in a Ni-contaminated environment, as plants would be exposed to such environmental conditions since seed germination. According to a study by Renella et al. (2002), regarding the effects of heavy metals on the soil microbial biomass, it was confirmed that the short-term exposure to heavy metals cannot be used to predict the effects of long-term field exposure. Therefore, the use of the results of the short-term assays might be unpredictable and have low similarity to the long-term impacts observed in the field (Giller et al., 2009), but also, the acclimation time in the short-term experiments may not be representative of the interactions in long-term events (Smolders et al., 2003). These reports lead to the decision to grown plants for a longer period of time. Prior research regarding the expression of the MT gene family tend to select one of two options: exposure of plants for a short period of time after they grew in non-stressful conditions; or exposure of the seedlings to the stress conditions since germination. Within the first group, W.-J. Guo et al. (2008) and Patankar et al. (2019) grew the seedlings for 3 and 4 days, respectively, in standard conditions before applying the stress treatment for 14 days. Additionally,

Dubey et al. (2021) and Kim & Kang, (2018) grew the seeds in control conditions for 2 weeks, then subjected the seedlings to stress conditions for 1 week or 1 day, respectively. Contrary to this, few researchers like , R. Benatti et al. (2014) and Xiong et al. (2018) grew the seedlings in contact with the different HMs for 8 and 12 days before analysing the gene expression. Even though these studies are more similar to the situations that occur in the environment, as the plants were in contact with the HMs from seed germination, they were only exposed to the stress conditions for a short period of time. So, in this study, the plants were exposed to Ni for a longer period of time: since germination until 21 days post-germination.

It has been previously shown that MTs are differentially expressed throughout *A. thaliana*, but how the varying Ni concentrations would influence the expression of these genes is still unknown. As so, the cDNAs from three different organs: ML, YL and R were used for this study. Before the RT-qPCR analysis, a qualitative PCR was performed using cDNA of the different organs of the WT plants grown in a non-stressful situation. There were some results that were noticeable when confronted with those depicted in the literature. MT type I have been reported to be expressed mainly in the R (W. Guo et al., 2003; Zhou & Goldsbrough, 1994), however the band for these MTs was more predominant in the leaves, indicating that these genes are expressed in both organs. Both type II and III MTs are mainly expressed in the leaves (W. Guo et al., 2003; Hsieh et al., 1996; Reid & Ross, 1997) and the band for these MTs was more intense in YL and ML, but also appearing in R, also indicating that these genes are also expressed in both organs. The type IV MTs are detected mostly in seeds (W. Guo et al., 2003) and, in contrast, in the PCR results, the band more prominent was obtained with the ML, indicating that they may be involved in HM translocation during leaf senescence. Nevertheless, as no loading control was performed in such analysis, these results do not allow a clear comprehension of how they are regulated and, therefore, quantitative RT-PCR analysis was in order.

According to the RT-qPCR, using cDNA from YL of plants exposed to increasing concentrations of Ni, a decrease in the expression of *AtMT1A*, *AtMT2A*, *AtMT2B* and *AtMT3* was detected, and *AtMT1B* and *AtMT1C* showed an increase in their expression. *AtMT1A* was the only gene to be impacted significantly by the 4 concentrations of Ni, revealing to be down-regulated by this HM. *AtMT2A* was down-regulated by the three highest concentrations, and the expression of *AtMT1B*, *AtMT1C*, *AtMT2B*, *AtMT3* was impacted by 7.5 and 10 mg/L Ni. These results indicate that this gene family is differentially regulated by the Ni dosage in YL, with the highest concentrations exerting

a negative effect on most genes, with the exception of *AtMT1B* and *AtMT1C*, whose gene expression increased and the corresponding products may be of major importance in controlling the high Ni levels reaching the YL.

