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Study of *HIPP25*, *HIPP26*, *HIPP27* gene expression and glucosinolate content in *Arabidopsis thaliana* and *Noccaea caerulescens* upon heavy metal treatments

Barsanti Gautam

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Supervisor: Atle M. Bones, IBI
Co-supervisor: Ralph Kissen, Biology

Norwegian University of Science and Technology
Department of Biology

Abstract

Hyperaccumulator plant species accumulate exceedingly high concentrations of metals in aerial parts and are increasingly studied because of their potential for cleaning up land contaminated with heavy metals. One such hyperaccumulator, *Noccaea caerulescens* has been recognized as a model species for its remarkable Zn, Cd and Ni hyperaccumulation capacity and shares 88% sequence similarity with non-hyperaccumulator *Arabidopsis thaliana* in the coding region sequence. HIPPs (Heavy metal-associated isoprenylated plant proteins), are characterized by the presence of one or two HMA (Heavy metal associated) domain and a C–terminal isoprenylation motif, present in both plants which have a possible role in hyperaccumulation. In the current study transcript levels of *HIPP25* along with its closely related gene *HIPP26* and *HIPP27* were monitored in roots, leaves, flower and siliques of *A. thalian*a and root and leaves of *N. caerulescens* when exposed to Ni, Cu, Zn and Cd for 14 days (long term) and 24 hours (short term). Effect of metal treatment under long term condition was performed with two concentrations. Furthermore, the effect of these metal treatments on the *HIPP25* protein level was also determined. Under long term treatments, in leaves of *A. thaliana HIPP25* gene expression was higher with Cd, Zn and Cu exposure as compared to the control plants. *HIPP25* gene in *A. thaliana* is possibly involved in the accumulation of Zn and Ni homeostasis as the expression of HIPP25 was higher in Zn treated leaves and lowered in the Ni treated leaves at both gene and protein level. In *N. caerulescens,* under short term treatments, Cu decreased *HIPP25* and increased *HIPP26* gene expression in roots. Under long term treatments, higher concentration of Ni reduced whereas lower concentration induced the *HIPP26* gene expression in roots. From the comparative study on the protein level, HIPP25 proteins were expressed more in *N. caerulescens* than *A. thaliana* under both long term and short term treatments. Apart from this, changes in glucosinolate content in both plants exposed to Zn under both long term and short-term treatments were also monitored where the glucosinolate content in leaves of *A. thaliana* increased upon lower concentration under long term treatments whereas decreased upon higher concentration under both long and short-term treatments. In *N. caerulescens* under both concentrations in long term treatment, the glucosinolate content decreased and remains unchanged for short term treatments.

Keywords: Hyperaccumulator, Noccaea caerulescens, Arabidopsis thaliana, Heavy metal-associated isoprenylated plant proteins, gene expression, protein level, glucosinolate

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Abbreviations

1Meo-I3M	1-methoxyindol-3-ylmethyl glucosinolate
2OH-2MeP	2-hydroxy-2methylpropyl glucosinolate
2P	2-propenyl
3B	3-butenyl
3MSP	3-methylthiopropyl glucosinolate
4MSB	4-methylsulfinylbutyl glucosinolate
4MeO-I3M	4-methoxyindol-3-ylmethylglucosinolate
7MSH	7-methylsulfinylheptyl glucosinolate
8MSO	8-methylsulfinyloctyl glucosinolate
At	*Arabidopsis thaliana*
CAX	Cation exchangers
Cd	Cadmium
CDF	Cation diffusion facilitators
cDNA	Complementary deoxyribonucleic acid
Cu	Copper
dNTP	Deoxynucleotides
dH2O	Sterile water
DNA	Deoxyribonucleic acid
DW	Dry weight
FW	Fresh weight
gDNA	Genomic deoxyribonucleic acid
GSH	Glutathione
GSL	Glucosinolate
GUS	β-glucuronidase
HIPP	Heavy metal isoprenylated plant protein
HIPP25	Heavy metal-associated isoprenylated plant protein 25
HIPP26	Heavy metal-associated isoprenylated plant protein 26
HIPP27	Heavy metal-associated isoprenylated plant protein 27
HMA	Heavy metal transporting ATPases
HPLC	High performance liquid chromatography
HPP	Heavy metal plant protein
IPTG	Isopropyl β-D-1-thiogalactopyranoside

I3M	Indol-3-ylmethyl glucosinolate
kDa	Kilo Dalton, measure unit for protein weight
LB	Luria Bertini
MATE	Multidrug and toxin efflux
mRNA	messenger ribonucleic acid
MTP	Metal transporter proteins
NA	Nicotianamine
NAS	Nicotianamine synthase
Nc	*Noccaea caerulescens*
NCBI	National center for biotechnology information
Ni	Nickel
NIP	Nodulin 26-like Intrinsic Proteins
PCR	Polymerase chain reaction
p-OH-bZ	P-hydroxy benzyl glucosinolate
RhaOxy-bz	4-α-rhamnosyloxybenzyl glucosinolate
RPM	Revolutions per minute
RNA	Ribonucleic acid
RT	Room temperature
RT-qPCR	Real-Time quantitative reverse transcription PCR
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TAIR	The Arabidopsis information resource
TBST	Tris-Buffered saline with Tween20
X-gal	Bromo-chloro-indolyl-galactopyranoside
YSL	Yellow strip 1 like
ZIP	Zn regulated transporter-, Iron regulated transporter-proteins
Zn	Zinc

1 INTRODUCTION

1.1 Heavy metals

Plants are sessile organisms which depends on the soil condition (temperature, pH, acidity, salinity etc.) and composition (minerals, water) to survive and reproduce. Soil contains both essential and non-essential elements. An element is considered essential if the plant grows and reproduce normally in the presence of suitable concentration but fails to grow normally and to complete its life cycle if it is grown in a medium where the element is adequately removed (Rajurkar et al., 1998). Some of the essential elements include macronutrients (nitrogen (N), phosphorus (P), potassium (K), magnesium (Mg), calcium (Ca), and sulphur (S)) and micronutrients (manganese (Mn), iron (Fe), cobalt (Co), copper (Cu), zinc (Zn), nickel (Ni), chromium (Cr), chlorine (Cl), bromine (Br), and iodine (I)) and the non-essential and the toxic elements includes aluminium (Al), rubidium (Rb), strontium (Sr), arsenic (As) , cadmium (Cd), tin (Sn), lead (Pb) and mercury (Hg) (Desideri et al., 2010). Heavy metals are naturally occurring metallic elements that have atomic number ≥ 20 and density greater than 5 gm/cm^3 (Tchounwou et al., 2012). Among the 90 naturally occurring elements, 21 are non-metals, 16 are light metals, and the remaining 53 (with As included) are heavy metals (Kannan et al., 2008). Certain heavy metals are required for plant growth, development, and metabolism such as Co, Zn, Cu, Fe, Mn, Ni (Appenroth, 2010) and while the metals, like Cd, Pb, and Hg, are generally toxic to plants (Clemens, 2006) and do not have known physiological and biochemical function in plant metabolism.

1.1.1 Sources of heavy metal, threat and removal

Heavy metals are found into the environment through natural and anthropogenic sources. The natural sources include earth's crust, weathering of minerals, erosion and volcanic activity while anthropogenic sources include mining, smelting, electroplating, use of pesticides and (phosphate) fertilizers as well as biosolids in agriculture, sludge dumping, industrial discharge, atmospheric deposition, etc. (Chehregani et al., 2007; Fulekar et al., 2009; Mehmood et al., 2009; Modaihsh et al., 2004; Wuana et al., 2011)

Heavy metal contamination into the environment has been a major global threat which not only has effect on the crop yield, soil biomass and fertility, but also has deep effect on the bioaccumulation of the food chain. These heavy metals cannot be easily destroyed and the increasing concentration of heavy metals has been a great challenge that pose a serious threat

to environment and human health with its long-term persistence (Gisbert et al., 2003; Halim et al., 2003).

The conventional remediation methods for removal of heavy metals include *in situ* vitrification, soil incineration, excavation and landfill, soil washing etc. (Sheoran et al., 2010; Wuana et al., 2011). Use of these physical and chemical method includes limitations as excessive cost, labor intensive, change in the soil properties and loss of indigenous and beneficial microflora. Among the different approaches to the restoration of heavy metals contaminated soils *in situ*, special attention is drawn to the technologies of phytoremediation.

1.2 Phytoremediation

Phytoremediation, the use of naturally or genetically modified plants for environmental clean-up purposes, has been proposed as an attractive concept for its low-cost and environmental friendly technique (Pilon-Smits, 2005). It is an emerging technique where plants take up the contaminants (organic and inorganic) from the soil and water through various processes. Toxic heavy metals and organic pollutants are being more targeted for the phytoremediation technique these days. The phytoremediation process can be divided into different classes as described below (Pilon-Smits, 2005) and represented in *Figure 1.1*:

(i) phytostabilisation – the contaminant gets stabilized in the root tissues and prevents its movement through the soil;

(ii) phytostimulation/rhizodegradation – the contaminant, generally organic, is degraded by root exudates and rhizosphere microorganisms;

(iii) phytovolatilisation – the contaminant (inorganic or organic), once absorbed, is physically changed to a gaseous state by the plant's metabolism;

(iv) phytodegradation – like phytostimulation/rhizodegradation but occurring in the aerial parts of the plant;

(v) phytoextraction – the contaminant is absorbed through roots, and high concentrations are transported to the aerial parts of the plant, making it possible to harvest the aboveground plant parts containing the contaminant.

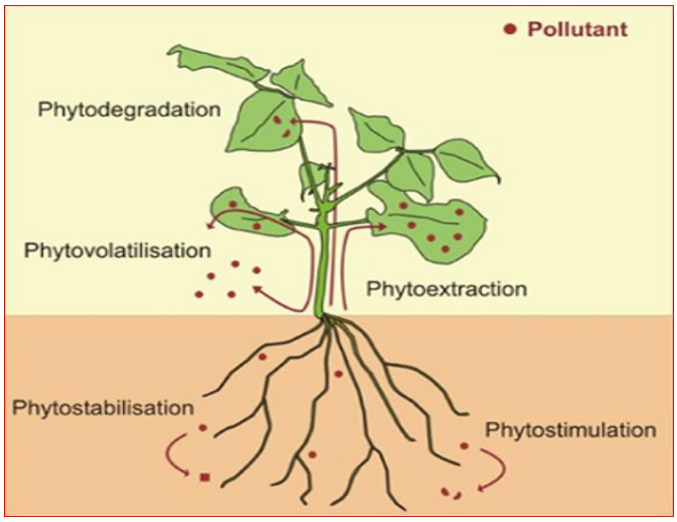


Figure 1.1 Possible Fates of Pollutants with the phytoremediation process (Pilon-Smits, 2005)

Regarding cost, phytoremediation has low installation and maintenance costs as compared to other conventional methods, as less as 5% of other cleanup methods (Prasad, 2003; Van Aken, 2009). Furthermore, due to the growth of plants in the field, the problem of erosion and metal leaching is also prevented (Chaudhry et al., 1998). Among all the techniques in phytoremediation, the most promising concept seems to be the phytoextraction (Sun et al., 2011), where plant absorbs the toxins like heavy metals and metalloids found in the soil and water and translocate to the aerial parts of the plant (Lombi et al., 2001; Salt et al., 1998) which can be harvested. It implies that the plants are able to concentrate metals to higher concentrations than in contaminated soils due to which total plant dry matter containing the metals will be less and easier to handle than the tons of soil to be treated by conventional ways of remediation (Hassan et al., 2011). The efficiency of phytoextraction depends on many factors like bioavailability of the heavy metals in soil, soil properties, speciation of the heavy metals and plant species concerned (Ali et al., 2013). The identification of hyperaccumulators has been an important task as the key to successful implementation of phytoremediation (Zhou, 2002; Zhou et al., 2004).

1.3 Hyperaccumulators

Hyperaccumulator plants are those plants when grown on the metal rich soils are able to concentrate greater than 100 mg/kg dry weight (0.01%) Cd, or greater than 1,000 mg/kg dry (0.1%) weight Ni, Cu and Pb, Co, Cr, As, Se, Tl, or greater than 10,000 mg/kg (1%) dry weight Zn and Mn in their the leaves without suffering its own phytotoxicity damage (without yield reduction) than the crop plants or common non-accumulator plants (Chaney et al., 2007; Mitch, 2002).

Plants can be heavy metal-tolerant non-hyperaccumulator, in which metals can enter root cells where they are sequestrated into root vacuoles, preventing translocation to shoots or they can be heavy metal-hypertolerant hyperaccumulator, in which metals are actively taken up through the root, and largely loaded into xylem for root to shoot transport (Lin et al., 2012). Hyperaccumulation of heavy metal occurs in approximately 0.2% of all angiosperms but it is particularly abundant in the *Brassicaceae* family and most of these species are Ni hyperaccumulators (BakerAJM et al., 2000; Krämer, 2010). Such hyperaccumulators might be good as candidates for phytoextraction purpose; however, they are found to be low-biomass and slow growing in nature. Despite having low biomass, heavy metal hyperaccumulators are suitable model plants to understand the molecular mechanisms of heavy metal hyperaccumulation and hypertolerance in plants by comparing with non-hyperaccumulators. Till date, the hyperaccumulation of different metals in various plant species has been investigated and it has been found that different mechanisms of metal accumulation, sequestration or detoxification exist in various plant species. Among all the hyperaccumulators, *Thlaspi/Noccaea sp*., *Arabidopsis halleri*, and *Sedum alfredii* are most extensively studied (Lone et al., 2008). *Thlaspi* genus are known to hyperaccumulate more than one metal, i.e., *T. caerulescens* for Cd, Ni, Pb and Zn, *T. goesingense* for Ni and Zn, *T. ochroleucum* for Ni and Zn, and *T. rotundifolium* for Ni, Pb and Zn (Vara Prasad et al., 2003).

1.3.1 Molecular mechanism behind hyperaccumulation

Three major process make a plant hyperaccumulator: (1) enhanced root uptake and loading into the xylem (2) efficient root to shoot translocation (3) efficient detoxification via chelation and sequestration mainly within leaf cell vacuoles (Clemens et al., 2002). Some transporters that are involved in the different process of hyperaccumulation are shown in *Figure 1.2*.

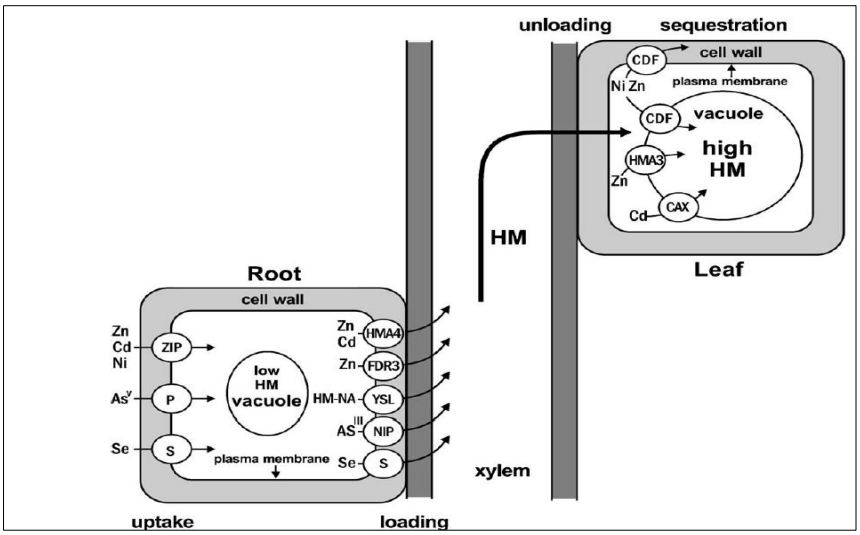


Figure 1.2 A scheme showing transport systems constitutively overexpressed and/or with enhanced affinity to heavy metals, which are thought to be involved in uptake, root-to shoot translocation and heavy metal sequestration traits of hyperaccumulator plants. (CAX = Cation Exchangers; CDF = Cation Diffusion Facilitators; FDR3 = a member of the Multidrug and Toxin Efflux family; HM= Heavy Metals; HMA= Heavy Metal transporting ATPases; NA= Nicotinamine; NIP = Nodulin 26-like Intrinsic Proteins; P = Phosphate transporters; S = Sulphate transporters; YSL = Yellow Strip 1-Like Proteins; ZIP = Zinc-regulated transporter Iron-regulated transporter Proteins (Rascio et al., 2011)

1.3.1.1 Heavy metal uptake from soil to the root

Metal availability and mobility in the rhizosphere can be influenced by root exudates, such as siderophores, organic acids and protons, as well as by the interaction with the rhizosphere microorganisms (Wenzel et al., 2003; Whiting et al., 2001; Zhao et al., 2001). The metals are then taken up from the soil to the root and several metal transporters are involved in this process which are found to be higher in hyperaccumulators as compared to non-hyperaccumulators. For example, some genes that belong to ZIP (Zinc-regulated transporter Iron-regulated transporter Proteins) family, which are responsible for Zn uptake is found higher in *N. caerulescens*, than non-hyperaccumulator species due to the constitutive overexpression suggesting that it might play key role in metal hyperaccumulation (Assunção et al., 2001). In most ecotype of *N. caerulescens* when supplied with Zn and Cd/Ni, demonstrates that Cd and Ni influx also depends upon Zn transporter with a strong preference for Zn over Cd and Ni (Zhao et al., 2002). Evidence exists that arsenic in the form of arsenate (AsV) through phosphate transporter and selenium in the form of selenate through sulphate transporter can enter the plant roots due to the chemical similarity between them (Caille et al., 2005; Meharg et al., 2002; Shibagaki et al., 2002).

1.3.1.2 Root-to-shoot translocation

Unlike non-hyperaccumulator plants, which retain metals in root cells, the hyperaccumulators take up most of the heavy metal from the soil, and rapidly and efficiently translocate these elements from the root to the shoot via the xylem retaining very low heavy metal in root vacuoles. Some of the metal transporters involved in this process are shown in *Figure 1.2*. *HMA4* gene from HMA (Heavy Metal transporting ATPases) family expression is up-regulated in root and shoots of *T. caerulescens* and *A. halleri* upon exposure to high levels of Cd and Zn, whereas it is down-regulated in non-hyperaccumulator relatives (Papoyan et al., 2004) and *HMA4* protein is involved in Cd and Zn efflux from the root symplasm into the xylem vessels, necessary for shoot hyperaccumulation (Rascio et al., 2011). Currently, *HMA4* is known to be the only gene which has a role in both Zn and Cd tolerance (Verbruggen et al., 2009). *FDR3* gene from MATE (Multidrug and Toxin Efflux) family, is constitutively overexpressed in roots of *T. caerulescens* and *A. halleri* (Talke et al., 2006) and FRD3 protein usually operates in the xylem influx of citrate as a ligand for Fe homeostasis and transport (Durrett et al., 2007; van de Mortel et al., 2006) and possibly in translocation of other metals, such as Zn (Krämer, 2010). Three genes; *YSL3, YSL5* and *YSL7* (Yellow Strip 1-Like), are constitutively overexpressed in roots and shoots of *T. caerulescens* and some YLS proteins are involved in the vascular loading and unloading of especially nicotinamine–metal complexes (Gendre et al., 2007). Most of selenium taken up by root cells of selenium hyperaccumulators remains as selenite and its root-to-shoot translocation occurs through sulphate transport systems (Sors et al., 2005). But in the case of the roots of hyperaccumulating ferns, most of arsenate (AsV) is quickly reduced to arsenite (AsIII) by the activity arsenate reductase (Duan et al., 2005). High expression of NIP (Nodulin 26-like intrinsic proteins) might account for the arsenite transfer from root cell cytoplasm to xylem vessels in arsenic hyperaccumulators (Zhao et al., 2009).