When using the cDNA from ML, a few complications in this procedure were encountered, as the reagent used was different and had specific requirements for the Ta of the primers, that did not exist with the reagent used for the YL, so the incoherence of the results could stem from this. So, the repetition of this analysis using the initial reagent would be advised in order to validate this data and allow a better comparison of the gene expression in the different organs and situations studied. Only three of the eight MTs genes, showed to be significantly reduced in their expression: *AtMT1A* and *AtMT2A* showed a reduction in expression in plants exposed to 7.5 and 10 mg/L of Ni, and *AtMT2B* had a decrease in its expression when exposed to 7.5 mg/L Ni. These results show that ML and YL behave different when it comes to respond to high Ni levels, as ML suffered less changes in MT gene expression than YL. This may be due to the fact that these leaves would be on the onset of the senescence period and there would be no need for the organ to spend energy in protecting it from such Ni levels. Nevertheless, the genes that were down-regulated were the same as in YL, with the exception of *AtMT3*, possibly revealing a regulation pattern of Ni in leaves for *AtMT1A*, *AtMT2A* and *AtMT2B*.

Contrary to what happened in the leaves, in the R the expression of the *AtMT1A*, *AtMT1C* and *AtMT3* significantly increase when exposed to Ni, thus indicating their responsiveness and participation in Ni homeostasis in R. While type I MTs were impacted by the 4 concentrations of Ni, the type III had a more significant reaction in the lowest concentration, 2.5 mg/L Ni. This reinforces the importance of type I MTs in controlling the high Ni levels in a concentration-independent way, while type III may play a more predominant role at lower concentrations. The Type IV MTs also showed a significant reduction in expression in plants exposed to different concentrations of Ni, which indicates that their expression is negatively affected by this HM. No variations in type II gene expression were detected in any situation, suggesting that these gene members may not play a significant role in Ni homeostasis in R, similarly to what happened in leaves. As such, this MT sub-family does not participate in Ni homeostasis at the whole plant level.

When studying the expression of *AtMTs*, most studies do not differentiate between the leaves developmental stages, since the plant is usually used as a whole (Dubey et al., 2021; Kim & Kang, 2018; R. Benatti et al., 2014; Zhu et al., 2009), but sometimes a separation between leaves and R is done (Khandekar & Leisner, 2011; Xiong et al.,

2018). The present study is, therefore, pioneer as it dissects the aboveground organs in to YL and ML, and studies their specific responses to high Ni levels. Also, it acts as an alert for other studies (already- or to be performed) that their corresponding conclusions may be dubious, as they do not explore which part of the shoot accounts more for such results. Also, prior studies focused on the impact of HM in plants and MTs, tended to stir their investigations towards the accumulation of HM, rather than the expression of these genes. Additionally, the existing research regarding the expression of MTs is in response to higher impact HMs, like: As, Cd, Cr, Pb (Dubey et al., 2021; Gu et al., 2014; Jaskulak et al., 2019; Lv et al., 2013). Khandekar & Leisner (2011) noted that when exposing *A. thaliana* with 35 days of growth for 3 days to high concentrations of Cu (30 μ M) the expression of MT1A, MT2A and MT2B increased in the leaves. When analysing *lMT2A*, an *AtMT2A* homologue from *Iris lactea*, Gu et al., (2014) stated that the gene was up-regulated by Cd. Similarly, Lv et al. (2013) showed that in 5 days-old *Brassica campestris* the expression of *BcMT1* and *BcMT2* was induced in leaves and R when exposed to 200 μ M CdCl₂ treatment, but only *BcMT1* showed an increase in expression when treated with 100 μ M CuSO₄. It has been shown that high concentrations of Cu and Cd cause an enhanced expression of different genes responsible for the resistance to the HM in question when using different species and tissues (Gu et al., 2015; Xiong et al., 2018; Zhigang et al., 2006). Unfortunately, little research has been done regarding the impact of Ni in plants and in the expression of the MT gene family. Kim & Kang (2018) stated that when exposing 2-week-old *A. thaliana* to 100 μ M Ni for 24 hours, MT1A, MT1C, MT2A, MT2B and MT3 showed a reduction in their transcript levels, although no alterations were noted in MT4A and MT4B. Since the author did not differentiate the tissues and used the plant as a whole and due to the weight of the different organs, the ratio between the quantity of shoot and root could be uneven, resulting in a higher concentration of RNA from leaves. Contrarily, Jaskulak et al. (2019) stated that in the shoots of *Lupinus luteus* exposed to 360 mg/kg and 720 mg/kg of Ni there was an increase in the expression of MT. Even though the plants used on both studies are different, these contradictory results further suggest the need to study the effect that Ni has on plants and in the expression of these genes in an organ-specific way.

As the R are the first organ to be in contact with the HM stress, the increase of MT gene expression could be explained by the need for these organs to protect themselves from such high HM doses. However, their response to the different concentrations is yet to be understood, since, in most studies, with the increase of concentration of the HM, the MT's gene expression would progressively either increase or decrease. The

decrease of the MTs observed in this study in the leaves could potentially be related to their translocation within the plant from R to shoots, particularly of MT1A and MT3, that have their expression enhanced in R in response to Ni and may be transporting it to the shoots as a mechanism to decrease the Ni content in R. Nevertheless, it would be necessary to determine the Ni levels present in YL, ML and R, to further test this hypothesis about the role MTs play in the plant's Ni homeostasis.

After the HM is accumulated in the plant, its toxicity can be deduced depending if the HM can be redox active or redox inactive. Ni is a redox inactive metal, that causes indirect mechanisms of oxidative stress such as by interacting with the antioxidant defence system, disrupting electron transport chains, and inducing lipid peroxidation (Rai et al., 2016).

The quantification of hydrogen peroxide levels showed that when *A. thaliana* was exposed to different concentrations of Ni, it had a tendency to decrease in the shoots and increase in the R. Similarly, Boominathan & Doran (2002) noted that H_2O_2 levels in *Alyssum bertolonii* and *Nicotiana tabacum* R' increased when exposed to Ni. However, when growing *Oryza sativa* exposed to different concentrations of Ni, Maheshwari & Dubey (2009) noted the H_2O_2 levels in the shoots suffered a higher increase than in the R. Previous studies have shown that this ROS molecule is involved in stress signalling pathways, which can activate several defence response mechanisms (Gill & Tuteja, 2010; Noor et al., 2022). So, the higher quantities of H_2O_2 in the R could mean that Ni is being accumulated in this organ, thus leading to an oxidative stress response.

Even though the variations of the concentration of hydrogen peroxide were not considered significant in this study, it could suggest an active participation of the antioxidant defence mechanism. Therefore, it would be interesting to analyse different scavenging mechanisms in plants, for example, catalase, ascorbate peroxidase, ascorbate, proline, sugars and polyamines, to see if other molecules than the ones analysed play a more important role in Ni-induced stress.

Resembling to the of H_2O_2 levels, the quantification of GSH showed that with the increase of the provided Ni concentration, the quantity of GSH in the shoots decreased and increased in the R. GSH is an essential metabolite in plants, since it's considered a crucial intracellular defence against ROS-induced oxidative damage and plays an important role in the antioxidant defence mechanism (Gill & Tuteja, 2010; Sun et al., 2007). Since the R are the first organ to be in contact with the HM stress, this could explain the observed tendency to increase the GSH levels in this organ. The similar

patterns of hydrogen peroxide and reduced glutathione levels obtained in this study was expected since the first molecule is responsible for signalling oxidative stress and the latter, in many cases, is one of the first antioxidant defence mechanisms to act.

When studying Cd tolerance of two populations of *Sedum alfredii*, Sun et al. (2007) noted an increase of GSH in the different organs of the plant: leaves, stems and R. Kukkola et al. (2000) noted that Ni caused more oxidative stress than Cu to *Pinus sylvestris* seedlings, since the GSH levels were higher in plants treated with Ni. In the Ni hyperaccumulator, *Thlaspi goesingense*, it has been reported that elevated quantities of GSH are involved in providing tolerance to Ni-induced oxidative stress (Freeman et al., 2004).