1.3.1.3 Sequestration in the shoot vacuoles

The ability to hyperaccumulate metals at least in part depends upon an enhanced capacity of metal storage in leaf vacuoles. Several families of transporters are involved in this process. CDF Family, also called metal transporter proteins (MTPs), contains members involved in the transport of Zn2+, Fe2+, Cd2+, Co2+ and Mn2+ not only from cytoplasm to organelles or apoplasm, but also from the cytoplasm to the endoplasmic reticulum (Peiter et al., 2007). The overexpression of *HMA3*, possibly involved in Zn compartmentation, and that of *CAX* (Cation Exchangers) genes seems to mediate Cd sequestration, have been noticed in *T. caerulescens*

and *A. halleri* and supposed to be involved in heavy metal hyperaccumulation (van de Mortel et al., 2008).

1.3.1.4 Role of Chelators in hyperaccumulation

It is assumed that most of the hyperaccumulated metals are bound to ligands, such as organic acids, amino acids, peptides and proteins (Verbruggen et al., 2009). For example, in the root levels, Histidine (His) forms stable complexes with Ni, Zn and Cd and it is present at high concentrations in hyperaccumulator roots (Persans et al., 1999). Another chelator, Nicotianamine (NA) are more expressed in *T. caerulescens* than in *Arabidopsis* (Hammond et al., 2006; van de Mortel et al., 2006) and has a role in Ni hyperaccumulation which form stable Ni–NA complex in Ni-exposed roots and facilitated its transport to the shoot (Mari et al., 2006; Vacchina et al., 2003). At the site of maximum metal accumulation inside leaf vacuoles, metals are chelated by organic acids such as malate or citrate (Krämer et al., 2000; Lee et al., 1978; Sarret et al., 2002), which are present in high concentrations in hyperaccumulators and favored in the acidic environment of the vacuole (Haydon et al., 2007). Glutathione (GSH), a major cellular antioxidant that can form complexes with several metals is the precursor of phytochelatins (metal-chelating compound present in all plants which is required for basic metal tolerance) (Verbruggen et al., 2009). Increased production of glutathione in *T. goesingense* and other Thlaspi Ni hyperaccumulators is believed to provide protection against oxidative damage due to high Ni concentrations (Freeman et al., 2004) when compared to closely related non-accumulator. Only few aspects of the metal detoxification by ligands have been discovered, a complete picture of the different chelators involved in the hyperaccumulation strategies are yet to be established.

1.4 Two model plants studied

1.4.1 *Noccaea caerulescens* (Hyperaccumulator)

Noccaea caerulescens (J. & C. Presl) F. K. Mey., formerly named *Thlaspi caerulescens* is a Zn, Cd and Ni hyperaccumulating species that has been at the forefront of research concerning hyperaccumulation and has been recognized as a model species to study hyperaccumulation (Assunção, Schat, et al., 2003; Krämer, 2010; Milner et al., 2008; Peer et al., 2003). This plant is also Pb-hypertolerant but not necessarily Pb-hyperaccumulating (Mohtadi et al., 2012). *N. caerulescens* is a member of the Brassicaceae family, self-compatible, biannual, genome size of approximately 0.7 pg, almost twice than that of *Arabidopsis thaliana* Col-0 (0.34 pg) and shares around 88% sequence identity in DNA coding regions with non-hyperaccumulator

model plant *Arabidopsis thaliana* (Peer et al., 2006; Peer et al., 2003). *N. caerulescens* is an exceptional species, as it is known to hyperaccumulate Zn (30,000µg/g DW), Ni (4000µg/g DW) and Cd (2700µg/g DW) in shoots, which are two orders of magnitude higher than other, non-accumulating, species generally accumulate (Brown et al., 1995; Lombi et al., 2000; McGrath et al., 1993; Reeves et al., 1983). There exists substantial natural variation regarding metal specificity and metal tolerance among different ecotypes. The most studied ecotype are: Prayon (Belgium) and Ganges (France), both of which hyperaccumulate Zn and Cd in different degrees; Monte Prinzera (Italy) which accumulates Zn and Ni; La Calamine (Belgium), a Zn/Cd hypertolerant population with low accumulation rates (Assunção, Bookum, et al., 2003; Assunção, Schat, et al., 2003; Verbruggen et al., 2009). *N. caerulescens* has higher uptake rate of Cd due to specific rooting strategy i.e. the Cd-specific transport channels exist in the root membrane within this species (Schwartz et al., 2003). Next to *N. caerulescens*, *Arabidopsis halleri* is developed as model metal hyperaccumulator (Claire-Lise et al., 2012). This species is also hypertolerant to Zn and Cd, and a strong Zn hyperaccumulator, but less of a Cd hyperaccumulator and not known to be adapted to Ni (Lin et al., 2014).

1.4.2 *Arabidopsis thaliana* (Non-hyperaccumulator)

A popular model organism in molecular biology whose genome has been fully sequenced, *A. thaliana*, is a member of Brassicaceae family, that has five chromosome and a genome size of approximately 135 megabase pairs (Mbp) (The Arabidopsis Information Resource (TAIR)). It is a metal non-hyperaccumulator plant but has genes involved in metal homeostasis. It has been revealed that most key steps in hyperaccumulation do not rely on novel genes, but depend on genes common to hyperaccumulators and non-hyperaccumulators, that are differently expressed and regulated in the two kinds of plants (Verbruggen et al., 2009). In *Arabidopsis thaliana*, 45 Heavy metal-associated isoprenylated plant proteins (HIPPs) and 22 Heavy metal associated plant proteins (HPPs) have been identified (Barth et al., 2009; Tehseen et al., 2010).

1.5 HIPP (Heavy metal-associated isoprenylated plant proteins)

Metallochaperones are generally soluble intracellular proteins which are involved in metal binding to prevent deleterious reactions with other cellular elements and transport of metallic ions safely through the cell (Abreu-Neto et al., 2013). In organisms like algae, fungi and animals they are present in small amount (Abreu-Neto et al., 2013). But in plants this metallochaperone like protein has been diversified to form large family comprising of two

group of proteins: heavy metal-associated plant proteins (HPPs) and heavy metal-associated isoprenylated plant proteins (HIPPs) (Barth et al., 2009; Tehseen et al., 2010).

HIPPs are characterized by the presence of one or two heavy metal associated domains (HMA, pfam00403.6) and an isoprenylation motif and in most HIPP there is presence of a flexible glycine-rich region between these elements (Barth et al., 2009; Gao et al., 2009). The HMA exhibits a core amino acid sequence motif, M/L/IxCxxC, with two conserved cysteine residues that are involved in heavy metal binding (Dykema et al., 1999; Hung et al., 1998). Some HMA domain-containing proteins from animals, yeast, bacteria and plant species specifically bind copper and are involved in copper homeostasis (Hung et al., 1998; Thomas et al., 1993). In addition to Cu, the HMA domain also showed binding activity with other heavy metals, such as nickel or zinc (Dykema et al., 1999; Suzuki et al., 2002). Analysis of several HMA-containing proteins from a variety of different organisms has demonstrated that they play an essential role in heavy metal transport and homeostasis (Chu et al., 2005; Suzuki et al., 2002).

Post translation modification can dramatically alter the function and physical properties of the proteins (Abreu-Neto et al., 2013). Isoprenylation, also known as farnesylation, is a post-translational protein modification, occurs in the cytosol, which involves the addition of 15 -carbon farnesyl or 20 carbon-geranylgeraniol to the thiol group of the cysteine residue present in a protein that terminates with C-terminal CaaX motif (where 'C' is cysteine, 'a' is an aliphatic amino acid, and 'X' is any amino acid) (Crowell, 2000; Tehseen et al., 2010). There are three enzymes that carry out prenylation in the cell, farnesyl transferase, Caax protease and geranylgeranyl transferase I (Casey et al., 1996). Then the prenylation of plant proteins as in animal and yeast cells is followed by a proteolytic cleavage of the aaX residues, and subsequent methylation of the a-carboxyl group (Clarke, 1992). The terminal X has been determined to confer isoprenylation specificity (Clarke, 1992; Kinsella et al., 1991). Polypeptides containing a carboxyl-terminal serine, alanine, methionine, or glutamine residue are farnesylated, whereas those containing a leucine residue are geranylgeranylated. Proteins containing a carboxyl-terminal CC or CXC motif are also geranylgeranylated (Farnsworth et al., 1991; Khosravi-Far et al., 1991; Kinsella et al., 1992). The exact role of isoprenylation is still uncertain but it seems to affect the membrane localization and the protein-protein interactions (Barbu, 1991). However, the role of isoprenylation in metal homeostasis is yet to be identified.

Evolutionary history of HIPP gene family is still not clear but HIPPs are found to act on three different ways: (a) in heavy metal homeostasis and detoxification mechanisms (especially cadmium tolerance) (Chandran et al., 2008; Gao et al., 2009; Suzuki et al., 2002; Tehseen et

al., 2010), and (b) as regulatory elements in the transcriptional response to cold and drought (Barth et al., 2009; Barth et al., 2004) (c) plant pathogen interaction (Abreu-Neto et al., 2013). A relevant characteristic of the HIPPs is their size, which ranges from 113 to 584 amino acids (Abreu-Neto et al., 2013).

In this report, we focused mainly on study of three HIPP genes, *HIPP25*, *HIPP26* and *HIPP27* and find its role in metal homeostasis.

1.5.1 *HIPP25, HIPP26, HIPP27*

Only a few HIPPs have been studied so far, and the more detailed studies focused on the model plant *Arabidopsis*. In *Arabidopsis thaliana*, whose genome is fully sequenced, sequence comparisons divided the proteins into seven major clusters (I–VII) by NJ (Neighbour-joining) analysis, where cluster IV is notable for the presence of a conserved Asp residue before the CysXXCys, metal binding motif, analogous to the Zn binding motif in E. coli ZntA (Tehseen et al., 2010). 45 Heavy metal-associated isoprenylated plant proteins (HIPPs) and 22 Heavy metal associated plant proteins (HPPs) have been identified, including AtATX1, AtCCH and AtCCS as HPPs in *A. thaliana* which has been represented in *Figure 1.3*.

Based on the information from *A. thaliana*, the role of HIPP genes in *N. caerulescens* is yet to be established. Higher expression of *HIPP25* gene involved in metal homeostasis in *N. caerulescens* than in *A. thaliana* is known from published cDNA microarray data (van de Mortel et al., 2006).

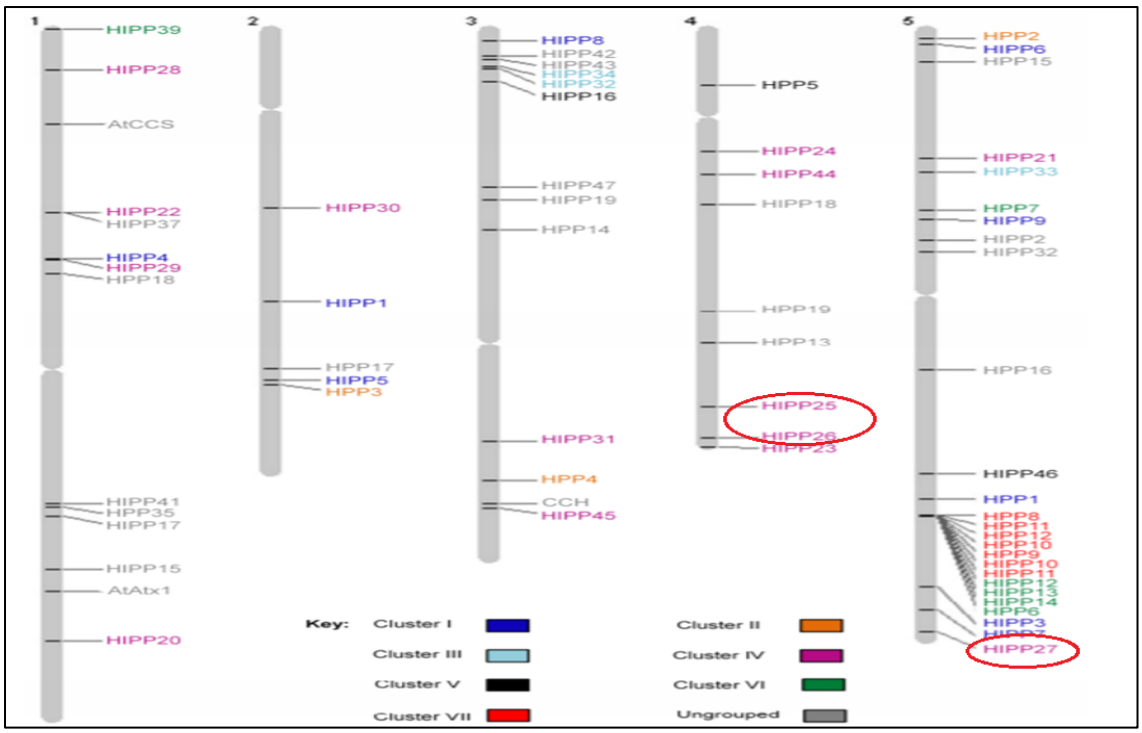


Figure 1.3 The relative locations of the Arabidopsis HIPP and HPP genes mapped on to the five *Arabidopsis* chromosomes using the Chromosome map tool at TAIR (Tehseen et al., 2010). *HIPP25, HIPP26, HIPP27* gene grouped on cluster IV is encircled.

1.5.1.1 Background

HIPP25, HIPP26 and HIPP27 are heavy metal binding proteins like other HIPP proteins as they have heavy metal binding domain. From the information obtained from "The Arabidopsis Information Resource (TAIR)", *HIPP25*, *HIPP26* and *HIPP27* gene is found on chromosome 4,4 and 5 respectively in cluster IV along with other HIPP genes in *A. thaliana*. The size of Coding DNA sequence (CDS), genomic DNA of these genes are mentioned in *Table 1.1*. As these genes are protein coding type, they produce proteins with the size and molecular weight as mentioned in the *Table 1.1*.

Table 1.1 General Information regarding *HIPP25*, *HIPP26* and *HIPP27* genes. Alternative protein names are mentioned in the brackets. Abbreviations: aa: amino acid, bp: base pair, CDS: Coding DNA Sequence

Organism		**Gene**				**Protein**		
Name	**Chromosome**	**Id**	**name**	**CDS**	**Genomic DNA**	**name**	**length**	**Mol. wt. (Dalton)**
Arabidopsis thaliana	4	AT4G35060	*HIPP25*	462 bp	984 bp	Heavy metal-associated isoprenylated plant protein 25 (Farnesylated protein 5)	153aa	17162.3
Arabidopsis thaliana	4	AT4G38580	*HIPP26*	462 bp	1459 bp	Heavy metal-associated isoprenylated plant protein 26 (Farnesylated protein 6)	153 aa	17023.5
Arabidopsis thaliana	5	AT5G66110	*HIPP27*	444 bp	711 bp	Heavy metal-associated isoprenylated plant protein 27 (Farnesylated protein 7)	147 aa	16651.3

1.5.1.2 Subcellular localization of proteins

Subcellular localizations determine the environments in which proteins operate. It influences protein function by controlling access to and availability of all types of molecular interaction partners. Thus, knowledge regarding the localization of a protein inside the cell is very important for elucidation and characterization of hypothetical and newly discovered proteins (Scott et al., 2005).

Two studies investigated the sub-cellular localization of HIPP26. From one study, it was confirmed that HIPP26 is localized in the nucleus consistent with the localization of its interacting partner, the transcription factor, ATHB29, which is known to play a role in dehydration stress (Barth et al., 2009) . From the same study, the functional relationship between HIPP26 and ATHB29 is also indicated by experiments with mutants of HIPP26, where the mutants showed altered expression levels of such genes which are known to be regulated by ATHB29. Another study reported HIPP26 localized at the plasma membrane consistent with the localization of its interacting partner, ACBP2 Arabidopsis thaliana acyl-CoA binding protein 2 (Gao et al., 2009).

Regarding HIPP25 and HIPP27, no literature has been published for its localization but based on the nature of the protein and putative function, its possible localization as found in the database is mentioned in *Table 1.2*.

Table 1.2 Subcellular location of HIPP25, HIPP26 and HIPP27 proteins (source: TAIR)

Gene	**Subcellular location**
HIPP25	cytoplasm, membrane, nucleus, plasmodesmata
HIPP26	Cytoplasm, nuclear membrane, nucleus, plasma membrane, plasmodesmata
HIPP27	cytoplasm, plasma membrane

1.5.1.3 Expression of HIPP25, HIPP26, HIPP27 proteins

Promoter (β-glucouronidase) - GUS reporter expression is an important technique to analyze the activity of promoter in terms of the expression of a gene which is under the promoter control. HIPPS in transgenic *A. thaliana (Col-0)* has been studied in more detail to visualize the cellular pattern which indicates the variable expression of HIPPs in *A. thaliana*.

HIPP25: In roots of *A. thaliana*, HIPP20 and HIPP25 show strong expression in the root vasculature while HIPP22 and HIPP26 are only expressed in lateral root tips (Tehseen et al., 2010). HIPP25p-GUS expression was high in older roots, the shoot apical meristem region, trichomes and flower buds but absent in lateral roots (Tehseen et al., 2010).

HIPP26: Previously published expression analysis using HIPP26p-GUS indicated expression in vascular tissues of the whole plant (Barth et al., 2009) . However, in another study HIPP26p-GUS expression was only detected in lateral roots, shoot apical meristem, petals of unopened flowers and weak expression in leaf vasculature (Tehseen et al., 2010).

HIPP27: No expression was detected in HIPP27p-GUS transgenic plants in study done by Tehseen et al., (2010) .

1.5.1.4 *HIPP* response to heavy metal stress

In 1999, HIPP proteins were described the first time for their capacity to bind metals like Cu^{2}+, Ni^{2+} and Zn^{2+} by Dykema et al., (1999). Later it was found that HIPPs can also bind to Cd2+, Hg2+, Pb2+ but not to co2+, Mn2+, Ca2+ (Gao et al., 2009; Suzuki et al., 2002). By using the Genevestigator tool based on the microarray data, At*HIPP05*, At*HIPP13*, At*HIPP14*, At*HIPP39*, At*HIPP43*, Os*HIPP14* and Os*HIPP44* in rice (*Oryza sativa*) were found to be up-regulated, and At*HIPP32*, Os*HIPP18*, Os*HIPP23* and Os*HIPP38* were found to be down-

regulated in the roots of Cd-treated plants indicating that the genes could be involved in Cd homeostasis (Abreu-Neto et al., 2013).

Apart from these HIPPs, *HIPP25, HIPP26* and *HIPP27* response to heavy metal stress has been discussed below:

***HIPP26* and *HIPP27* binds cadmium and mediates Cd tolerance**

The transcription of many HIPPs is altered in response to heavy metal stresses, which indicates that HIPPs may be involved in homeostasis of these elements (Abreu-Neto et al., 2013). In a study, it was found that expressing AtHIPP20, AtHIPP22, AtHIPP26 and AtHIPP27 proteins in yeast conferred Cd-resistant to a Cd-sensitive strain. It was suggested that the resistance conferred to yeast was more due to the binding of Cd in the cytosol rather than influencing the transport of Cd into the vacuole or out of the cell (Tehseen et al., 2010). In addition, from another study done by Gao et al. (2009), showed that *HIPP26* can bind Cd2+, Cu2+ and Pb2+ and overexpressing *AtHIPP26* in *A. thaliana* enhanced tolerance to Cd2+, which indicates that members of this protein family are able to bind Cd2+ invivo and can have a role in Cd detoxification in planta.

***HIPP25* can have a role in metal homeostasis and tolerance:**

Apart from the importance of At*HIPP26* and At*HIPP27*, another gene from the same cluster IV of HIPPs gene family, At*HIPP25*, is still functionally unknown. The possible involvement of At*HIPP25* in metal tolerance is indicated by a higher gene expression of At*HIPP25* in *N. caerulescens* than in *A. thaliana* roots from published cDNA microarray data under control and Zn (100µM for Nc, 25µM and 2µM for At) treated condition for 7 days (van de Mortel et al., 2006).