The concentration of the photosynthetic pigments analysed did not vary significantly in either of YL or ML of the plants exposed to the increasing concentrations of Ni, but some tendencies were noticed. Lutein was negatively impacted by 2.5 mg/L and 5 mg/L of Ni in ML and YL, respectively. In ML, chlorophyll a and chlorophyll b seemed to be affected the most by 5 mg/L. Chlorophylls and carotenoids play an important role in photosynthesis and HM can affect their biosynthesis; therefore, chlorosis and the reduction of the accumulation of the different photosynthetic pigments are frequently associated with HM-induced stresses (Gajewska & Skłodowska, 2007; Pandey & Sharma, 2002; Rai et al., 2016). Kaamoush et al. (2022) confirmed that in *Spirulina platensis* cultures, low concentrations of Ni (<2.0 mg/L) caused a significant increase in the concentration of chlorophyll a and carotenoids, while higher concentrations of the HM (≥ 2.0 mg/L) suppressed the levels of these pigments. When studying the effects of Ni nanoparticles in *Coriandrum sativum*, it was noted that the photosynthetic pigments reacted differently to the varying concentrations of the HM. For example, chlorophyll a and b had opposing reactions to the different concentrations, while the concentrations of the first was increased with 20 and 80 ppm, it decreased with 40 ppm; however, the concentration of the second pigment was reduced with 20 and 80 ppm and increased with 40 ppm. Meanwhile, carotenoids displayed similar levels to the control group, but had a reduction when exposed to 40 ppm Ni and increased at 80 ppm (Miri et al., 2017). After exposing *Psidium guajava* to increasing concentrations of Ni, total chlorophyll content in fully expanded leaves did not differ between treated and control plants, but the concentrations of carotenoids in leaves increased with increasing Ni^{2+} concentrations (Bazihizina et al., 2015).

The research regarding the impact of Ni in *A. thaliana* is in an early stage so there are few results to compare with. However, similarly to Miri et al. (2017), the different

pigments studied in this work also had varying reactions to the increasing concentrations of Ni. It would be interesting to evaluate the biosynthetic pathways of these pigments to understand the oscillation in their content in response to the increasing concentrations of Ni supplied.

Since it has been shown that HMs can affect the growth and development of plants, the foliar area and the R length were measured. Even though no significant alterations were noted, the area of ML seemed to increase with the concentrations of Ni and the root length seemed to increase in plants exposed to 5 mg/L. Since Ni tends to be accumulated in the R, the growth of this organ is more affected by the HM than the shoots (Cempel & Nikel, 2006; Chen et al., 2021). After six days of As treatment, *WT A. thaliana* had a reduction in the biomass (Dubey et al., 2021). When exposing *Brassica campestris* to 200 μ M CdCl₂ or 100 μ M CuSO₄ a reduction in the root elongation was noted (Lv et al., 2013). Shukla & Gopal, (2009) exposed *Solanum tuberosum* to increasing concentrations of Ni (0.05, 0.1, 0.2, 0.3, 0.4, and 0.5 mM) and reported a reduction in the biomass and in the growth of shoots and R. Overall, when most plants are exposed to Ni, the biomass tends to decrease (Bazihizina et al., 2015; Gajewska & Skłodowska, 2007; Pandey & Sharma, 2002).

However, the opposite of what was expected in plants exposed to Ni seems to have happened in this study, as there was an increase in the foliar area and in the root length. Further research is needed to clarify these results. Also, a reduction of the number of seeds sown in the culture medium could be favourable, as it would increase the HM available for each plant because of a lower “competition” between plants, plus ensuring that each seedling is exposed to similar quantities of Ni. Additionally, the biochemical parameters studied would benefit from extra analysis using more biological replicates in order to confirm if the tendencies observed could have a statistical meaning.

Regarding the genetic transformations, unfortunately it was not possible to obtain a positive recombinant colony with any of the attempts made. The size of the inserts could have been the main implication for the unsuccessful attempts, since the inserts were approximately 150 bps while the smaller vector is over 10-fold the size of those fragments: pSMART is 1788 bps, pSK is 3000 bps and pMDC83 is 10779 bps. When using pSK for cloning there was a possibility that the inserts entered along the reading frame as the *LacZ'*, meaning that the resulting colonies would maintain their blue colour. Even though some of such colonies were tested for the presence of insert, none proved to be recombinant. Therefore, a colony screening could have been useful to identify the colonies transformed with the recombinant plasmid. Other methods that could be applied

are cloning through Gateway recombination or Gibson assembly. Since the inserts are small, primer designing corresponding to the entire cDNA sequences with subsequent ligation to the plasmid would be another alternative. However, due to time restrictions, these strategies were not performed. These transformations would have contributed with important knowledge regarding eventual translocations of MTs throughout the vertical axis of the plant. Regarding the present study, the decrease in the expression of MTs in the shoots could be related to their potential translocation from R. Therefore, continuing with the cloning techniques to obtain a fusion protein with mNeonGreen will be advantageous to clarify this hypothesis.

Other HM defence mechanisms, not analysed in this study, may also be involved in Ni tolerance, such as PCs, as they are also responsible for the chelation of HM. Citric acid has been previously described as a predominant chelator for Ni transport in several hyperaccumulating plant species (Kersten et al., 1980; Lee et al., 1977, 1978; Montargès-Pelletier et al., 2008), and so its quantification could provide further insights in the defence mechanisms of *A. thaliana* to this HM. GS is also a precursor for the synthesis of PCs (Noctor et al., 2011), and evaluating its biosynthesis could be useful to understand the impact of Ni in the synthesis of this molecule.

Due to the time available and the weight of the biomass produced by the plants, it was not possible to quantify the concentration of Ni present in the different tissues analysed. This would be a valuable experiment since it would provide more insights regarding the accumulation of this HM as well as the *A. thaliana*'s tolerance to Ni. It would also be worthwhile to repeat the gene expression experiments using knockdown mutants of certain MTs, to understand if gene redundancy is in order or if each MT plays its own role in HM homeostasis.

5. Conclusion

This study helped to understand how the increasing concentrations of Ni: 2.5 mg/L; 5 mg/L; 7.5 mg/L; 10 mg/L, influenced the defence mechanisms of *A. thaliana* with a special focus on the gene expression of MTs, plus some oxidative stress-related endpoints, like hydrogen peroxide, photosynthetic pigments and GSH levels in WT plants.

Regarding the gene expression of *AtMTs*, the eight genes reacted differentially to the increasing concentrations of Ni. Some genes' expression increased in the R: MT1A, MT1C and MT3, while the others' remained unchanged; the response in the leaves was more fluctuating, as some increased: MT1B and MT1C in YL, and others decreased: MT1A, MT2A, MT2B and MT3 in YL and MT1A, MT2A and MT2B in ML. This means that the effect of the different concentrations of Ni on the expression of these genes varied for each gene and depended on the organ analysed.

The results obtained through the analysis of the biochemical parameters were not considered significant. This could indicate that the concentrations of Ni used aren't inducing significant oxidative stress response, hence why further testing should be made in order to validate our results. Besides expanding the biological replicates, reducing the number of seeds placed in the petri dishes to safeguard that the HM concentration that each seedling is exposed is similar.

The genetic transformations were unsuccessful as we were not able to obtain a positive recombinant colony. The small size of the insertion fragment could be hindering its insertion in the reading frame.

By exposing the seedlings to the HM stress from germination it allowed the obtainment of similar reactions of plants as if they had been sown in a polluted environment. Therefore, through an attempt to imitate the conditions the plants can be exposed if it were to grow in the environment, the seedlings were exposed to the HM for a longer period of time from germination until 21 days post-germination.

MTs have shown to be important in regulating cellular metal homeostasis and detoxifications process, due to their ability to chelate metals, providing protection against HM toxicity and oxidative stress, especially in R. The increase of expression of *AtMT1A*, *AtMT1C* and *AtMT3* in the R could potentially indicate their active role in the homeostasis of Ni, reducing the toxicity effects of this HM. Additionally, the decrease of the MTs in the

leaves could potentially be related to these MTs' translocation within the plant from R to shoots, particularly MT1A and MT3.

Plants possess several defence mechanisms, such as PCs and Citric acid, that are known chelators that could be involved in the protection of the plant against Ni. The evaluation of these two chelating molecules, along with the quantification of Ni in the different organs would provide further insight to the defence mechanism and accumulation of this HM. It would also be interesting to repeat this experiment using mutants with the knockdown gene expression of certain MTs, to understand how the rest of the MTs would be affected and would participate in the defence against high Ni levels.

As main conclusion, the increase in the expression of MTs in the R, especially MT1A, MT1C and MT3, indicates their importance in reducing Ni toxicity. Meanwhile, the reduction of the expression of MTs in the leaves could be linked to their eventual translocation. Since the research regarding the impact of Ni in *A. thaliana* is at an early stage, further research, coupled with the results obtained in this study, could be beneficial for the understanding of plants' the defence mechanisms against this HM.