1.5.1.5 Other functions of *HIPP25, HIPP26 and HIPP27*

In addition to the simple function of chelating metals in the cytosol, HIPPs may act in a more elaborate mechanism which are described below.

***HIPP25* and *HIPP26* is induced in response to cold treatment**

In a study done by Barth et al. (2009), the cold treatment to *A. thaliana* resulted in the induction of four gene, *HIPP23, HIPP24, HIPP25* and *HIPP26. HIPP23* and *HIPP26* within the first 6 h of the cold treatment but expression level decreased in the later stages of the stress treatment again indicating a fast and transient expression pattern during cold stress. *HIPP25* showed induction only in the early phase. *HIPP26* fast and transient induction in early phases of the stress treatment and the presence of a nuclear localization signal (NLS) in addition to the two

conserved domains (HMA and isoprenylation motif), hint at a possible regulatory function of this protein (Barth et al., 2009) .

***HIPP26* interacts with ACBP2 and mediates Cd tolerance:**

Gao and colleagues showed that *HIPP26* in *A. thaliana* (At*HIPP26*) upon interaction with plasma membrane acyl-CoA-binding protein 2 (ACBP2) via ankyrin repeats (which mediate protein-protein interaction) is involved cadmium Cd2+ tolerance. From the reverse transcriptase polymerase chain reaction and northern blot analyses, it was revealed that *HIPP2P6* mRNA was induced by cadmium Cd2+ (1 mM) in *A. thaliana* roots and less extent in shoots. Further, northern blot analysis indicated that *HIPP26* expression, was not induced by Pb2+ and Cu2+, but it was induced by Cd2+ and Zn2+ in both roots and shoots (Gao et al., 2009).

***HIPP26* and *HIPP27* interacts with *HB29* (drought stress related gene):**

A publication of Tran et al. (2007), proves a role of zinc-finger homeodomain box transcription factor At*HB29* in dehydration stress response of plants and found to be induced by drought, high salinity and abscisic acid. In the study done by Barth et al. (2009), confirmed that At*HIPP26* strongly interacts with At*HB29*, with an intact heavy metal associated domain including the two central cysteines. *HIPP26* expression was not induced by ABA, while drought stress showed only a slight effect but, salt stress clearly resulted in increased transcript levels of *HIPP26*. Copper treatment and leaf senescence rather inhibited expression of *HIPP26*. Further, loss-of-function of *HIPP26* in mutant line At*HIPP26* specifically inhibited expression of stress responsive genes upregulated by At*HB29*.

Other proteins from the same cluster (At*HIPP20, 21, 22, 23, 24, 27* and *30*) were also able to interact with this same transcription factor (At*HB29*), indicating that additional HIPPs could play a role in transcriptional regulation (Barth et al., 2009) .

***HIPP27* interacts with *UBP16* indicating a role in cadmium tolerance:**

Deubiquitinating enzymes (DUBs) are proteases that reverse the modification of proteins by ubiquitin which are divided into two groups, ubiquitin C-terminal hydrolases (UCHs) and ubiquitin-specific proteases (UBPs) based on their amino acid sequence and substrate specificity (Wilkinson, 1997; Yan et al., 2000).

It was found that *ubp16* mutant seedlings is more sensitive to $CdCl_2$ (30 µM) where the shoot growth of the *ubp16* mutant was more severely impaired and the anthocyanin level was also

significant higher than that of wild-type Col-0 (Yan et al., 2000). It was also reported that *UBP16* interacts with *HIPP27 (HIPP27* has been reported to play an important role in cadmium detoxification (Tehseen et al., 2010)) and this implies that HIPP27 and *UBP16* interaction modulates cadmium tolerance, although other evidences are needed (Zhao et al., 2013)

Table 1.3 *HIPP25, HIPP26, HIPP27* IN BRIEF (Information obtained from databases like Uniprot (Consortium) and TAIR

Protein	Function	Interaction and Induction	Expression
HIPP25	➢ Heavy-metal-binding protein. Binds cadmium. May be involved in cadmium transport and play a role in cadmium detoxification. (source - UNIPROT)	Not known	➢ Expressed in roots, shoot apical meristem, trichomes and flower buds (Tehseen et al., 2010)
HIPP26	➢ Heavy-metal-binding protein. Binds lead, cadmium and copper. (source: UNIPROT) ➢ May be involved in heavy-metal transport (Gao et al., 2009) ➢ May be involved in cadmium transport and play a role in cadmium detoxification (Tehseen et al., 2010)	➢ Up-regulated by cadmium and zinc, but not by lead or copper (Gao et al., 2009) ➢ Up-regulated by cold, drought and salt stress. Not induced by abscisic acid or by leaf senescence (Barth et al., 2009; Gao et al., 2009) ➢ Interacts with HB29 (Barth et al., 2009) and ACBP2 via ankyrin repeats (Gao et al., 2009)	➢ Expressed in roots, stems and flowers. Lower expression in siliques and leaves. Expressed in the vascular tissues. Detected in lateral roots, shoot apical meristem, petals of unopened flowers and weak expression in leaf vasculature. (Barth et al., 2009; Gao et al., 2009; Tehseen et al., 2010)
HIPP27	➢ Heavy-metal- binding protein. Binds cadmium. May be involved in cadmium transport and play a role in cadmium detoxification (source: UNIPROT)	➢ Interacts with UBP16 (Zhao et al., 2013) ➢ Interacts with HB29 (Barth et al., 2009)	➢ Expressed in leaves at four nodes, other than the cotyledon node (source: TAIR)

1.6 Glucosinolates

Glucosinolates are nitrogen and sulfur containing plant secondary metabolites typically found in members of the Brassicaceae and a few other plant families of the order Capparales (Radojčić Redovniković et al., 2008). Plants that produce glucosinolates commonly accumulate them in all vegetative and reproductive parts throughout development (Louda et al., 1991). The primary biological function of glucosinolates is unknown but a role in plant defense against bacterial and fungal pathogens and insect predators has been suggested (Fenwick et al., 1983).

The chemical structure of GSL contain a β-D-glucopyranose residue linked via a z-sulfur atom to a (Z)-(or Cis)-N hydroximino sulfate ester, and each GSL is distinguished from by a variable R group as shown in *Figure 1.4*. This R-group is synthesized from different amino-acids, which further sub-divide GLS into different structural groups referred as aliphatic, indole and aromatic, or benzylic (Agerbirk et al., 2012; Fahey et al., 2001). Aliphatic glucosinolates are derived from methionine, isoleucine, leucine or valine, aromatic glucosinolates are derived from phenylalanine or tyrosine, and indole glucosinolates derived from tryptophan (Radojčić Redovniković et al., 2008). The biosynthesis of glucosinolates comprises three phases: (i) amino acid chain elongation, in which additional methylene groups are inserted into the side chain, (ii) conversion of the amino acid moiety to the glucosinolate core structure, (iii) and subsequent side chain modifications (Radojčić Redovniković et al., 2008).

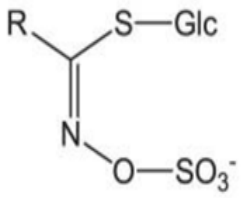


Figure 1.4 A chemical structure of Glucosinolates, β thioglucoside N hydroxy sulfate with a sulfur linked β-D-glucopyranose residue and R represents the variable side chain (Ishida et al., 2014).

Apart from being naturally present in the plant, glucosinolates are also induced as a result of tissue damage (Asad et al., 2013). Abiotic stresses, such as salinity, drought, extreme temperatures, light and nutrient deprivation, also alter the glucosinolate profiles of plants (Del Carmen Martínez-Ballesta et al., 2013). 132 different glucosinolates have been identified till date (Agerbirk et al., 2012) and most of them are chemically and thermally stable so hydrolysis is mainly enzymatically driven (Polat, 2010). Intact glucosinolates exhibit very little defense

role, but the hydrolysis products provide effective defense against herbivores and pathogens (Asad et al., 2013). Upon cell disruption, Myrosinase (E.C. 3.2.3.1), a β-thioglucosidase, enzyme present in most of the species along with the glucosinolates, catalyzes the hydrolysis of GSL to biologically active products, including isothiocyanates, thiocyanates and nitriles, depending on the reaction conditions and presence of associated proteins (Fenwick et al., 1983; Rask et al., 2000) (*Figure 1.5*). The hydrolysis products have different biological activities as defense compounds and attractants (Halkier et al., 2006). Mainly isothiocyanate exhibits biocidal activity including insecticidal, nematicidal and fungicidal effects (Aires et al., 2009; Larkin et al., 2007; Manici et al., 1997). GSL are of special interest in medical research after the discovery of anticarcinogenic properties in Broccoli (Zhang et al., 1992). They are also responsible for the sharp and bitter taste in the Brassica vegetables (Oerlemans et al., 2006).

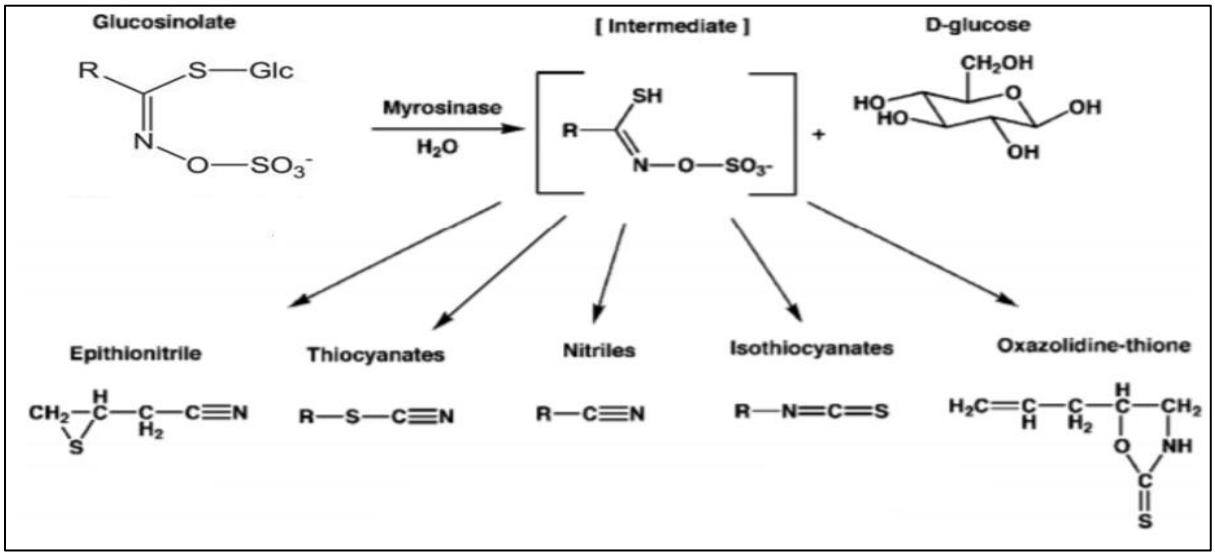


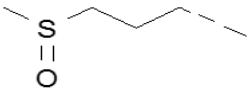
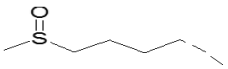
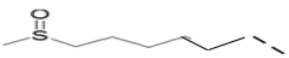
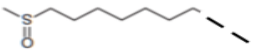
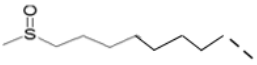
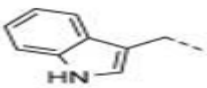
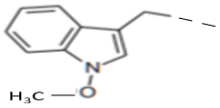
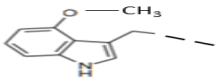
Figure 1.5 Various hydrolysis products after enzymatic hydrolysis of glucosinolate by myrosinase upon cell disruption. Modifed from Rask et al. (2000).

1.6.1 GSL in non-hyperaccumulator

In the model plant *Arabidopsis thaliana*, a member of the Brassicaceae, around 30 different members of glucosinolates have been described from the Columbia (Col-0) ecotypes (Haughn et al., 1991; Kliebenstein et al., 2001; Reichelt et al., 2002). The glucosinolate content, both the composition and concentration in various organs of *Arabidopsis thaliana* (Col-0) was studied at different stages by Brown et al. (2003), where the dormant and germinating seeds had the highest concentrations of GSL (approximately 2.5–3.3% by dry weight), Inflorescences and siliques had the next highest levels (0.6–1.2%) followed by roots, stems and cauline leaves, and rosette leaves (0.3–1.0%). Regarding the composition, aliphatic glucosinolates predominated in photosynthetic tissues, 2-Phenylethyl glucosinolate and compounds with

benzyloxy substituents glucosinolates were most abundant in the seed and indole glucosinolates were most prominent in the root and mature rosette leaves, nearly to the half of the total composition (Brown et al., 2003). An overview of some common glucosinolates found in *A. thaliana* is mentioned in *Table 1.4.*

Table 1.4 some common GSL present in *Arabidopsis thaliana* and their classification. The glucosinolates are listed with increasing R group chain length. The abbreviations are used throughout the paper (Kissen et al., 2009; Radojčić Redovniković et al., 2008).

GSL	Type of side chain	Semi systematic name	Trivial name	R group	Abbreviation
Aliphatic	Alkenyl Side chain	2-Propenyl-	Sinigrin		2P
		3-Butenyl-	Gluconapin		3B
		4-Pentenyl-	Glucobrassicanapin		4P
	Methyl thioalkyl Side chain	4-Methylthiobutyl-	Glucoerucin		4MTH
		7-Methylthioheptyl	Glucoarabishirsutain		7MTH
	Methyl Sulfinyl alkyl Side chain	3-Methylsulfinylpropyl-	Glucoiberin		3MSP
		4-Methylsulfinylbutyl-	Glucorafanin		4MSB
		6-Methylsulfinylhexyl-	Glucoalissin		6MSH
		7-Methylsulfinylheptyl-	Glucoibarin		7MSH
		8-Methylsulfinyloctyl	Glucohirsutin		8MSO
Indolic	Heterocyclic side chain	Indole 3yl-methyl-	Glucobrassicin		I3M
		1-methoxyindol-3-ylmethyl	Neoglucobrassicin		1MeO-I3M
		4-Metoxyindol-3-ylmethyl-	4-Methoxyglucobrassicin		4MeO-I3M

1.6.1.1 GSL in *A. thaliana* in response to metal treatment

In response to heavy metals like cadmium (50µM and 100µM), the total concentration of GSL was significantly decreased both in the leaves and roots of *A. thaliana* (which was mainly due to the decrease of indolyl-glucosinolates) (Sun et al., 2009). This has been hypothesized as a mechanism to make more sulfur available for Phytochelatin synthesis which involves the chelation of heavy metals (Herbette et al., 2006).

The role of zinc effect in glucosinolates in *A. thaliana* will be identified in our study.

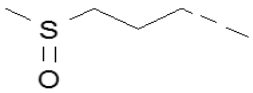
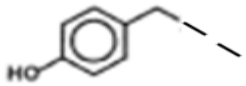
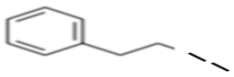
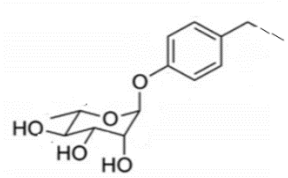
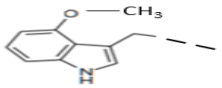
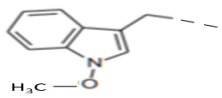
1.6.2 GSL in hyperaccumulators

Production of Glucosinolates is quite common feature with the Brassicaceae family and most of the hyperaccumlators like *Alyssum* and *Thlaspi* belongs to this family (Tolra et al., 2001). High glucosinolate levels in Brassicaceae species that hyperaccumulate Ni (*Alyssum bertolonii*) (Sasse, 1976) or Zn (*Thlaspi alpestre)* (Mathys, 1977) have been described. On one hand, it was found that Zn resistant plants contained twice the amount of benzylglucosinolate as the sensitive ones and it has been suggested that glucosinolates might play a role in Zn tolerance mechanisms (Mathys, 1977) or these compounds may serve as a source for Ni-binding substances (Sasse, 1976). On the other hand, the "Trade off " hypothesis in hyperaccumulators seems to be the most interesting which suggests that heavy metals provides an ecological advantage to hyperaccumulators by protecting them against herbivores and pathogen while lowering the glucosinolate content and this metal based defense may have been evolved to reduce the level of energy demanding organic defense like GSL (Rascio et al., 2011; Tolra et al., 2001). Few data in support of trade off hypothesis are available, for example; lower level of defensive GSL were found in Ni hyperaccumulator *Streptanthus polygaloides* (Davis et al., 2000) and in Zn hyperaccumulator *T. caerulescens,* when compared to their respective non-hyperaccumulators. However, the relationship between GSLs and metal accumulation, and these relationships regarding defense against herbivores in hyperaccumulator species, are not clear and they are often based on the total GSL content while the individual GSL content may have different activity in biological interactions. Therefore, further investigation in the metal uptake and glucosinolate pattern in the hyperaccumulators is required.

The most abundant glucosinolate in *N. caerulescens* reported by Tolra et.al (2001) within both roots and shoots was p-hydroxybenzyl glucosinolate (aromatic glucosinolate). The concentrations of 3-butenyl-(aliphatic GSL) and 2-phenylethyl-glucosinolates (aromatic GSL) were about one order of magnitude lower and the lowest concentrations were found for indolyl

glucosinolates. An overview of some common GSL found in *Noccaea caerulescens* are mentioned in *Table 1.5.*

Table 1.5 Some common GSL present in *Noccaea caerulescens* and their classification (De Graaf et al., 2015; Fahey et al., 2001; Tolra et al., 2001).The glucosinolates are listed with increasing R group chain length. The abbreviations are used throughout the paper.

GSL	Side chain	Semi systematic name	Trivial name	R group	Abbre viation
Aliphatic	Alkenyl Side chain	3 butenyl	Gluconapin		3-B
	Methyl Sulfinyl alkyl Side chain	3-Methylsulfinylpropyl	Glucoiberin		3MSP
Aromatic		P-hydroxybenzyl	Sinalbin		P-OH-bz
		2-Phenylethyl	Gluconasturtin		2-Pe
		4-α-rhamnosyloxy benzyl	Glucomoringin		RhaOxy-bz
Indolic		4-methoxy-3indolylmethyl	4-Methoxyglucobrassicin		4-MeO-I3M
		1-methoxy-3indolylmethyl	Neoglucobrassicin		1-MeO-I3M

1.6.2.1 GSL in *Noccaea caerulescens* in response to metal treatment

From one study, it was demonstrated that GSL production in *Noccaea caerulescens* increased in shoot tissue with Zn treatments (Foroughi et al., 2014). However, another research group Tolra et al. (2001), identified that that total GSL content in *Thalpsi caerulescens* exposed to Zn, decreased in shoots with increasing Zn concentrations, whereas in roots it increased with

increasing Zn concentrations. The root data supports the “trade off” hypothesis whereas, the shoot data are not in line with “trade off” hypothesis. It has been expected that roots could have increased defense against pathogens (Tolra et al., 2001). With other metals apart from zinc, total GSL content in *Noccaea* leaves increased with the concentration of Ni but reduced with Cd in the soil supporting the “trade off” hypothesis partially (Asad et al., 2013).

1.7 Aim of the study

Comparative transcriptional profiling reveals that *HIPP25* expression is higher in the hyperaccumulator *N. caerulescens* than in the non-hyperaccumulator, *A. thaliana* (van de Mortel et al., 2006; van de Mortel et al., 2008). *HIPP25* belongs to a family of metallochaperone-like proteins containing a heavy metal-associated domain. Some members of this family have already been implicated in heavy metal tolerance. In this project, we aim to find out the gene expression level of *HIPP25* along with its phylogenetically closely related genes *HIPP26* and *HIPP27* from both plants under different developmental stages (root, leaves, flower and siliques), and under different metal treatments (Zn, Ni, Cu and Cd) by RT-qPCR (real-time reverse transcription-PCR). Furthermore, the expression of HIPP25 proteins will also be detected by western blot. This will give an indication of the possibility of these genes in metal mechanisms like accumulation and tolerance. Apart from this, the effect of metal (Zn) treatment to the glucosinolates content present in both plants will be identified.