6. References

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7. Supplements

S1. Result of the alignment of the mRNA sequences of *Arabidopsis thaliana* MTs

>MT4A

```

-----GCTTC----TC---TTGAG-----CTTTACGAAAC-----
CCGAACTCTCTTAGG---
CTGCCCCGAGAGTCGTCTTTATGTATATTTGAGTTTCCTTCATGACTCTAATTACC
GATCATGTCTGTTCACTCTCTG-----
CCGGAGACCGAGTCGACTCACTGTTGGATGCTTTGTCACGACTGAGAGGGA-----
CTTTAAGATTGAAAA-CGAAAAT-----GGCAGATACAGGCAAAG-----
GAAGCTCTGTGCT-----
GGCTGCAACGATAGCTGTGGCTGCCCTTCTCCCTGTCCCGGTGGGAATTCCTGC
AGGTG----CAGGA-----
TGAGAGAAGCATCTGCTGGGGATCAAGGGCACATGGTGTGCCCGTGTGGGGAGC
ACTGCGGATGCAA---CCCCTGCAACTGCCCAA-----GACCCAAACCCA-----
-----AAC--
CTCCGCCAAGGGCTGCACCTGTGGTGAGGGTTGCA-----
CATGCGCCTCTTGCGCCACTTAGACATCCTTATCTCTGTC--
TGTACCACACTTTGTATAATA---GTACTAGCTAGTCAG-ATC----
TGAAAAATAAATCTGTTTATTAGGTCCAGGTACAAGAAAACATATGTTTCGTATGCT
CTGC-----TC-----TCG-TCTTATGACTCATCAAGAATCTGAAAATCCT-----
-----GAAGGTCTT----GGACT-----
-----
-----

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>MT4B

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-----
-----
-----AAAAAGGA-----A--GAGAAAACAAAAAAT-----
GGCAGACACAGGCAAAG----GAAGTGCAAGCGCT-----
AGCTGCAACGATCGTTGTGGCTGCCCTTCTCCATGTCCCGGTGGCGAATCTTGCA
GGTG----CAAGATGA-----
TGAGCGAAGCATCTGGTGGGGATCAAGAGCACAAACACGTGTCCATGTGGGGAGC
ACTGTGGCTGTAA---CCCTTGCAACTGCCCAA-----GACTCAAACCTCA-----
-----AAC--

```

CTCTGCTAAGGGTTGCACTTGTGGTGAGGGCTGCA-----
 CCTGTGCCACTTGCGCTGCTTAGACATCCTTAAGTATGGT--TATACCATACT-
 TGTATAATAGGTACTACTAGCTAGTC-G-ATC---TGAAA--TAAAGCT-----
 GCACAACAAAATATATGTATCGTATGCTCTGC-----TTCTTGTGTTTCCT-
 TTTTATGAATCACCGAGAGTTTGTAAATCCTTATAACT-----
 TCAAGCCTTCAAGGTCTT----GCACCAATACAATCCAC-----TATGTAT---
 AGAACAA-----TTCTCTTTTT---TTTTTTACTCTAT-----
 ---TCTAGAGTA-AAATATAGAATAAGATTAGAGA--GAA-----
 TATTACTCTA---TAATAAAGTTTAGAGGAAAAAATAGAGTTGTAGATATATACTTTT--