We also aim to clone a full-length coding region sequence (CDS) of *HIPP25* from both *A. thaliana* (At*HIPP25*) and *N. caerulescens* (Nc*HIPP25*) to overexpress At*HIPP25* and Nc*HIPP25* in *A. thaliana* and compare their metal tolerance and accumulation.

1.8 Hypothesis

In concordance with the information obtained from the literatures on the expression of *HIPP* genes and glucosinolates content in hyperaccumulator and non-hyperaccumulators, under different metal treated conditions, the following hypotheses were generated:

1. At*HIPP25*, which is more highly expressed in the hyperaccumulator, *N. caerulescens* roots than non-hyperaccumulator *A. thaliana* under control as well as zinc treated condition, was identified from published cDNA microarray data (van de Mortel et al., 2006) indicating the possible role in metal homeostasis. In our study, effect of *HIPP25* gene/protein is expected to express higher in *N. caerulescens* than *A. thaliana*.
2. It was suggested that At*HIPP26* and At*HIPP27* genes could have a role in Cd detoxification (Gao et al., 2009) and Cd tolerance (Gao et al., 2009; Zhao et al., 2013). In our study, the gene expression of *HIPP26* and *HIPP27* is expected to increase in response to Cd treatment in roots of *A. thaliana* indicating the possibility of its role in metal tolerance.
3. Based on the information from the databases and its phylogenetically closely related genes (*HIPP26* and *HIPP27), HIPP25* gene/ protein expression is expected to response to Cd and other heavy metals as these genes contain heavy metal binding domain.
4. Based on the information obtained in other Brassica species (Coolong et al., 2004) and the preliminary results from the experiment performed in *A. thaliana* at NTNU, Glucosinolates content in *A. thaliana* is expected to increase after zinc treatment.
5. Glucosinolates content in *N. caerulescens* is expected to decrease after zinc treatment supporting the "tradeoff" hypothesis in hyperaccumulators indicated by previous researches (Tolra et al., 2001).

2 MATERIALS AND METHODS

2.1 Seed sterilization

For all the experiments performed, *Arabidopsis thaliana* Columbia Col-0 and *Noccaea caerulescens* La calamine accessions were used. *Arabidopsis thaliana* seeds and *Noccaea caerulescens* seeds were surface sterilized on gas chamber with chlorine (100ml) and 37% HCl (3ml) for 3 hours and imbibed with 0.1% agarose solution.

2.2 Plant material and growth conditions

A. thaliana and *N. caerulescens* seeds were sterilized and imbibed with 0.1% agarose as mentioned in *section 2.1*. Then, imbibed seeds were sown on the agarose filled Eppendorf tubes dipped in the box containing (0.5 lt) modified Hoagland's medium on dark room at 4°C for 3 days. The plants were then transferred to growth chamber (16:8 hours light: dark photoperiod, 22°C, light intensity 120 µmol/m^2/s) and different heavy metal (Ni, Cu, Zn, Cd) treatments was given to plants on 5l box (one box per treatment) at 22nd day. The plants were grown there until harvested (day 36). Medium was replaced once a week. For the glucosinolate analysis, the same Arabidopsis and Noccaea plant materials treated with zinc were used.

2.3 Compare target genes and protein expression in different tissues and under different metal treatments

Organism's gene expression level can be estimated by the number of copies of gene transcripts (mRNA), which is determined by the rates of its synthesis and degradation. To have an overview of possible gene function of *HIPP25* along with its phylogenetically closely related genes *HIPP26* and *HIPP27,* gene expression in *A. thaliana* and *N. caerulescens* was studied by RT-qPCR and the expression of AtHIPP25 and NcHIPP25 were also detected at protein level by western blot.

2.3.1 Preparation of hydroponic medium and experimental set up

0.5X Hoagland's medium modified from Schat et al. (1996) was prepared by mixing stock solutions of macro- and micronutrients (*Appendix A.2)* as mentioned in *Appendix A.3*. The solution was buffered upon the addition of 2mM MES (0.39 g/L) and pH was adjusted to 5.5 with 5M KOH. The lids and bottom from 0.5ml microcentrifuge tubes were cut and inverted into adhesive tape. Each of the cut tubes were filled with 0.55% agarose solution until the dome shaped droplet was seen at the top and left to solidify for 15 mins. The tubes with solid agarose

were then kept at empty pipette tips boxes which could fit the tubes on upright position. The sterilized seeds of *Arabidopsis thaliana* and *Noccaea caerulescens* imbibed with 0.1 % agarose solution (*section 2.1*) were then added dropwise on the 0.5ml microcentrifuge tube containing 0.55% agarose medium keeping one seed per tube and different box was set up for Arabidopsis and Noccaea *plants*. Then the box was filled with the Hoagland's medium, covered with aluminum foil and then placed for 3 days at 4°C in the dark condition for germination. After 3 days, the germinated seedlings were transferred to growth chamber (*Figure 7.9)* with an average light intensity of 120µmol/m^2/sec, 22°C, 16-hour photoperiod, and kept there until harvested. The medium was replaced after a week in these small boxes. All the growth conditions i.e. light and temperature was made similar for both plants but grown on two different places within the growth room due to limited availability of space and time. Since the plant takes 36 days for one round completion, and only one round could be performed in a provided space, two separate places were thus chosen for two plants to speed up the experiment.

2.3.2 Heavy metal treatment to *A. thaliana* and *N. caerulescens*

A. thaliana and *N. caerulescens* were exposed to four heavy metals under short term and long-term conditions as described below.

a. Short term (24 hr)

On day 22 after sowing out Arabidopsis and Noccaea, well grown plants on small boxes were selected and transferred into the 5 L black box containing 0.5X modified Hoagland's medium with 12 plants on each box *(Figure 7.11*). The plants were grown under these conditions until day 35 when they were exposed to Hoagland's medium supplemented with different heavy metals for 24 hours. Each plant was exposed individually to heavy metal solution in a small pot (ca. 125 cm^3) *(Figure 7.10*). The different heavy metal with their concentrations used for short term treatment for both plants were: $NiSO_4$ (100 µM), $CuSO_4$ (25 µM), $CdSO_4$ (30 µM), $ZnSO_4$ (50 µM). The control treatment consisted of 0.5x Hoagland's solution without any heavy metal supplement i.e. Control (0 µM).

b. Long term (14 days)

Similarly, Arabidopsis and Noccaea plants grown for 22 days on small boxes were transferred to 5L black box (12 plants per box) containing 0.5X Hoagland's Medium supplemented with one of several heavy metals (1 box per treatment) and grown for another 14 days before harvesting. Medium was replaced once a week. Due to time and space restrictions, Arabidopsis and Noccaea plants were grown independently and each was grown in two different batches at

different time points. A control, consisting of 0.5x Hoagland's solution without any heavy metal supplement was included in each round. The first batch plants were treated with higher metal concentration than the second one. Since the Ni (100µM) treated plants in the first round was highly affected, the plants had reduced growth and the tissues could not be harvested from them, two lower concentrations of Ni were thus used in the second round for *A. thaliana*. The concentrations of the different heavy metal used in two batches for two plants were:

- *A. thaliana*
  a. First Batch (At1): $CuSO_4$ (25µM), $CdSO_4$ (10µM), $ZnSO_4$ (25µM), Control (0µM)
  b. Second batch (At2): $NiSO_4$ (80 µM), $NiSO_4$ (50 µM), $CuSO_4$ (15µM), $CdSO_4$ (5 µM), $ZnSO_4$ (15µM), Control (0µM)
- *N. caerulescens*
  a. First Batch (Nc1): $NiSO_4$ (80µM), $CuSO_4$ (25µM), $CdSO_4$ (10µM), $ZnSO_4$ (25µM), Control (0µM)
  b. Second Batch (Nc2): $NiSO_4$ (50µM), $CuSO_4$(15µM), $CdSO_4$ (5µM), $ZnSO_4$(15 µM), Control (0µM)

An overview of the major stages of the treatment is given in *Table 2.1*.

Table 2.1 Overview of the activities done at different time point. Abbreviations HS: Hoagland's solution

Treat-ment	Day 1	Day 4	Day 16	Day 22	Day 29	Day 35	Day 36
Short Term	Grown plants at 4°C, dark in 0.5X HS	Transferred plants to growth chamber	Medium Replacement on each small box (0.5L)	Transferred plants to big black boxes containing 0.5X HS (5 L)	Medium replacement on big box (5 L)	Medium supplemented with Heavy Metal treatment for 24 hr	Harvesting
Long Term	Grown plants at 4°C, dark room in 0.5X HS	Transferred plants to growth chamber	Medium Replacement on each small box (0.5L)	Transferred plants to big black boxes containing 0.5X HS supplemented with Heavy metal (5 L)	Medium supplemented with heavy metal replacement on big box (5 L)	-	

2.3.3 Harvesting

a. Short term treated plants

On day 36, four plants exposed to heavy metals for 24 h and four control plants were harvested individually into liquid nitrogen, each plant being considered as a biological replicate. For Arabidopsis, roots, leaves, and flower+siliques (together) were harvested. For Noccaea, only root and leaves were harvested as the plants did not produce flowers at that stage. The harvested tissues were kept at -80°C in the freezer.

b. Long term treated plants

On day 36, plants exposed to each of the 4 heavy metals and control plants were harvested into liquid nitrogen. For this purpose, tissue from 3 plants was pooled to constitute a biological replicate (giving in total four biological replicates). Root, leaves, and flower+siliques (together) were harvested from Arabidopsis, while root and leaves were harvested from Noccaea. The tissue was kept at -80°C in the freezer until further processing.

2.3.4 RNA isolation

Harvested tissues from both plant species were first coarsely crushed using a spatula and weighted (approximately 80-90mg) tissue were then crushed using a TissueLyser (QIAGEN, Hilden, Germany) at 25 Hz for 2 min. Total RNA was isolated from the crushed tissue using Spectrum Plant Total RNA kit (#STRN250-1KT, Sigma-Aldrich) with the instructions provided by the supplier, except that RNasin was not added to the extracted RNA. The protocol followed is described below.

80-90 mg of frozen tissue of each sample was kept into 2 ml tubes and tissuelyser was run with stainless bead (2 X 1 min 25 Hz). In fume hood, 500µl of lysis buffer was added and tissuelyser was run again (2 X 1 min at 25 Hz). The samples were then incubated at 56°C for 1-3 mins, centrifuged for 3 mins and supernatant was transferred to the tube with filtration column, avoiding the pellet. The tube was centrifuged again for 1 min. Filter was removed from the tube and 500µl of binding solution was added to each sample which was then mixed thoroughly by pipetting. 700µl of the mixture was added into the tube with binding column and centrifuged (1 min). The flow through was discarded. (If the sample volume was above 700µl, the aliquots were centrifuged in the same spin column and flow- through was discarded). 300µl Wash buffer1 was added, centrifuged for 1 min and flow-through was removed. 70µl buffer RDD with 10µl DNAse was mixed in a separate tube and 80µl of DNAse+RDD was added directly

to the center of the column membrane and incubated for 15 mins at room temperature. 500µl Wash Buffer1 was added again to the column, centrifuged and flow-through was discarded. 500µl Wash Buffer2 was added and centrifuged for 30 seconds, flow through was discarded and repeated this process once more. Then the column was spin for 1 min to dry and placed in a new 2 ml collection tube. 50µl elution buffer was added directly to the middle of the spin column membrane and left for 1 min and centrifuged for 1 min to elute. The column was removed and the eluted RNA was kept in ice. The concentration of total RNA was measured with a Nanodrop-1000 Spectrophotometer (Thermo Fischer) and kept at -80°C until further use.

2.3.5 RNA agarose gel

A denaturing agarose-formaldehyde gel was prepared as described in *Appendix A.5* and the gel was left to solidify for 30 mins. Meanwhile, FA gel running buffer (*Appendix A.6*) was prepared and was poured on the solidified gel and equilibrated for 30 mins in the electrophoresis tank. 1µg RNA samples was prepared and 5X RNA loading dye(3µl) (*Appendix A.7*) was added to each sample. The samples were incubated at 65°C for 3-5 mins and kept immediately over ice. The samples were then loaded and electrophoresis was run (75v, 1 hr). The pictures were then photographed using the G: BOX (Syn gene).

2.3.6 cDNA synthesis and RT-qPCR

The cDNA synthesis reactions were performed on 1 µg of total RNA with QuantiTect Reverse transcription kit (#205310, Qiagen). The RNA was first mixed with gDNA Wipeout buffer (2µl) and RNase-free water (total volume of 14 µl) and incubated for 2 min at 42°C to remove gDNA. The mastermix for cDNA was prepared containing Quantiscript reverse transcriptase (RT), RT buffer and RT primer mix *(Table 2.2).* 6µl of mastermix was added to each RNA sample and incubated at 42°C, 15 min followed by a 3-minute incubation at 95°C. The cDNA was diluted in the 1:5 ratio with autoclaved MQ H2O prior to the RT-qPCR reactions.

For each target gene (*HIPP25, HIPP26, HIPP27*) a mastermix for RT-qPCR was prepared containing the RT-qPCR Primers (forward and reverse), LightCyler 480 SYBR Green I Master and PCR grade water as shown in *Table 2.2.* 15µl of the mastermix was pipetted to LightCycler 96-well plates and 5µl of diluted cDNA was added to it. RT-qPCR reactions were run on a Roche LightCycler 96 system using the cycling conditions described in *Table 2.3*. In addition to the target genes that were assessed the three reference genes Tip41like (At4g34270), PP2A (At3g25800) and Actin2 (At3g18780) were used and their stability was tested using geNorm

(Vandesompele et al., 2002) in qBase+ version 2.7 (Hellemans et al., 2007). The primers used for each of the target and reference genes are mentioned in *Appendix B.1.* The primers for target genes were designed in such a way that should work both for *A. thaliana* and *N. caerulescens* as they have high sequence similarity (88%). The fluorescence values were imported into LinRegPCR software (Ramakers et al., 2003; Ruijter et al., 2009) to calculate the Cq values for each sample and mean PCR efficiency for each amplicon group. Further analysis was done by exporting these data to qBase+ version 2.7 software. The statistical analysis was performed either by Mann-Whitney test (for pair-wise comparisons) or One-way ANOVA to obtain the p-values and calculate the significance, p value being less than 0.05.

Table 2.2 Mastermix for cDNA and RT-qPCR

Type	**Components**	**Volume/reaction (µl)**
cDNA	Quantiscript RT	1
	RT buffer	4
	RT-primer mix	1
	Total volume per reaction	6
RT-qPCR	PCR grade H_2O (vial 2)	3
	Primer pair mix (5µM each)	2
	2x conc. LightCycler 480 SYBR Green I Master (vial 1)	10
	Total volume per reaction	15

Table 2.3 condition for RT-qPCR

Step	**Temperature (°C)**	**Time (seconds)**	**Cycle**
Pre-Incubation	95	600	1
Amplification	95	10	45
	55	10	
	72	10	
Melting	95	5	1
	65	60	
	97	1	
Cooling	37	30	1

2.3.7 Confirming the identity of RT-qPCR products

To confirm the identity of the amplicons obtained for the *HIPP25*, *HIPP26* and *HIPP27* target genes, some of the samples amplified with these target genes were submitted to sequencing. For that purpose, RT-qPCR products (Ni treated (50µM) and control leaves amplified with

three target genes) were run on 2%agarose gel (80V, 1 h) and then photographed (G: BOX). The amplicon bands were excised from the gel, kept in the separate eppendorf tube for each sample and equal volume of membrane binding solution was added. The tubes were kept on the heater at 60°C for 10 mins until the gel was properly dissolved and the mixture was purified using the Wizard SV gel and PCR clean-up system kit (#A9282, Promega) following the supplier's protocol for each of the sample in the tube as described below:

The mixture was added to the SV minicolumn fitted with the collection tube, incubated for 1 min at RT and centrifuged at 13,000 rpm for 1 min. The liquid passed through the column was discarded from the collection tube. Then, the column was washed by adding 700µl of membrane wash solution and centrifuged for 1 min at 13,000 rpm. The collection tube was emptied as before and repeated the wash with 500 µl of membrane wash solution, centrifuged for 5 mins at 13,000 rpm. The collection tube was made empty again and the column assembly was recentrifuged for 1 min. The SV minicolumn was carefully added to a clean 1.5 ml microcentrifuge tube. Finally, 50 µl of the Nuclease free water was added directly to the center of the column. Then, it was incubated at RT for 1 min and centrifuged for 1 min at 13,000 rpm. The eluted DNA was stored at -20°C.

Sequencing reaction was carried out on 5 µl of the eluted DNA (template) using BigDye Terminator v3.1 Cycle Sequencing Kit (#4337455, Applied Biosystems, Foster City, USA) as shown in *Table 2.4* with each of the RT-qPCR primers, see *Appendix B.1.* The PCR conditions for sequencing are mentioned in *Table 2.5.*

Table 2.4 Components for sequencing reaction

Components	**Volume**
Template	5 µl
H_20(deionized)	10
Sequencing buffer	3 µl
Primer 3.2 pmol	1 µl
Big-Dye V.3.1	1 µl
Total	20 µl

Table 2.5 PCR condition for sequencing

Step	**Temperature**	**Time**	**Cycle**
Denaturation	96°C	5 min	1
Annealing	96 °C	10 secs	25
	50°C	5 secs	
Extension	60° C	4 min	1
	4° C	∞	

2.3.8 Protein extraction and quantification

After RNA isolation for RT-qPCR, the tissues were insufficient for extracting proteins from each replicate. For protein extraction, root and leaves of control plants and those treated plant tissue of the four replicates was pooled. Approximately 200 mg of tissue was crushed using a TissueLyser (QIAGEN, Hilden, Germany) at 25 Hz for 1 min. Then 200µl of protein extraction buffer (50 mm Tris-HCl pH 8, 150 mm NaCl, 1 mm EDTA,) containing complete EDTA-free Protease Inhibitor Cocktail (Roche Diagnostics GmbH, Mannheim, Germany) (*Appendix A.8*) was added to each sample, vortexed until all the samples get well mixed with the extraction buffer and stored on ice. The tubes were centrifuged at 13,000 RPM at 4° C for 10 mins. The supernatant was transferred to the new 1.5 ml Eppendorf tubes.

Protein quantification was performed by Bradford Assay (Bio-Rad Protein Assay Dye Reagent) with Bovine Serum Albumin (BSA) as standard. The absorbance was measured at 595 nm with a Spectrophotometer. Proteins were stored at -20°C until use.

2.3.9 Protein separation using SDS PAGE

Protein extracts were separated by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis using a Bio-Rad Mini-PROTEAN 3 system. For this, 15% Running gel was prepared as described in *Appendix A.9.* The gel was left for around 40 mins to set. Then, Stacking Gel (*Appendix A.9)* was prepared, the comb was added in between the plates and the remaining part inside the glass plates was filled with the stacking gel and left for around 30 mins to set.

Depending on the samples concentration obtained from Bradford assay, either 15µg or 50µg proteins were used and water was added to a final volume of 20 µl. Protein extracts were denaturated in 4µl of 5X loading dye (*Appendix A.11*) at 95°C for 10 minutes before being placed on ice. Glycine-Tris-SDS running buffer (25 mm Tris, 190 mm glycine, 0.1 % SDS) was used (*Appendix A.10).* The samples were then loaded on the wells and electrophoresis was

run (120V, 90 mins).