>MT3

-----GGACACGAGAGGT--
 TGGCAACGAAAGGCTTTTCTCTCCATAACCCAAAAAA---GATTCGGTTGTC-----
 ACAT-----AATTTGCAG---AGATTCTCCACTCTCTCTATCCTTCTATAAATT---
 ACATAGCC-ATCGGAT----C---ACAAAACAGAA-----C-AACAACAACAA-----
 -----CAACAAACAA-----GAACTCAAACACT-----TCATAAC-----
 TAA-----ACATCCTTTA-----AAGCCTTTT-----
 CAAAAACTCAATCATGT-----
 CAAGCAACTGCGGAAGCTGCGACTGTGCTGACAAGACCCAGTGCGTAAAG-----
 -AAGGGAACCAGCT-----
 ACACCTTCGACATCGTCGAGACTCAGGAGAGCTACAAGGAGGCCATGATCATGGA
 CGTTGGTGCCGAGGAGA---A-C-----AACGCAAA-----TT-----
 GCAAGTGCAAGTGCGGCTCCTCTTGCAGCTGCGTCAACTGCA---CTTGCTGCCCC-
 -----AACTAATGAAG--CTTCTTTAATCAAAAT--GTAAT-----
 ---ATGAATAAAAGT-----T--GA-TGT-----GGGCTCAT--CT--
 ATTGAGCTCATGTCTCTC-----T-----TATTACTACTCTCT--
 AGTATGGTGTGATGTAATGGGTTATGACCCTTCTTT---CCCTTCC-----
 CTATAAACTA--AAGAACT-----TGC---
 AAGATA-----

>MT2B

 -----AT--TCACTGAT-----
 --TATCGTTTAAG--GCAAA-----TTAAGA-----TCAT-CTTCATAAATCTTC-----

TCAGATCTCTTCC-----AATTTTCTAGAAA-----AAACATGT-----
CTTGCTGTGGTGGGAAGCTGTGGTTGTGGATCTGCCTGCAAGTGCGGCAATGGTT
GCG--GAGGTTGCAAAAGGTACCCTGACTTGGAG-----AA--
CACCGCCACCGAGACTCTTGTCTCGGTGTTGCTCCGGCGATGAACTCTCAG-----
-----TACGAGGCTTCCGGCGAGACTTT-C-----GTTGCCGAGAA---TG-----
ATGCTTGCAAATGCGGATCTGACTGCAAGTGCAACCCTTGTA--
CCTGCAAATGAAG-----AACTT--CATAAACCC-----TA-----AGTC----T-----
---G-----TAATAACCCTAATG---T---TA-TGTTAGGTTTG-
CTTATATGTAATAATTGGCTGATTTTTCCGGTAG-
TTTTGCCGGCGACGTTGGTCTTTCTCTTCTTCTGTGTGTGTTTTATGGTTTGGTCA
TTAAGATAT-----CTCTGCAAAGTTTTATCTTTGTGACTTTA--TTAATCCTA---A-
GACTATTATGGGTTTGTATT-----AAAGTTTGCTTCTTTCTTGCTCA-----
CTAC---ACAATTAAGATTCAAGCCC-----

>MT2A

ATAAAAACACGAAATACCAAGTGGACGACTGAGGTTAA-----
TAGATCTAGCCGTAGAATAAAGATCTGCATGAAAGGCGGTGAGAATCTAAACGGT
GATAAGACCATAACACACGGAACATCGGTACGC-TCTC-----
GAACGTACAAGAATCGACG-----
ACACACAAACACTCCACAATTATTTGAACACTGGACAAT-----TATT-----
GAACCGACGTACGAGAATCAATGCGCTGAGG-GTAAAGACGTAA-
ATGAAGAACTAGTTTTGGAGATAAGAGCGGAGAAAGA---
TTGCGACACATGTATGGTCAATATTAATCTCATTTAGCTTATAAA-----TTTGGGA--
GCTTCCTCTATCATTAATTTTCATTCATAAATTTTCTTCAATTTGAATTTTCT-
CGAG-----AAAAATGT-----
CTTGCTGTGGAGGAAACTGCGGATGTGGATCTGGCTGCAAGTGCGGCAACGGTT
GTG---GAGGTTGCAAATGTACCCTGACTTGGGATTCTCCGGCGAGACAA-C--
CACAAGTGAAGTCTTTGTCTTGGGCGTTGCACCGGCGATGAAGAATCAG-----
TACGAGGCTTCAGGGGAGAGTAA-C-----AACGCTGAGAA---CG-----
ATGCTTGCAAGTGTGGATCTGACTGCAAGTGTGATCCTTGCA---CCTGCAAGTG----
---AAGAAGCCTTTTTTAA-----TAAGCAGAGATAATCGAG---
TCTCTTTAATTAATTA--AGTTATTCAATAAGT-----AAACCATATATAGGATGGTGT-
TTTAGGTTTGGTTTATGTGTAATAATGGCTTCAGCTTATC---T-TTTAGC-CGAT-
CA-----TTGTCTTTTGTGTTTGTGTTTGATCATA---
TCTTTTAGATGTCTAGCAACTCTGCCATGTGATGAGTTTGTACTTCCAGTGGAATG
ATA---A-