2.3.10 Wet transfer and western blots

Proteins from SDS-PAGE gels were transferred onto a 0.2 μm nitrocellulose membrane (Bio-Rad, 1620112) by wet transfer with the Trans-Blot Cell (Bio-Rad) as per the the supplier's instructions. Methanol transfer buffer (25 mm Tris, 190 mm glycine, 20% (v/v) methanol, 0.05 % SDS) was used and the transfer was carried out at 110V for 1 h (*Appendix A.10).* After transfer, the gels were stained with SimplyBlue SafeStain (Life Technologies, CA, USA) to assess the completion of protein transfer to the nitrocellulose membrane. Membranes were blocked overnight at 4°C in TBST buffer (10 mm Tris- HCl pH 7.4, 150 mM NaCl, 0.05% (v/v) TWEEN20) supplemented with 5% w/v Oxoid Skim Milk Powder (*Appendix A.10*).

On the next day, membranes were transferred to a clean 50 ml centrifuge tube and incubated with the 20mL milk-TBST buffer for three hours at room temperature on a laboratory roller mixer. Primary and secondary antibody was diluted in milk-TBST buffer and streptavidin was diluted in TBST. Overall incubation procedure was one hour primary anti HIPP25 antibody incubation (1:5000, X-O49613-N, Abmart), 4 times washing with TBST buffer for 5 mins, two-hour secondary antibody incubation (1:4000, biotinylated polyclonal rabbit anti-mouse immunoglobulins; DakoCytomation), 4 times 5mins TBST washing, one hour incubation with streptavidin-horseradish peroxidase (1:4000; Vector Laboratories, Burlingame, USA), and 4 times 5 mins TBST washing. The enhanced chemiluminescent (ECL) SuperSignal West Pico kit (#34080, Thermo Fisher) was used and the substrates present in the kit were mixed in equal amount and spread over the membranes for 10 mins. Chemiluminescence was recorded with the built-in chemiluminescence detection module of the G: BOX Chemi-XRQ gel imaging system (Syngene International Ltd.).

2.4 Analysis of glucosinolates level by HPLC

The glucosinolates profiles of Arabidopsis and Noccaea zinc treated leaves from the long-term treatment with first batch (Zn 25μM, 14 days), second batch (Zn 15μM, 14 days) and short-term treatment (Zn50μM, 24 h) with their respective controls were analyzed by High Performance Liquid Chromatography - UV detection (HPLC-UV).

2.4.1 Glucosinolates extraction

Approximately 100 mg of leaf tissue harvested from Arabidopsis and Noccaea plants grown on Hoagland's solution for 36 days and stored at -80°C were transferred to 2 ml pre-weighted

eppendorf tube and freeze dried for 48h (temp: -110°C, pressure: 100 mTorr), after which the dry weight was measured. A steel bead was added to the plant tissue, followed by the addition of 20 µl of a 5mg/ml solution of sinigrin as internal standard. 750 µl of 70% methanol preheated at 80°C was added and incubated at water bath for 3 minutes. The material was then crushed with the Tissue Lyser (Qiagen, Hilden, Germany) at 25 Hz for 1 minute. The tubes were then incubated back to water bath at 80°C for another 3 minutes and centrifuged further for 1 min at 13,000 rpm. The supernatant was collected in the new tube (2ml) and placed on ice. 750 µl of boiling (80°c) 70% methanol was added again to the pellet and vortexed and centrifuged for 1 min at 13,000 rpm. The supernatant was added on to the previous collected supernatant into the 2 ml Eppendorf tube. The samples were kept on the ice until the Sephadex column was prepared.

2.4.2 Glucosinolates extracts purification

Sephadex DEAE A25 (GE Healthcare 117-0170-01) was equilibrated in 0.2M sodium acetate buffer (pH 5) for 2 days and kept at 4°C until use. The Sephadex matrix two hours before the extraction process, was equilibrated in an equal volume of 0.02M acetate buffer pH5 for at least 2 hours in shaker at RT. 2 ml equilibrated Sephadex was added to Poly-Prep Chromatography Columns (Bio-Rad, 731-1550) which after compacting yielded approximately 1 ml of matrix. The Sephadex column was then washed three times with 1 ml autoclaved MQ H_2O.

200 µL 0.4M barium acetate was added to the glucosinolates extract and centrifuged for 10 min at 4000 rpm. Then the extracts were loaded onto the columns, and the matrix were rinsed twice with 1 ml H_2O and twice with 500µL of 0.02M sodium acetate buffer, pH5. 75 µl of the *Helix pomatia* sulphatase (Sigma S9626) prepared by following the protocol described by Graser et al. (2001) was added directly to the center of each column and incubated for 18 h (overnight) at room temperature in the dark. The desulfoglucosinolate extracts were then eluted with 3 times 500 µl of autoclaved MQ H_2O, put at -20°C overnight and freeze-dried for 24 hours (-110°C, 100 mTorr). The freeze-dried samples were then weighed and resuspended in autoclaved MQ H_2O (200µl/mg of dry extract).

2.4.3 Analysis of glucosinolates profile by HPLC

The desulfoglucosionolates were separated by an Agilent HPLC 1200 series with a UV diode array detector (G1315C DAD SL; Agilent Technologies) at 25°C on a C-18 reversed phase HPLC column (Supelcosil LC-18, 2.1 mm I.D., 250 mm L; particle size 3 µm; Sigma Aldrich, 57942) with the following H_2O-acetonitrile (solvent A- solvent B) mobile phase gradient

program: 0-2 min 3% B, 2-17 min 3-40% B, 17-22 min 40% B, 22-22.1 min 40-100% B, 22.1-32 min 100% B, 32-32.1 100-3% B, 32.1-60 min 3% B (all steps at a flow rate of 300 µl/min). The quantification of the desulfoglucosinolates was based on the peak area at 229 nm normalized to the internal standard and response factors published in the literature (Brown et al., 2003).

2.5 Cloning *HIPP25* genes from both *N. caerulescens* and *A. thaliana*

Five cDNA samples (one flower and one siliques from *Arabidopsis* and one shoot and two root tissues from *Noccaea* were kindly provided to me by Ya-Fen Lin. The cDNA obtained was then used for cloning purpose.

2.5.1 PCR

The gene of interest *HIPP25* had to be amplified by PCR before cloned into a vector. The PCR reactions set up is shown in *Table 2.6.* In our case enzyme DNA polymerase: Ex taq (#RR001A, TaKaRa Ex Taq) was used for two purposes: a) high fidelity amplification of the fragment of interest to be cloned (efficient 3' to 5' exonuclease activity) b) 80% of the PCR products contain 3'-A overhangs that can be cloned into T- vectors.

All the PCR components were well mixed, centrifuged and first set PCR was run for five cDNA templates*; Arabidopsis* flower (*At_F), Arabidopsis* silique *(At_S)* and *Noccaea shoot (*Nc_S), *Noccaea* root (Nc_R1, Nc_R2) based on the conditions described for respective plant species in *Table 2.7* and using the primers designed for respective plant gene *(Appendix B.2).*

Table 2.6 PCR components

Components	**Volume**
H_20	33.75 µl
10X Ex Taq Buffer	5 µl
2.5 Mm dNTPs	4 µl
10µM Primer(F)	2.5 µl
10 µM Primer(R)	2.5 µl
Takara Ex Taq	0.25 µl
cDNA template	2 µl
Total	50 µl

Table 2.7 PCR conditions for *AtHIPP25* and *NcHIPP25*

Set	Samples	Step	Temp	Time	Cycle
First	At_F At_S	Initial Denaturation	94°C	1 min	1
		Denaturation	98°C	10 secs	32
		Annealing and Extension	68°C	52 secs	
		Hold	4°C	∞	
First	Nc_S Nc_R1 Nc_R2	Initial Denaturation	*94°C*	*1 min*	1
		Denaturation	*98°C*	*10 secs*	32
		Annealing	50°C	30 secs	
		Extension	72°C	50 secs	
		Hold	*4°C*	∞	

Table 2.8 PCR conditions for *NcHIPP25*

Set	Sample	Step	Temp	Time	Cycle
Second *	Nc_R2	Initial Denaturation	*94°C*	*1 min*	*1*
		Denaturation	*98°C*	*10 secs*	*32*
		Annealing	64°C	30 secs	
		Extension	72°C	50 secs	
		Hold	*4°C*	∞	
Third *	Nc_R2	Initial Denaturation	*94°C*	*1 min*	*1*
		Denaturation	*98°C*	*10 secs*	*32*
		Annealing	55°C	30 secs	
		Extension	72°C	50 secs	
		Hold	*4°C*	∞	

*Second and third set were performed later in the experiment (*section 3.4.3*)

2.5.2 Agarose gel electrophoresis

1.5% agarose gel was prepared with 1X TAE. Out of 50µl PCR products for each sample, an aliquot of the reaction (10µl) with 10X DNA loading dye (1µl) were run on agarose gel at 100V for 30mins along with the marker (Gene ruler 1 Kb plus DNA ladder, Thermofisher sceintific) to verify the amplicons of the expected size. The resulting gels were photographed using Gel Doc 2000 (Bio Rad).

2.5.3 PCR cleaning

From Arabidopsis, At_F(40µl) and At_S(40µl) PCR products were merged together to make one sample and named as At_HIPP25 and equal volume of membrane binding solution (80µl) was added to the PCR amplified product. PCR cleaning was carried out by Wizard SV gel and PCR clean-up system kit following the supplier's protocol (#A9282, Promega) as described in *section 2.3.7* to clean up the excess nucleotides and primers.

For Noccaea, the PCR amplicons from electrophoresis (100v, 30 mins) on 1.5% agarose gel were cut and kept in the tube and equal volume of membrane binding solution was added. Instead of using PCR product directly, gel cutting and elution was done to purify the amplicon of the expected size. The tube was kept on the heater at 60°C for 10 mins until the gel was properly dissolved. Then, the same clean up procedure as described in *section 2.3.7* was performed.

2.5.4 A-Tailing

This protocol was used to improve the TA-cloning efficiency so that 3'-A overhangs is contained in each PCR product if Takara Ex Taq Polymerase fails to do so. A-Tailing reaction was performed with the PCR clean DNA for At_*HIPP25* and Gel clean DNA for Nc_*HIPP25* as shown in *Table 2.9*. The mixture was incubated at 72°C for 15 min and thereafter kept at 4°C.

Table 2.9 Components for A-Tailing

Components	**Volume**
PCR clean DNA/ Gel clean DNA	7µl
10xDNAzyme Buffer	1µl
2 mM dATP	1µl
DYNAzymeII DNA polymerase1	1µl
Total	10 µl

2.5.5 Topo TA cloning

TOPO TA cloning system provides a highly efficient coning strategy for the direct insertion of *Taq* polymerase-amplified PCR products into a plasmid vector, without ligase, or requirement of PCR primers containing specific sequences (Scientific).

Topo cloning was performed using INVITROGEN TOPO TA cloning kit (#k453020, Thermofisher scientific) for At_*HIPP25* and Nc_*HIPP25* separately in which A-tailed PCR

product was mixed with salt solution and pCRII Topo vector (*Table 2.10*) and the samples were kept at RT for about 30 mins.

Table 2.10 Components for Topo cloning

Components	**Volume**
A-Tailing PCR	2 µl
Salt solution	0.5 µl
pCRII TOPO® vector	0.5 µl
Total	3 µl

2.5.6 Transformation

The pCRII-Topo vector containing the probable desired insert was transformed to the competent *E. coli* DH5α cells. For this, the TOPO TA reaction (3 µl) was added to the competent cells DH5α (100 µl) and kept on ice for about 20-30 min, before it was submitted to a thermal shock at 42°C for 45 seconds followed by the immediate placement on ice for 1-2 min. 1 ml of prepared LB medium without antibiotics (*Appendix A.1*) was added onto the heat shocked product on the eppendorf tube and wrapped with the parafilm and placed on the shaker at 37°C for 45 mins at 220 rpm. Meanwhile, the LB plates were prepared. The antibiotic kanamycin (50 mg/l), IPTG(0.5mM), Xgal (80 mg/ml) was added in the prepared LB plates left it for 10 mins to get dried. The Eppendorf tubes after recovering from the shaker were centrifuged for 30 seconds. The supernatant was discarded and the pellet was dissolved with pipette and transferred into the LB plates. Using the sterile L-shaped rod the samples were properly spread on the plate and it was incubated at 37°C overnight (16-20 hrs).

From the plate containing blue (without insert) and white (with insert) colonies, 6 white colonies obtained with each of the two *HIPP25* constructs were cultured. For each of the samples, a loop of single colony was taken with the sterilized inoculating loop and cultured on culture tubes containing 3 ml LB (kan 50 mg/L) and placed at 37°C overnight on the shaker at 220 rpm.

2.5.7 Miniprep

The gene of interest after cloned into the vector and transformed into the bacteria, miniprep was done to isolate the plasmid DNA from bacteria (*E. coli*) culture grown from a single colony. The protocol was followed as described by the Plasmid DNA purification QIAprep Spin Miniprep kit (#27106, Qiagen). For this, 1.5 ml from each of the 3 ml *E. coli* culture tubes grown overnight were transferred to the 1.5 ml separate eppendorf tube and the remaining

sample was kept at 4°C to perform sequencing later. The sample in eppendorf tube was centrifuged at 13.3*g* for 1 min. The supernatant was discarded and the last drop was removed into the tissue. The pellet containing *E. coli* cells were resuspended in 250µl Buffer P1 and vortexed until the pellet dissolved completely. Then 250µl Buffer P2 (lysis buffer) was added and left for the lysis reaction for 3 mins. The tubes were inverted 3-5 times in between. Then 350 µl of Buffer N3 was added and mixed immediately by inverting it few times. Then the samples were centrifuged for 10 min at 13.3 *g* and the supernatant was transferred to the column fitted with the collection tube and was centrifuged for 1 min. The flow through was discarded from the collection tube. 0.75 ml of Buffer PE (ethanol added) was added to the column and centrifuged for 1 min. The sample flow through was discarded again from the collection tube and additional 1 min centrifugation was done to remove buffer PE which remained may inhibit subsequent enzymatic reactions. The column was kept on the new eppendorf tube and 50µl of EB buffer was added into the middle of the membrane. The column was left open for 1 min and centrifuged 1 min again to elute plasmid DNA which was kept in the ice box till the concentration was measured. The plasmid DNA concentration was then measured with a NanoDrop-1000 spectrophotometer (Thermo Fisher).

2.5.8 Restriction enzyme digestion check

Enzyme digestion check was carried out with the restriction enzyme EcoR I (< 10% of total volume), Plasmid DNA (450 ng), 10X EcoR I buffer and deionized water as mentioned in *Table 2.11 t*o verify the presence of insert of expected size. The samples were then kept at 37°C for 1 hr for enzyme digestion. Then the 20 µl of product from the restriction enzyme digestion were loaded on 1.5% agarose gel (100v, 30 mins) and the photos were taken with Gel Doc 2000 (Bio Rad).

Table 2.11 Components for enzyme digestion

Components	**Volume**
Plasmid DNA (450 ng)	X µl (depending on PD concentrations)
H_20(deionized)	17- X µl
E.coR I 10X Buffer	2 µl
E.coR I (enzyme) (<10% of total volume)	1 µl
Total	20 µl

2.5.9 Sequencing reaction

Once the presence of insert was confirmed, then the exact sequence of the insert was identified by performing the sequencing reaction with the sequencing primers appropriate for the pCRII-TOPO vector. For At_*HIIPP25* only the M13 forward (M13_For) primer was used whereas for Nc_*HIPP25* both M13_For and the M13 reverse (M13_Rev) primers were used. M13_For and M13_Rev annealing sites are present on the pCRII-TOPO plasmid backbone, on either side of the insert integration site (*Appendix C.3*). The sequencing reactions were performed with the BigDye Terminator v3.1 Cycle Sequencing Kit (#4337455, Applied Biosystems, Foster City, USA) using 200 ng of plasmid. Samples were well mixed (*Table 2.12*) and PCR was carried out in the conditions mentioned in *Table 2.5* with the primers showed in *Appendix B.3*.

Table 2.12 Components for sequencing reaction

Components	**Volume**
Template (2oo ng)	X µl (based on size of bp)
H_20(deionized)	15-X
Sequencing buffer	3 µl
Primer 3.2 pmol	1 µl
Big-Dye V.3.1	1 µl
Total	20 µl

The sequencing was performed at the DNA sequencing core facility of the Universitetssykehuset Nord-Norge (Tromsø, Norway).

3 RESULTS

3.1 The effect of heavy metal treatments on the expression levels of selected *HIPP*s

3.1.1 Phenotypic change in plants under heavy metal exposure

Arabidopsis and Noccaea plants were grown under different heavy metal exposure (Ni, Cu, Zn and Cd) and normal condition (without metal exposure) on modified Hoagland's medium. To minimize variation in the bioavailability of micronutrients, we used a hydroponic rather than soil-based culturing system. To observe the effect of these metals on expression of target gene (*HIPP25*) and its closely related genes (*HIPP26, HIPP27*) as compared to the normal condition, both plants were grown under two conditions: short term (24 hr) and long term (14 days) exposure to heavy metals as described in *section 2.3.2*. Usually the first visible symptoms caused by heavy metal toxicity include not only stunted growth related, but also reduced root growth and changes in root morphology (Feigl et al., 2013). Normally roots are more affected than leaves as they are the organs in direct contact with the toxic element. Since *A. thaliana* is a non-hyperaccumulator, it can be clearly seen the higher effect of metal treatment in *A. thaliana* than *N. caerulescens (Appendix Figure 7.13-7.16)* under long term exposure. *N. caerulescens* was well able to withstand the metals (preferentially Zn, Cd, Ni). Under short term, the tissues harvested after 24 hr exposure from both plants showed no altered phenotype that could be attributed to excess metal exposure.

3.1.2 Assessment of the quality of RNA

The plant material from different tissues of *A. thaliana* and *N. caerulescens* were harvested (*section 2.3.3*), RNA was extracted from them and before the gene expression analysis, the integrity of RNA was assessed on running denaturing agarose-formaldehyde gels as described in the *section 2.3.5*. For most of treatments, at least three replicates were used except for Cu 15µM where the tissues were not enough and two replicates was used for the analysis. Although the intensity of the bands is not in the ratio of 2:1, but the upper rRNA band 25S and the lower rNA band 18s for *A. thaliana* and *N. caerulescens* is seen distinct for most of the tissues. In the roots and flower and siliques only two bands were seen but, with the leaves additional bands below 18s rRNA is seen in both plants. These additional bands correspond the chloroplast ribosomal subunits which are absent in the root tissues.

Under short term treatment, the smearing below the rRNA was seen in roots treated with Cu25 µM in *A. thaliana (Figure 3.1)* which usually indicates the inferior quality of RNA. In *N. caerulescens*, intensity of band seen was lower again in case of roots treated with Cu 25µM as seen in *Figure 3.2*, whereas in the leaves the band were distinct. Results for the RNA quality of all the remaining metal treated and control tissues from both plants are shown in *Appendix C.1* which does not seem to have degraded RNA.

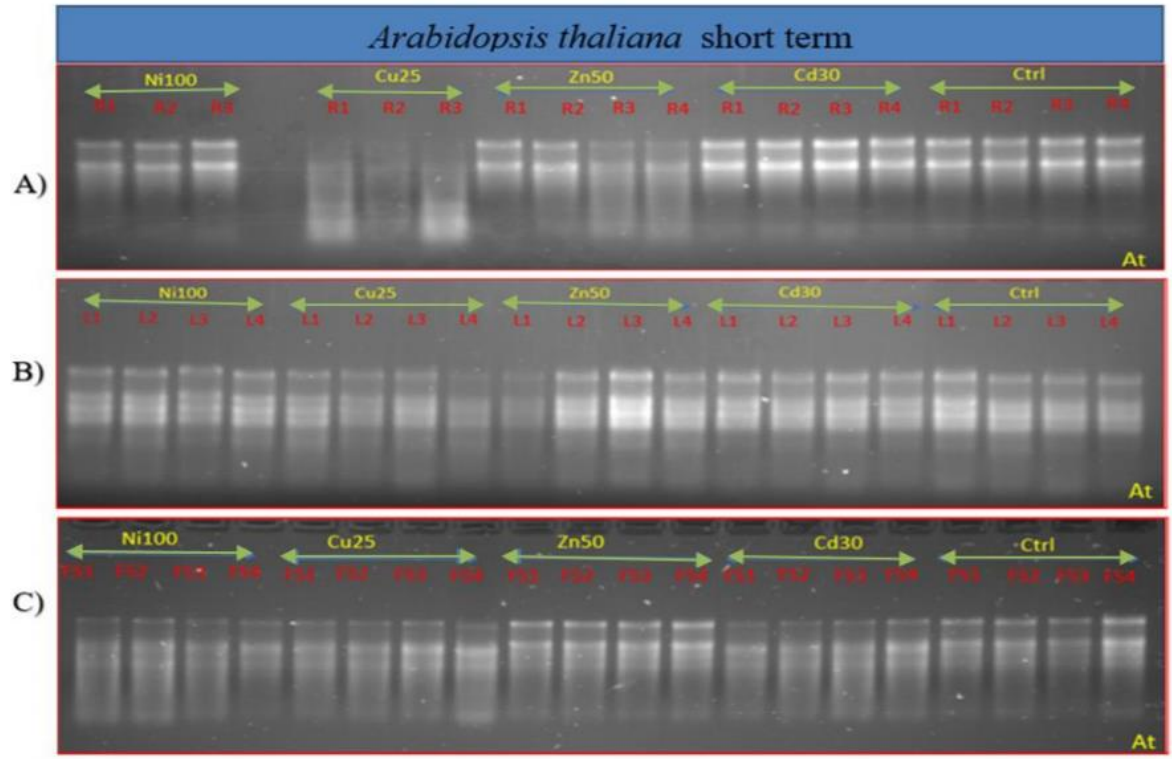


Figure 3.1 Total RNA isolated from root, leaves and flower+siliques of *Arabidopsis thaliana* exposed to different heavy metals (Ni 100µM, Cu 25µM, Zn 50µM, Cd 30µM) and without any metal treatment (control) for short term (24 hr). A) R1-R4 represents root samples in four replicates B) L1-L4 represents leaf samples in four replicates C) FS1-FS4 represents flower and siliques in four replicates.

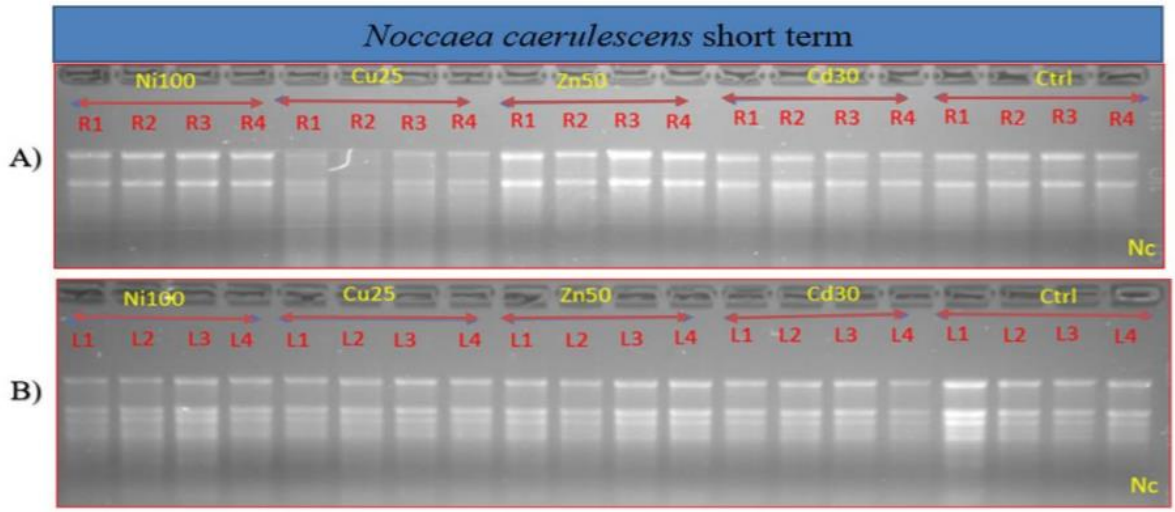


Figure 3.2 Total RNA isolated from root and leaves of *Noccaea caerulescens* exposed to different heavy metals (Ni 100µM, Cu 25µM, Zn 50µM, Cd 30µM) and without any metal treatment (control) for short term (24 hr). A) R1-R4 represents root sample in four replicates B) L1-L4 represents leaf samples in four replicates.

3.1.3 RT-qPCR analysis *of HIPP25, HIPP26* and *HIPP27* gene expression

The expression level of the gene *HIPP25*, *HIPP26* and *HIPP27* from *Arabidopsis thaliana* and *Noccaea caerulescens* grown under metal exposure for short term and long term were investigated by RT-qPCR according to the protocol described in *section 2.3.6* and using the primers mentioned in *Appendix B.1*. Though our primary target gene is *HIPP25*, but *HIPP26* and *HIPP27* genes were also studied because the gene sequences are very closely related as they belong to the same cluster of the HIPP family. At least three replicates for each treatment and control plants were used for analysis except in few cases where two replicates (for Cu15µM roots) were used.

3.1.3.1 Gene expression of *HIPP25, HIPP26* and *HIPP27* under short term metal treatment in *A. thaliana*

Arabidopsis plants treated with different heavy metals (Ni 100µM, Cu 25µM, Zn 50µM, Cd 30µM) for short term when compared with respective controls, none of the metal treated plants gene expression were found to be significant as seen in *Table 3.1*. Though, the gene expression level was not significant enough, the very first thing that could be analyzed was that all the treatments had decreased *HIPP25 gene* expression in the roots than in other tissues. The gene was induced in flowers+siliques by Ni, Cu and Zn treatment and leaves by Cu, Zn whereas, upon Cd treatment, the gene expression decreased in all tissues.

HIPP26 gene expression was reduced in the presence of all heavy metals in all tissues except Zn. With Zn treatment, the gene expression was found to be induced only in flower and siliques.

In contrast to this, *HIPP27* gene expression was increased in all the tissues with all the treatments except zinc, where it had the ratio of 0.99 in root tissues which means there was almost no change in the expression.

Table 3.1 Expression level of target genes on root, leaves, flower+siliques of *Arabidopsis thaliana* grown on Hoagland's medium supplemented with four different heavy metals: Ni 100µM, Cu 25µM, Zn 50µM, Cd 30µM and control (CTRL) plants for 24hr. A ratio higher than 1 indicates a higher expression of the gene (red color) while a ratio lower than one indicates a lower expression of the gene under (green color) as compared to the control plants. Significance was calculated based on p values obtained ($p<0.05$) in qBase+ using one-way ANOVA.

Target Genes	*Gene expression ratio between heavy metal and control treatment in A. thaliana under short term exposure*			
	Comparision	***Root***	***Leaves***	***Flowers+Siliques***
HIPP25	*Ni100 /CTRL*	*0.444*	*0.869*	*1.913*
	Cu25 /CTRL	*0.219*	*2.65*	*1.444*
	Zn50/CTRL	*0.933*	*1.297*	*1.636*
	Cd30 /CTRL	*0.794*	*0.743*	*0.921*
HIPP26	*Ni100 /CTRL*	*0.234*	*0.804*	*0.974*
	Cu25 /CTRL	*0.168*	*0.634*	*0.883*
	Zn50/CTRL	*0.595*	*0.975*	*1.327*
	Cd30 /CTRL	*0.784*	*0.873*	*0.74*
HIPP27	*Ni100 /CTRL*	*6.878*	*1.534*	*1.837*
	Cu25 /CTRL	*2.316*	*1.362*	*1.543*
	Zn50/CTRL	*0.991*	*1.358*	*1.329*
	Cd30 /CTRL	*1.111*	*1.135*	*2.029*

3.1.3.2 Gene expression of *HIPP25, HIPP26* and *HIPP27* under long-term metal treatment in *A. thaliana*

There were two rounds of 14 days' exposure of heavy metal treatments in *A. thaliana* grown in Hoagland's medium where the first round (Cu 25µM, Zn 25µM, Cd 10µM) had the higher concentration of heavy metals than the second (Ni 80µM and 50µM, Cu 15µM, Zn 15µM, Cd 5µM). Since the Ni (100µM) treated plants in the first round was highly affected, the plants were quite destroyed and the tissues could not be harvested from them, two lower concentrations of Ni were thus used in the second round for *A. thaliana*. Upon Cu 25µM treatment, the root tissues were highly affected and they were not enough for RNA extraction so the data for roots is not presented while for Cu 15µm only two replicates was available.

As can be seen on the *Table 3.2*, on the first batch, *HIPP25* gene expression was induced by all metal treatments in the leaves but significant increase on *HIPP25* expression was seen with

only one metal treatment i.e. Cu 25µM with almost twice the ratio (1.9) as compared with the control (1.0). Although not significantly, but Zn (25µM) and Cd (10µM) treatment also lead to increase in *HIPP25* expression in the leaves whereas in flower and siliques the expression remained almost the same as that of control plants. In the root tissue, Zn increased whereas Cd decreased the *HIPP25* expression.

HIPP26 expression decreased with all the metal treatments in all the tissues experimented but significant gene expression was found only with Cd treatment in flowers+siliques, leaves and Zn treatment in flower+siliques. With Cu 25µM treatment, *HIPP27* expression was significantly upregulated in leaves whereas, it was significantly downregulated in flower and siliques. Zn increased *HIPP27* expression in leaves and flower+siliques but Cd treatment decreased the gene expression in both flower+siliques and leaves.

In the second batch, *HIPP25* gene expression followed the similar pattern as seen in the first round where the expression was higher with Cu 15µM, Zn 15µM and Cd 5µM in the leaves. The only treatment that changed *HIPP25* gene expression significantly in this round was for leaf tissues treated with Cd 5µM and Ni 80µM resulting in the upregulation (2.5) and downregulation (0.167) of the gene respectively. In the root tissues, Zn 15µM increased whereas Cd 5µM decreased *HIPP25* expression similar to the first round. With Zn and Cd treatment, in the flower and siliques the gene expression remained almost same with the ratio being closest to 1, whereas it was slightly induced with Cu treatment.

HIPP26 gene was significantly downregulated in flower and siliques with Zn treatment like that of the first round. Cd treatment upregulated the gene in flower and siliques this time while it downregulated in the first round. *HIPP27* gene was significantly induced by Ni (80µM) in roots and significantly downregulated by Cd (5µM) in leaves.

Table 3.2 Expression level of target genes on root, leaves, flower+siliques of *Arabidopsis thaliana* grown in modified Hoagland's medium supplemented with four different heavy metals Ni, Cu, Zn, Cd for 14 days and compared with their respective controls. The first batch plants (At1) were treated with higher metal concentrations, and the concentration was decreased to lower on the second batch (At2). The two batches were grown at different time period but the growth conditions like temperature, light and the place used was same for both batches. A ratio higher than 1 indicates the higher expression of the gene (red color), a ratio lower than one indicates the lower expression of the gene (green color) as compared to the control plants. Significance was calculated based on p values obtained ($p<0.05$) in qBase+ using one-way ANOVA. Significant results are marked with an *.

Target Genes	***Gene expression ratio between heavy metal and control treatment in A. thaliana under long term exposure***							
	First batch(At1)				***Second batch(At2)***			
	Comparision	***Root***	***Leaves***	***Flowers + siliques***	***Comparision***	***Root***	***Leaves***	***Flowers+ Siliques***
HIPP25					*Ni80 /CTRL*	*0.516*	***0.167 ****	*1.903*
					Ni50 /CTRL	*0.983*	*0.665*	*0.746*
	Cu25 /CTRL	-	***1.926 ****	*0.9*	*Cu15/CTRL*	*0.408*	*1.45*	*1.108*
	Zn25/CTRL	*1.291*	*1.435*	*0.954*	*Zn15/CTRL*	*1.801*	*2.257*	*0.908*
	Cd10 /CTRL	*0.681*	*1.449*	*1.051*	*Cd5 /CTRL*	*0.751*	***2.453 ****	*0.94*
HIPP26					*Ni80 /CTRL*	*2.808*	*0.498*	*0.743*
					Ni50 /CTRL	*1.262*	*1.412*	*1.136*
	Cu25 /CTRL	-	*0.803*	*0.821*	*Cu15/CTRL*	*1.895*	*2.522*	*1.052*
	Zn25/CTRL	*0.85*	*0.747*	***0.492 ****	*Zn15/CTRL*	*0.796*	*0.854*	***0.623 ****
	Cd10 /CTRL	*0.968*	***0.341 ****	***0.537 ****	*Cd5 /CTRL*	*1.207*	*0.734*	*1.216*
HIPP27					*Ni80/CTRL*	***2.874 ****	*0.382*	*1.379*
					Ni50/CTRL	*1.683*	*1.066*	*0.777*
	Cu25 /CTRL	-	***2.765 ****	***0.734 ****	*Cu15/CTRL*	*2.301*	*1.46*	*1.002*
	Zn25/CTRL	*0.913*	*1.894*	*1.173*	*Zn15/CTRL*	*0.919*	*0.614*	*1.015*
	Cd10 /CTRL	*1.065*	*0.785*	*0.974*	*Cd5 /CTRL*	*1.389*	***0.37 ****	*0.745*

3.1.3.3 Gene expression of *HIPP25, HIPP26* under short term metal treatment in *N. caerulescens*

Similarly, Noccaea plants treated with different heavy metals (Ni 100µM, Cu 25µM, Zn 50µM, Cd 30µM) for short term when compared with respective controls, it was found that both *HIPP25* and *HIPP26* gene expression in roots was changed significantly upon Cu treatment (*Table 3.3), HIPP25* being reduced but *HIPP26* being induced. Although insignificant, Ni treatment also led to the decrease of *HIPP25* and *HIPP26* gene expression in roots.

In leaves, *HIPP25* and *HIPP26* gene were hardly affected by any of the metal treatments. The target gene *HIPP27* could not be analyzed as more than one melting peaks were identified which indicates that multiple products had been amplified.

Table 3.3 Expression level of target genes on root and leaves of *Noccaea caerulescens* grown on modified Hoagland's medium supplemented with four different heavy metals: Ni 100µM, Cu 25µM, Zn 50µM, Cd 30µM and control plants for 24hr. A ratio higher than 1 indicates the higher expression of the gene (red color), a ratio lower than one indicates the lower expression of the gene (green color) as compared to the control plants. Significance was calculated based on p values obtained ($p<0.05$) in qBase+ using one-way ANOVA. Significant results are marked with an *.

Target genes	*Gene expression ratio between heavy metal and control treatment in N. caerulescens under short term exposure*		
	Comparision	***Root***	***Leaves***
HIPP25	*Ni100 /CTRL*	*0.956*	*1.072*
	Cu25 /CTRL	***0.214 ****	*1.529*
	Zn50/CTRL	*1.327*	*1.062*
	Cd30 /CTRL	*1.024*	*1.158*
HIPP26	*Ni100 /CTRL*	*0.826*	*1.437*
	Cu25 /CTRL	***2.925 ****	*0.924*
	Zn50/CTRL	*0.985*	*1.153*
	Cd30 /CTRL	*1.283*	*1.499*

3.1.3.4 Gene expression of *HIPP25, HIPP26* under long-term metal treatment in *N. caerulescens*

HIPP25 gene expression ratio in different tissues exposed to heavy metal treated and control conditions under long term metal exposure is represented in *Table 3.4*. There were two rounds of 14 days' exposure of heavy metal treatments in *N. caerulescens* grown in Hoagland's medium where the first round (Ni 80µM, Cu 25µM, Zn 25µM, Cd 10µM) had the higher concentration of heavy metals than the second (Ni 50µM, Cu 15µM, Zn 15µM, Cd 5µM).

In first batch, *HIPP25* gene was almost not affected by any metal treatments in both leaves and root tissue as the values ae too close to 1. *HIPP26* gene in root tissues of all three metals were

downregulated but only roots with Ni (80 µM) treatment could produce significant result. In the leaves, gene expression was slightly increased by Ni.

In second batch, *HIPP25* gene in roots of Cu (15µM) treated plants decreased 5-fold whereas in Ni (50µM), Zn (15µM), and Cd (5µM) roots gene expression was hardly affected. In the leaves, Cu and Cd treatment again decreased the expression while Ni and Zn slightly increased the gene expression. The *HIPP26* gene was upregulated to some extent by Ni and Cu in roots, although not significantly.

Table 3.4 Expression level of target genes on root and leaves of *Noccaea caerulescens* grown in modified Hoagland's medium supplemented with four different heavy metals Ni, Cu, Zn, Cd for 14 days and compared with their respective controls. The first batch plants were treated with higher metal concentrations, and the concentration was decreased on the second batch. The two batches were grown at different time period but similar growth conditions (temp, light, place) was maintained. A ratio higher than 1 indicates higher expression of the gene (red color), a ratio lower than one indicates the lower expression of the gene (green color) as compared to the control plants. Significance was calculated based on p values obtained ($p<0.05$) in qBase+ using one-way ANOVA. Significant results are marked with an *.

Target Genes	***Gene expression ratio between heavy metal and control treatment in N. caerulescens under long term exposure***					
	First Batch(Nc1)			***Second Batch(Nc2)***		
	Comparision	***Root***	***Leaves***	***Comparision***	***Root***	***Leaves***
HIPP25	*Ni80 /CTRL*	*0.903*	*1.147*	*Ni50 /CTRL*	*1.143*	*1.205*
	Cu25 /CTRL	-	*0.994*	*Cu15 /CTRL*	*0.182*	*0.9 62*
	Zn25/CTRL	*1.108*	*0.927*	*Zn15/CTRL*	*1.245*	*1.113*
	Cd10 /CTRL	*0.876*	*0.884*	*Cd5 /CTRL*	*1.169*	*0.968*
HIPP26	*Ni80 /CTRL*	***0.516 ****	*1.229*	*Ni50 /CTRL*	*1.645*	*0.999*
	Cu25 /CTRL	-	*1.033*	*Cu15 /CTRL*	*2.028*	*0.844*
	Zn25/CTRL	*0,594*	*1.005*	*Zn15/CTRL*	*1.178*	*0.830*
	Cd10 /CTRL	*0.667*	*1.000*	*Cd5 /CTRL*	*1.341*	*1.110*

3.1.3.5 Gene expression of *HIPP25, HIPP26, HIPP27 in Arabidopsis thaliana* grown on MS media with Ni 8oµM and Cu 25µM

Arabidopsis thaliana (col-0) was grown on solid half-strength MS media with Ni (80µM) and Cu (25µm) treatment along with control plants for 21 days by one of the student at NTNU (Léa, 2016). It was interesting to compare the results of our experiment with these plant tissues as the concentration of heavy metals used in this experiment was similar to mine though there was changes in growth conditions like duration of exposure, medium used and place in the growth

chamber. RNA from root and leaves tissues from these plants were extracted by Ana Fernandez Repetto during her bachelor thesis and provided to me to check the *HIPP25*, *HIPP26* and *HIPP27* gene expression under this different experimental set up.

As seen in *Table 3.5*, none of the comparisons were found to be significant. Although insignificant, it was found that Ni decreased *HIPP25* gene expression in roots and increased in leaves, whereas Cu increased *HIPP25* gene expression in roots while remains almost same in leaves which is not in line with my results. In my experiment, Ni decreased gene expression in both leaves and roots (significant in leaves but not in roots) and Cu increased gene expression in leaves significantly (shown in right). The gene expression of *HIPP27* were induced in roots and reduced in leaves with Ni treatment similar with my results. The gene expression of *HIPP26* does not seem to be affected by the treatments much in her samples whereas, it was induced in roots and reduced in leaves by Ni in my results.