TAATATTATAGTTTTAAATCTCTTTGTTTCGTTGGGTTTTCTTCATTGGTCCTCCATTG
TTTCTAAATTTTGA---AGAATTAATTTTTTATGGATTATTTGGCTTGG

>MT1B

ACTCTGTTGTCTTGCTAAAACTCTAACATTAAATTACTTTCTTGAATG-
AAGCTGGAACAAATCTAAC-----
ATAAAAAGAAAATGATGGGCAAGTTGATGTTATTCGT-----
AAATTTATTTGGATTATGTATAAAGTGATCCGAATCTCTCTCTTCTTCTATAAATAGT
GGCCATTCCC-ATTGGTT-----GAAATCACA-AA-AGCATCA-----TAAG-
AAGAAGAAGAA-ACTACAGAAG---TT-
AATCAATCAAAGACAAGTAAGAGAAATGGGACTGAT-TCTAACCAGGAT-----
GATTCTAAT-----
TGTGGATGTGGCTCCTCCTGCAAATGTGGTGACTCTTGCAGTTG----
CGAGAAGAACTACAA-CAAGGAGTGCGACAAC-----GTAGCTGTG---
-----GGTCAAACGTAACTTTTGATGAAATTATT---ATGGTCTAAAATCAT-----

ATATATGGCAGAAAAATTGGGGAAAATATGTGTTTTATGCTAAGAGATGTGTGTGT-
TGTTGGAATAAA-----GACGTGACCGTTGTGTTGCGTAT-C-----AAC-----
TCTCTT-----AAGCTT--TTGACTTTTCCCAGCT---TTGTAT-----
CAAAGCTGAGGCATACATACCCTT-GTAACGTCTC-----TTCAC--
ATTCGAACAAGTC-CCACTCTCACCAATTC---
GTCATTTGAGAATTGATATAAACCTAATTACGAACTGGT-TT-----T--
CTCAAACCTGGCC-----C-ATTAACCAGACCCA-----TCAGCTCTTGCGTTTA--
---CCAAT--TCT---ATTTCTT-----TCAAAGATAGGGATA--A-TAT---
-----TTGT---AAAATAACA-----

>MT1A

-----ATGGCA-----
-----GATTCTAAC-----
TGTGGATGTGGCTCCTCCTGCAAATGTGGTGACTCTTGCAGTTG----
CGAGAAGAACTACAA-CAAGGAGTGCGACAAC-----
GTAGCTGTGGATCAAACCTGCAGCTGTGGGTCAAACCTGTAACCTGTTGA-----

>MT1C

AAAAGATAATA-----TATATATATAGG-----AAATGTATG-----GTGGAT--
ATAAAACCGATATTTTATATTCTTCTATATATACTGGCCATTCCC--TTAGTT-----
GAAATCACATAA-AGCATCA-----GAAG-AAGAAAAAGAAAACCTACAA-----
AATCAATCAAAGAGAAGTAAGAGAAATGGCA-----GGTTCTAAC---
---TGTGGATGTGGCTCCTCCTGCAAATGTGGTGATTTCGTGCAGTTG----
CGAGAAGAACTACAA-CAAGGAGTGTGATAACT-----
GTAGCTGTGGATCAAACCTGCAGCTGCGGGTCAAGCTGTAAGTGTGAAGAAATTA
TCAGCATGGTCTCAAACCAA-----
GGAAAATGGGAAAAAAATGTGTTTTATGCTAAGAGATCTTTGTGTATGTTGGAATA
AA-----GACTTGG-CATTGTGTTGCGTAA-T-----GAGTAAGAGTAACTATT-----
-----AAGCTTGCTTGACTTGTTCTGCT---GTGTAATGTTTTTA---
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ATGTGTATATGTCTCCTCTCTAACGAAAAC--CATAATTTAAAAGGCTATACAT-----
ATTT---TCACGT-TT-----TGAATAAA-----