Table 3.5 Expression level of target genes on root and leaves of *Arabidopsis thaliana* grown in half strength MS medium supplemented with two heavy metals Ni, Cu for 21 days and compared with their respective controls. The results on the left (in 0.5X MS medium) are performed by one of the student at NTNU, on the right are the results from my experiment with the same concentration of metal exposed to *A. thaliana* grown in modified 0.5X Hoagland's medium for 14 days. A ratio higher than 1 indicates the upregulation (red color), a ratio lower than one indicates the downregulation (green color) compared with the control plants. Significance was calculated based on p values obtained ($p<0.05$) in qBase+ using one-way ANOVA. Significant results are marked with an *.

Target Genes	***Gene expression ratio between heavy metal and control treatment in A. thaliana under long term exposure***				
	In 0.5X MS medium for 21 days			***In 0.5X Hoagland's medium for 14 days***	
	Comparision	***Root***	***Leaves***	***Root***	***Leaves***
HIPP25	*Ni80 /CTRL*	*0.841*	*1.488*	*0.516*	***0.167****
	Cu25 /CTRL	*1.226*	*0.970*	-	***1.926****
HIPP26	*Ni80 /CTRL*	*1.141*	*0.941*	*2.808*	*0.498*
	Cu25 /CTRL	*1.017*	*0.986*	-	*0.803*
HIPP27	*Ni80 /CTRL*	*1.370*	*0.767*	***2.874****	*0.382*
	Cu25 /CTRL	*1.318*	*0.952*	***2.765****	***0.734****

3.1.4 Confirmation of target genes amplification

It was not possible to calculate the PCR efficiencies by LinRegPCR for *HIPP27* from any of the Noccaea material based on the RT-qPCR fluorescence data. From the melting curve of *HIPP27* in *N. caerulescens (Appendix C.2)*, multiple peaks could be observed, which *usually* means that multiple products were formed in the reaction. One of the possible reasons for such observation could be the primers used (which was designed based on Arabidopsis *HIPP27*

gene) being not completely specific for the cDNA template of Noccaea. In this case, a product other than the desired target could have been amplified, due to the sequence similarity or multiple forms of the same gene sequence. Another reason could be the primer dimer formation because the gene *HIPP27* might be lowly expressed in *N. caerulescens*. Hence, *N. caerulescens HIPP27* was not included in the analysis.

To verify the sequence of the target genes amplicons, the RT-qPCR reactions obtained on leaves samples (control and Ni treated) from both Arabidopsis and Noccaea were run on 2% agarose gel. Amplicons of the expected size were obtained for *HIPP25* (70 bp) and *HIPP26* (137 bp) in *A. thaliana* and *N. caerulescens*, see *Figure 3.3A*. In the case of *HIPP27* an amplicon of the correct size (138 bp) was only obtained for *A. thaliana*, while RT-qPCR on *N. caerulescens* generated a lower sized band consisting probably of primer-dimer. Bands with higher molecular weight obtained for *HIPP27* in *A. thaliana* represent probably amplification on gDNA. Only lower molecular bands of appropriate size on the gel (*Figure3.3A*) were cut, cleaned and rerun on gel for verification (*Figure 3.3B*) before being submitted to sequencing.

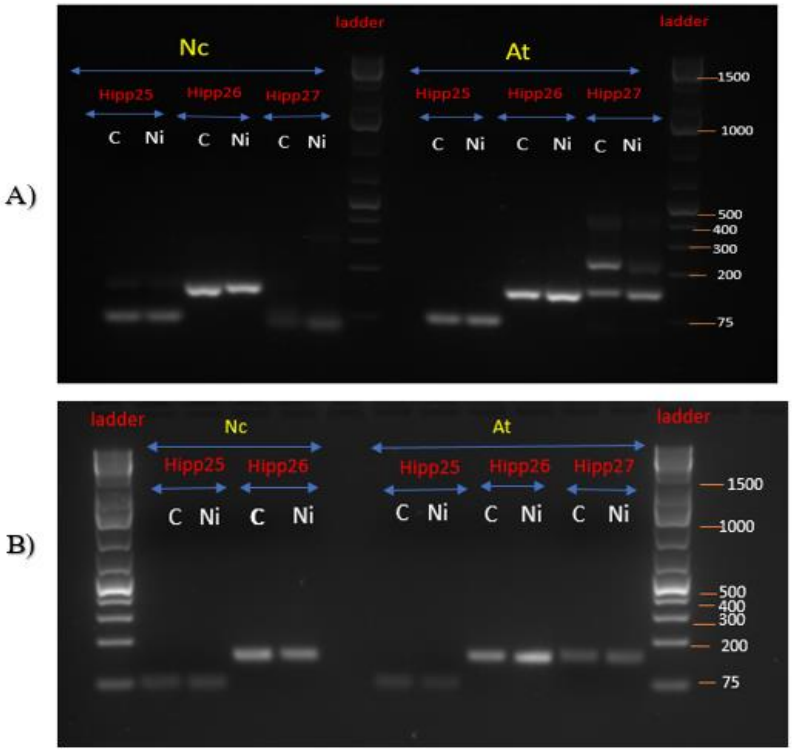


Figure 3.3 A) Bands after RT-qPCR amplification of control and Ni treated leaves on target genes *HIPP25, HIPP26, HIPP27* (B) gel electrophoresis of aliquots after gel purification of amplicons of the expected size (70bp, 137bp, 138bp for *HIPP25*, *HIPP26* and *HIPP27* respectively) from the gel in (a) Abbreviations: At: *A. thaliana*, Nc: *N. caerulescens*, C: control leaves, Ni: Ni50µM leaves

The Sequencing results verified that all the RT-qPCR primers, *HIPP25*, *HIPP26* and *HIPP27* amplified the correct gene in Arabidopsis and *HIPP25*, *HIPP26* in Noccaea. *HIPP25*, *HIPP26* and *HIPP27* gene sequences from each of the samples were aligned with their CDS of the respective gene found in the database (TAIR for *A. thaliana* and NCBI for *N. caerulescens*) and it was found that these sequences obtained from the plant samples was similar with the CDS of the respective gene.

3.2 Western blots

3.2.1 With anti-HIPP25 antibody

Gene expression level of *HIPP25* was known from RT-qPCR. To know the expression of HIPP25 proteins, western blot was carried with the protein extracts from leaf tissues of Arabidopsis and Noccaea plants treated with different heavy metals and compared with their untreated plants. After RNA extraction from the harvested tissues, the tissues of root samples from both plants and flower and siliques from *A. thaliana* were not enough to carry out protein extraction. The time limitations opposed us to grow plants further which takes 36 days for harvesting the tissues. Thus, protein extraction was performed with all the leaf tissues from both *A. thaliana* and *N. caerulescens* plants with 200 mg of tissues (50 mg from each of the four replicates).

The molecular weight of the AtHIPP25 protein (without post translational modifications) was predicted to be 17.16 kD through the Compute pI/MW tool at expasy.org. The molecular weight that we obtained from the western blot was more than 30 kD. The possibility for this increased band size are explained further in *section 4.4*.

3.2.2 With anti-actin antibody

Actin is a ubiquitous component of the plant cytoskeleton that plays an important role in cell elongation, cell shape determination, cell division, organelle movement and extension growth (Kobayashi et al., 1987; Seagull, 1990; Wernicke et al., 1992). Preferentially actin is expressed in young and expanding tissues, floral organ primordia, developing seeds and emerging inflorescence. The expected protein molecular weight is 41.6 kDa (antibodies). The protein on the membranes produced band size slightly higher than 40 kD which is thus the expected size.

The primary objective of doing this was to use the actin antibody as the loading control, i.e. to check that the lanes in the gel have been evenly loaded with sample, and that the proteins have been evenly transferred from the gel to the membrane across the whole gel. Since, Actin 2gene

was used as the reference gene for RT-qPCR, so the actin protein was chosen as the reference protein which might give some confirmative results. For comparing the expression levels of a protein in different samples, membranes containing protein mixture from each of the plant was blotted with anti-actin antibody. By far, the reference protein actin should not be affected with any metal treatments.

3.2.3 Metal treated leaves Vs control leaves in *A. thaliana* under long term exposure

Figure 3.4A represents the comparison within the leaves of both first (At1) and second batch (At2) in *A. thaliana*. HIPP25 protein expression is very low in leaves of first batch. In second batch, proteins are expressed higher in leaves of control plants as well as Cd and Zn treated plants than first batch. No bands could be seen for the leaves treated with Ni (80µM), Ni (50µM) and Cu(15µM). In both batches, proteins in leaves are higher expressed with Zn treatment than that of control leaves.

From *Figure 3.4B,* it can be seen that actin proteins were found abundantly in the leaves of *A. thaliana*. The bands intensity observed was not same for all the samples and very low for Cu treated leaves.

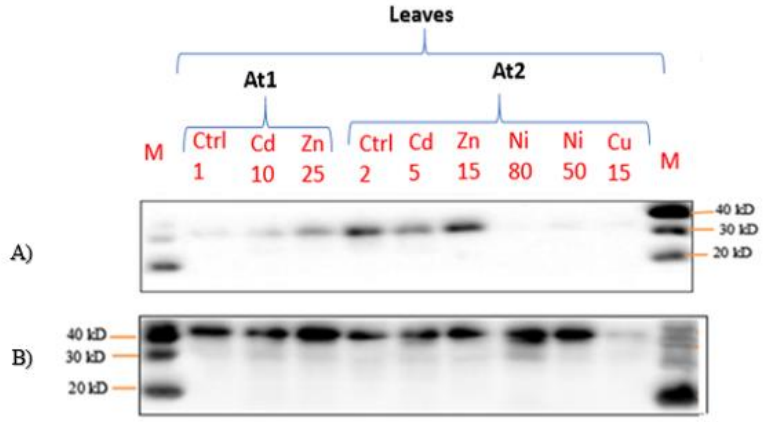


Figure 3.4 The representative nitrocellulose western blot on leaves of *A. thaliana* grown on modified half strength Hoagland's medium supplemented with different heavy metals on first batch At1 (Cd 10 µM, Zn 25µM) and second batch At2 (Cd 5µM, Zn 15µM, Ni 80µM, Ni 50µM, Cu 15µM) and without any metal treatment (control) in their respective batches for long term (14 days). 50µg total protein for each sample was used**. A)** Primary antibody was **anti-HIPP25** (X-O49613-N, Abmart) at 1:5000 dilution incubated for two hours. Secondary antibody was biotinylated polyclonal rabbit anti-mouse at1:4000 dilution and incubated for two hours. Two minutes chemiluminescence exposure on the Chemidoc Chemi-XRS (Bio-Rad). **B)** Primary antibody was **anti-actin** (AS13 2640) at 1:5000 dilution incubated for two hours. Secondary antibody was biotinylated polyclonal goat anti-rabbit at 1:4000 dilution and incubated for two hours. Two minutes chemiluminescence exposure on the Chemidoc Chemi-XRS (Bio-Rad). Abbreviations: M: Magic Marker, At1: *A. thaliana* first batch, At2: *A. thaliana* second batch, Ctrl1: Control first batch, Ctrl2: Control second batch.

3.2.4 Metal treated leaves Vs control leaves in *N. caerulescens* under long term exposure

Figure 3.5A represents the HIPP25 protein expression in control and metal treated leaves of *N. caerulescens*. In first batch (Nc1), the intensity of bands was higher for Zn (25µM) and Cd (10µM) treated leaves than for Ni (80µM) and control. No bands were seen with Cu (25µM) treatment. Related results were seen with the second batch, where the intensity of band was seen higher for Cd (5µM) and Zn (15µM) leaves, followed by Ni (50µM) and lowest on leaves of control plants. No bands were seen with Cu (15µM) treatment again.

Figure 3.5B represents the actin proteins expressed in leaves of *N. caerulescens* when treated with anti-actin antibody. The intensity of band for leaves treated with Cu was lower as seen in blots of *A. thaliana (Figure 3.4).*

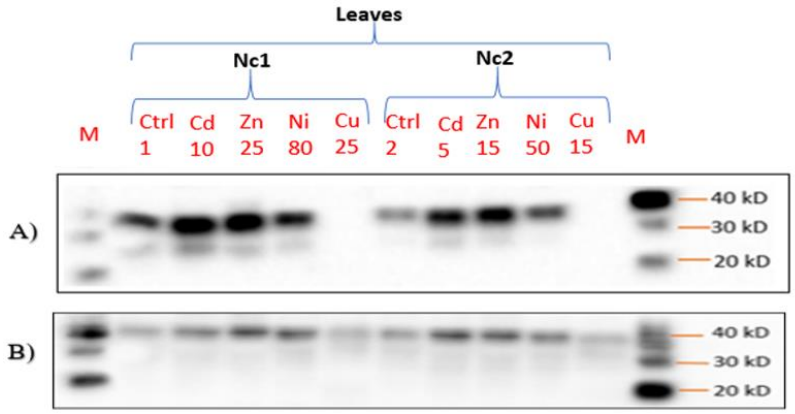


Figure 3.5 The representative nitrocellulose western blot on leaves of *N. caerulescens* grown on modified half strength Hoagland's medium supplemented with different heavy metals on first batch Nc1 (Cd 10µM, Zn 25µM, Ni 80µM, Cu 25µM) and second batch Nc2 (Cd 5µM, Zn 15µM, Ni 50µM, Cu 15µM) and without any metal treatment (control) in their respective batches for long term (14 days). 50µg total protein for each sample was used. **A)** Primary antibody was **anti-HIPP25** (X-O49613-N, Abmart) at 1:5000 dilution incubated for two hours. Secondary antibody was biotinylated polyclonal rabbit anti-mouse at1:4000 dilution and incubated for two hours. Two minutes chemiluminescence exposure on the Chemidoc Chemi-XRS (Bio-Rad). **B)** Primary antibody was **anti-actin** (AS13 2640) at 1:5000 dilution incubated for two hours. Secondary antibody was biotinylated polyclonal goat anti-rabbit at 1:4000 dilution and incubated for two hours. Two minutes chemiluminescence exposure on the Chemidoc Chemi-XRS (Bio-Rad). Abbreviations: M: Magic Marker, Nc1: *N. caerulescens* first batch, Nc2: *N. caerulescens* second batch, Ctrl1: Control first batch, Ctrl2: Control second batch.

3.2.5 *A. thaliana* Vs *N. caerulescens* leaves under long term metal exposure

In *Figure 3.6A and 3.6C,* when comparison is made in leaves between *N. caerulescens* and *A. thaliana,* HIPP25 proteins were expressed higher in *N. caerulescens* than in *A. thaliana* in control conditions as well as metal treated condition. HIPP25 proteins were expressed very low

in the leaves treated with Cu for both the plants. The presence of lower additional band in Noccaea with molecular weight around 24 kD represents some other proteins detected by anti-HIPP antibody. However, both of these bands sizes do not meet the expected size of HIPP protein which is below 20 kD.

Figure 3.6B and *3.6D* represents the high expression of actin proteins in *A. thaliana* than *N. caerulescens.* However, in the control plants in *A, thaliana*, the expression was very low than in metal treated condition. This possibly indicates that actin proteins are not good loading control which seems to be affected by metal treatments.

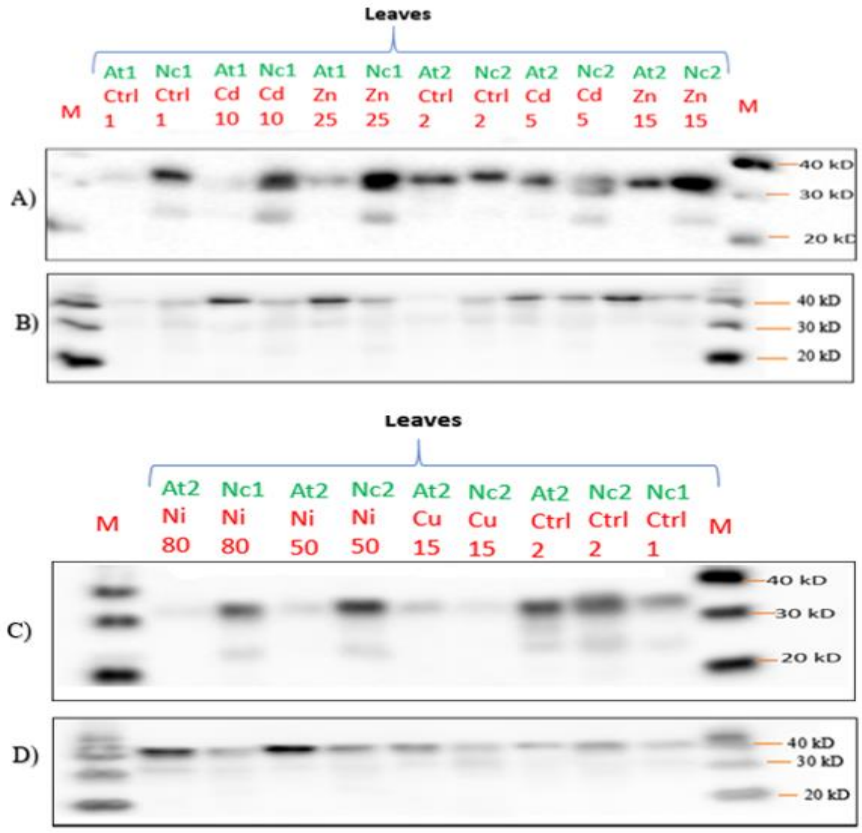


Figure 3.6 Four representative nitrocellulose western blots on leaves of *A. thaliana* and *N. caerulescens* grown on modified half strength Hoagland's medium supplemented with different heavy metals on first batch At1 (Cd 10µM, Zn 25µM), Nc1(Cd 10µM, Zn 25µM, Ni 80µM, Cu 25µM) and second batch At2 (Cd 5µM, Zn15µM, Ni 80µM, Ni 50µM, Cu 15µM), Nc2(Cd 5µM, Zn15µM, Ni50µM, Cu 15µM) and without any metal treatment (control) in their respective batches for long term (14 days). 50µg total protein for each sample was used. **A and C)** Primary antibody was **anti-HIPP25** (X-O49613-N, Abmart) at 1:5000 dilution incubated for two hours. Secondary antibody was biotinylated polyclonal rabbit anti-mouse at 1:4000 dilution and incubated for two hours. Two minutes chemiluminescence exposure on the Chemidoc Chemi-XRS (Bio-Rad). **B and D)** Primary antibody was **anti-actin** (AS13 2640) at 1:5000 dilution incubated for two hours. Secondary antibody was biotinylated polyclonal goat anti-rabbit at 1:4000 dilution and incubated for two hours. Two minutes chemiluminescence exposure on the Chemidoc Chemi-XRS (Bio-Rad). Abbreviations: M: Magic Marker, At1: *A. thaliana* first batch, At2: *A. thaliana* second batch Nc1: *N. caerulescens* first batch, Nc2: *N. caerulescens* second batch; ctrl1: control first batch, ctrl2: control second batch.

3.2.6 A. thaliana Vs *N. caerulescens* under short term metal exposure:

From *Figure 3.7*, several comparison and study on HIPP25 protein expression can be performed. Within the leaves of *A. thaliana*, HIPP25 proteins are higher expressed in Cd than control. No bands could be seen for Ni and Cu treated leaves. Within the leaves of *N. caerulescens*, again the protein expression was higher in Cd than control and other metal treatments. Within the roots of *A. thaliana*, expression is higher in control roots than the Ni treated. Similar, with *N. caerulescens*, control treatment had higher expression, followed by Ni and least at the Cu. Overall comparison shows that roots and leaves of *N. caerulescens* had higher HIPP25 protein expression than *A. thaliana* under short term metal exposure.

The same membrane blots when treated with the anti-actin antibody, no bands were seen when only 15µg protein for each sample was used unlike 50µg in other blots. One probable reason for the actin protein bands not to be seen on the blots was that actin might have been expressed very low under 15µg of protein.

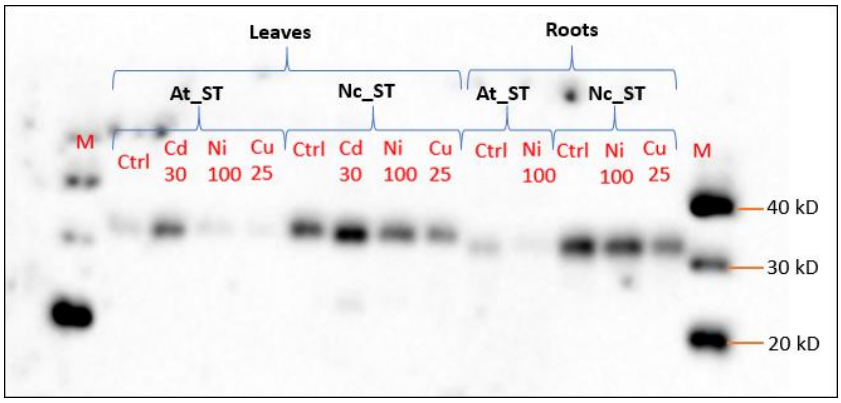


Figure 3.7 The representative nitrocellulose western blots on leaves and roots of *A. thaliana* and *N. caerulescens* grown on modified half strength Hoagland's medium supplemented with different heavy metals (Cd 30µM, Ni 100 µM, Cu 25µM) and without any metal treatment (control) for respective plants under short term meta exposure (24 hr). 15µg total protein for each sample was used. Primary antibody was **anti-HIPP25** (X-O49613-N, Abmart) at 1:5000 dilution incubated for two hours. Secondary antibody was biotinylated polyclonal rabbit anti-mouse at 1:4000 dilution and incubated for two hours. Two minutes chemiluminescence exposure on the Chemidoc Chemi-XRS (Bio-Rad). Abbreviations: M: Magic Marker, At_ST: *A. thaliana* short term, Nc_ST: *N. caerulescens* short term, Ctrl1: Control first batch, Ctrl2: Control second batch.

3.3 Glucosinolate profiles of Arabidopsis *thaliana* and *Noccaea caerulescens* Zn treated plants compared to their respective controls

Preliminary results from glucosinolate analysis of *A. thaliana* plants grown for 21 days on solid half-strength MS medium supplemented with 25µM $ZnSO_4$, performed during a previous master thesis, indicated that Zn increased the glucosinolate content compared to the control

treatment. To confirm the impact of the zinc treatment on glucosinolates, the leaf glucosinolate profiles of Arabidopsis and Noccaea plants after long term and short-term Zn treatment were analyzed by HPLC according to the procedure described in the *section 2.4.3*. A total of 7 glucosinolates were detected in the leaves of Arabidopsis plants; 4 aliphatic (3MSP, 4MSB, 7MSH, 8MSO) and 3 indolic (I3M, 4MeO-I3M, 1MeO-I3M) (refer to *Table 1.4* for full and trivial names). These were identified based on retention time and previous LC-MS analysis.

For *N. caerulescens*, this information was not available and we did not have the opportunity to run the glucosinolate extracts on LC-MS during this project. Glucosinolates from *N. caerulescens* could therefore be only putatively identified based on their retention times, UV spectra and/or expected profiles reported in the literature (Brown et al., 2003; De Graaf et al., 2015; Wathelet et al., 2004). A total of 4 glucosinolates were detected in leaves of *Noccaea* plants; one aliphatic (20H-2MeP, 2 -hydroxy-2 methylpropyl glucosinolate) and 3 aromatic (P-oH-bz, arylalkayl, RhaOxy-bz), refer to *Table 1.5* for full and trivial names.

The leaves tissue from the control and zinc treated plants used for glucosinolates analysis are from the same plants which were used for RT-qPCR and western blots. The Zn concentration used for both Arabidopsis and Noccaea for long treatment first batch (At1, Nc1) was 25µM and the second batch (At2, Nc2) was 15µM while for the short term it was 50µM.

3.3.1 Glucosinolates content in leaves of *A. thaliana* under short-term Zn exposure (24 hr)

Almost all the glucosinolates was found to be decreased in Arabidopsis leaves when exposed to 50 µM Zn for 24 hr) as compared to their respective controls, see *Figure 3.8*. Only the decrease in one aliphatic GSL, 3MSP was found to be significant. 7MSH and 8MSO were slightly increased upon the zinc treatment. The high error bars indicate the variation between the replicates used.

Referring to *Figure 3.9,* overall, total GSL content decreased with zinc treatment (19µmol/g DW) in the leaves as compared with control leaves (27µmol/g DW). Total amount of Aliphatic GSL was around 22µmol/g DW while the Indolic GSL was below 5µmol/g DW which decreased to around 16µmol/ g DW and 2µmol/g DW respectively upon the Zn 50µM treatment. It can be seen that, the total GSL content is mainly due to the contribution of total aliphatic GSL.

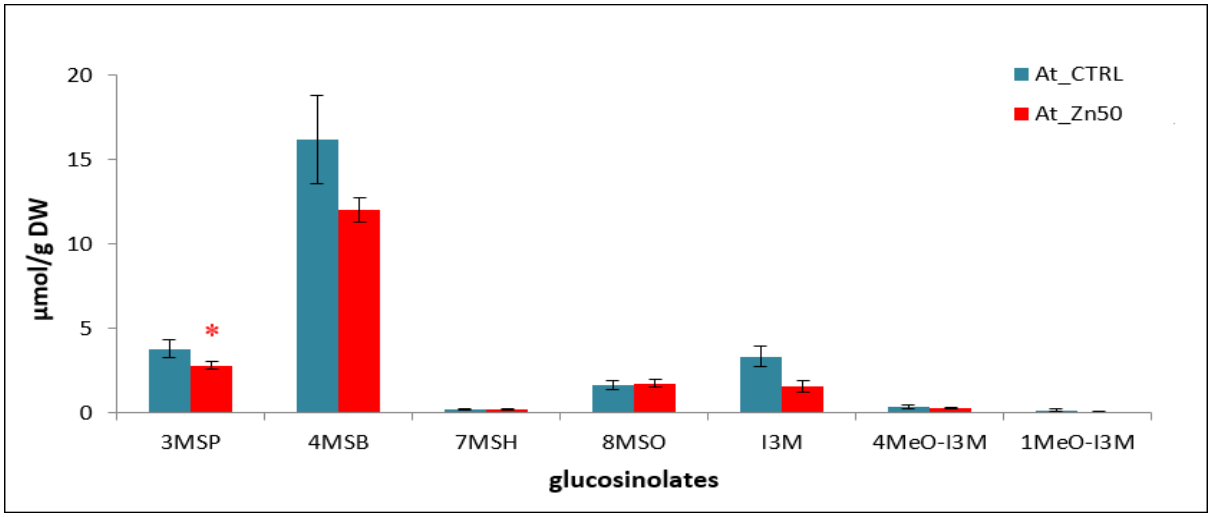


Figure 3.8 Individual glucosinolate profiles of 36 days old *A. thaliana* treated with Zn 50µM and control (CTRL) plants grown on modified half strength Hoagland's medium under short term (24 hr) condition before harvesting. The glucosinolates content presented here are the average of three replicates and the standard deviation is represented by the error bar. Statistical treatment of data was performed using PC program Sigma plot13. Data were treated by ANOVA, significance ($p<0.05$) was calculated with Tukey test. The significant change in Zn treated leaves when compared with control plants are marked with an *. DW: Dry Weight, 3MSP: 3-Methylsufinylpropylglucosinolate, 4MSB: 4-methylsulfinylbutylglucosinolate, 7MSH: 7-methylsulfinylheptylglucosinolate, 8MSO: 8-Methylsufinyloctylglucosinolate, I3M: Indol-3-ylmethylglucosinolate, 4MeO-I3M: 4-methoxyindol-3-ylmethylglucosinolate(4MeO-I3M), 1MeO-I3M: 1-methoxyindol-3-ylmethylglucosinolate

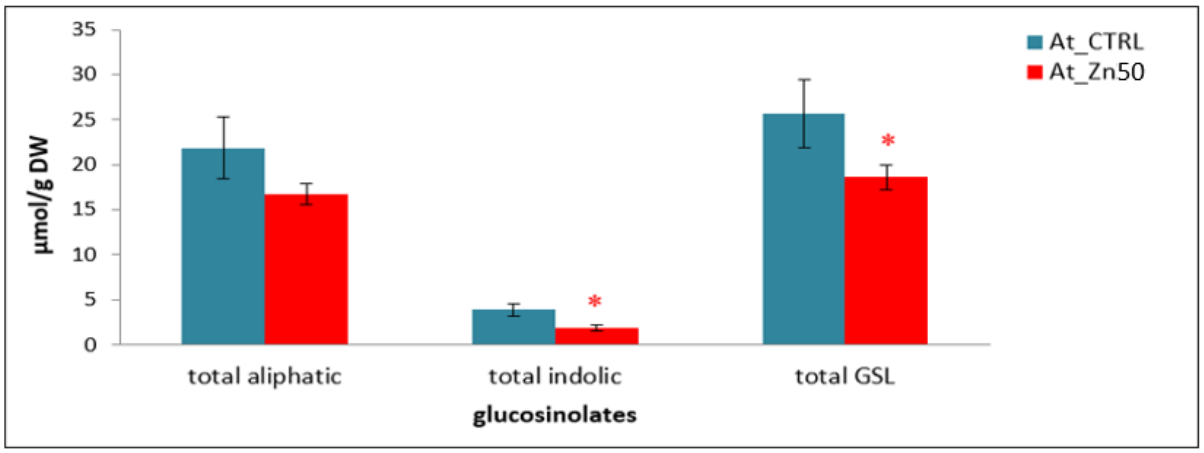


Figure 3.9 Aliphatic, aromatic and total glucosinolates content of 36 days old *A. thaliana* Zn treated (50µM) and control (CTRL) plants grown on modified half strength Hoagland's medium under short term (24 hr) condition before harvesting. The glucosinolates content presented here are the average of three replicates and the standard deviation is represented by the error bar. Statistical treatment of data was performed using PC program Sigma plot13. Data were treated by ANOVA, significance ($p<0.05$) was calculated with Tukey test. The significant changes in Zn treated leaves when compared with control plants are marked with an *. DW: Dry weight

3.3.2 Glucosinolates content in leaves of *A. thaliana* under long term Zn exposure (14 days)

As seen in *Figure 3.10*, most of the glucosinolates were hardly affected in Arabidopsis leaves on the first batch (Zn 25µM) treatment while the glucosinolates content increased on the second batch (Zn 15µM) as compared to their respective controls. 4MSB (aliphatic) was the main glucosinolate observed on both the batches while, 3MSP (aliphatic) being the next. In the second batch, all GSL increased but only the increase of 3MSP was significant. The high error bars indicate the variation between the replicates used. Also, the glucosinolate levels in control leaves differed quite substantially between the two batches.

From *Figure 3.11,* it can be observed that the total GSL content in the first batch for untreated leaves was 30µmol/g DW while, upon the zinc treatment (25µM) the GSL content remained quite stable (28µmol/g DW). In contrary to this, in the second batch total GSL content increased from around 15µmol/g DW to 32µmol/g DW upon Zn (15µM) treatment. Here, the total GSL content is mainly due to the contribution of total aliphatic GSL.

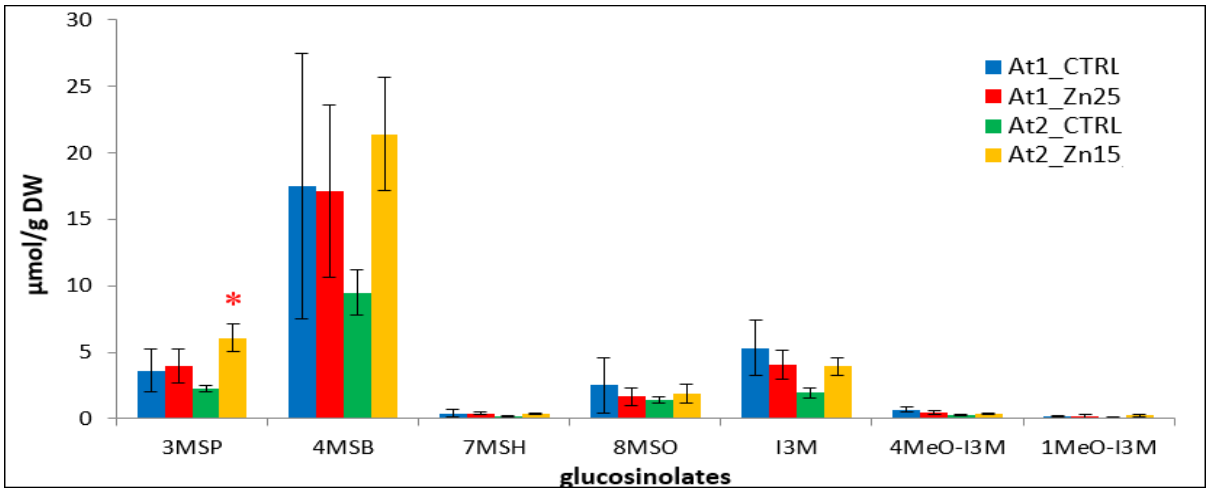


Figure 3.10 Individual glucosinolate profiles of 36 days old *A. thaliana* Zn treated (25µM and 15µM) and control (CTRL) plants from two batches (labelled At1 and At2) grown on modified half strength Hoagland's medium under long term (14 days) condition before harvesting. The glucosinolates content presented here are the average of four replicates and the standard deviation is represented by the error bar. Statistical treatment of data was performed using PC program Sigma plot13. Data were treated by ANOVA, significance ($p<0.05$) was calculated with Tukey test. The significant changes in Zn treated leaves when compared with control plants are marked with an *. DW: Dry Weight, 3MSP: 3-Methylsufinylpropylglucosinolate, 4MSB: 4-methylsulfinylbutylglucosinolate, 7MSH: 7-methylsulfinylheptylglucosinolate, 8MSO: 8-Methylsufinyloctylglucosinolate, I3M: Indol-3-ylmethylglucosinolate, 4MeO-I3M: 4-methoxyindol-3-ylmethylglucosinolate(4MeO-I3M), 1MeO-I3M: 1-methoxyindol-3-ylmethylglucosinolate

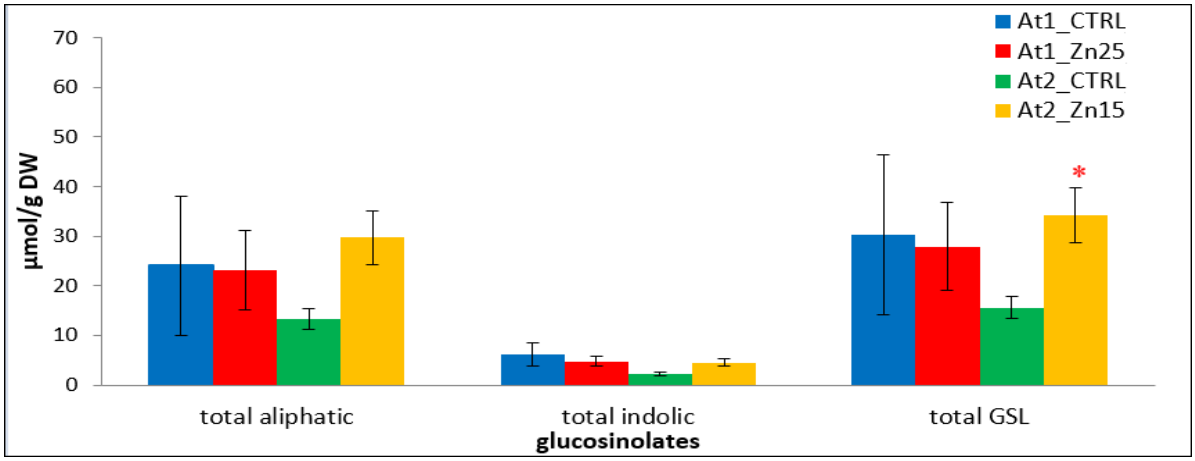


Figure 3.11 Aliphatic, aromatic and total glucosinolates profiles of 36 days old *A. thaliana* Zn treated (25µm, 15µm) and control (CTRL) plants from two batches (labelled At1 and At2) grown on modified half strength Hoagland's medium under long term (14 days) condition before harvesting. The glucosinolates content presented here are the average of four replicates and the standard deviation is represented by the error bar. Statistical treatment of data was performed using PC program Sigma plot13. Data were treated by ANOVA, Significance ($p<0.05$) was calculated with Tukey test. The significant changes in Zn treated leaves when compared with control plants are marked with an *. DW: Dry weight

3.3.3 Glucosinolates content in leaves of *N. caerulescens* under short term Zn exposure (24 hr)

As seen in *Figure 3.12*, with the zinc treatment on Noccaea, the glucosinolate(p-OH-bz) content in the leaves was found to be decreased by very less amount under short term condition when compared to their respective controls. The other three GSL also remained almost unchanged by the zinc treatment. High error bars indicate the variation between the replicates used.

From *Figure 3.13,* it can be seen that the total GSL which is the contribution mainly from aromatic GSL also gets decreased with very less amount and aliphatic GSL content remains the same.

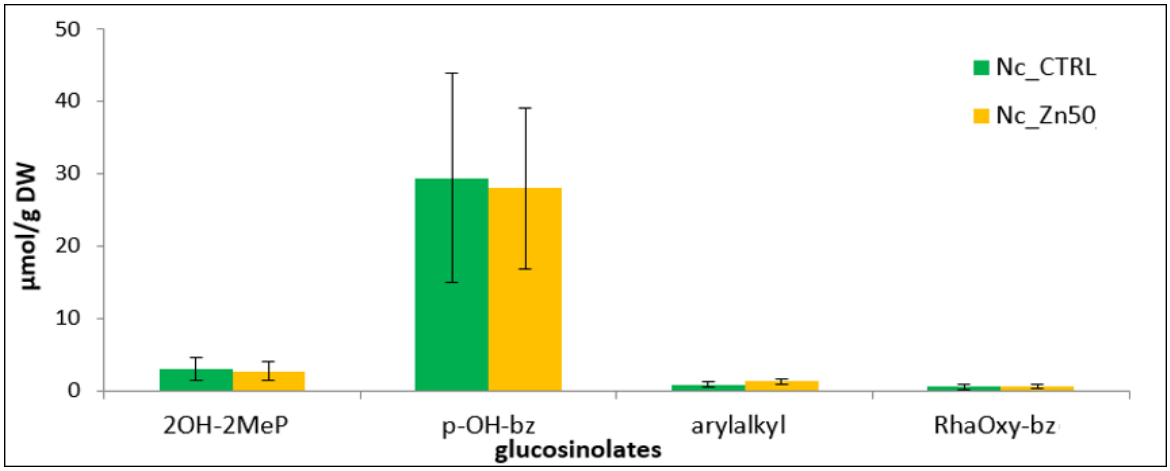


Figure 3.12 Individual glucosinolates profiles of 36 days old *N. caerulescens* Zn treated (50µM) and control (CTRL) plants grown on modified half strength Hoagland's medium under short term (24 hr) condition before harvesting. The glucosinolates content presented here are the average of four replicates and the standard deviation is represented by the error bar. Statistical treatment of data was performed using PC program Sigma plot13. Data were treated by ANOVA, significance ($p<0.05$) was calculated with Tukey test. DW: Dry weight, putatively identified glucosinolates: 2OH-2MeP: 2-hydroxy-2-methylpropylglucosinolate (glucoconringiin), p-OH-bz: p-hydroxybenzylglucosinolate (glucosinalbin), arylalkayl (unidentified GSL with UV spectrum indicating an arylalkyl structure), RhaOxy-bz: 4-α-rhamnosyloxybenzyl glucosinolate (glucomoringin).

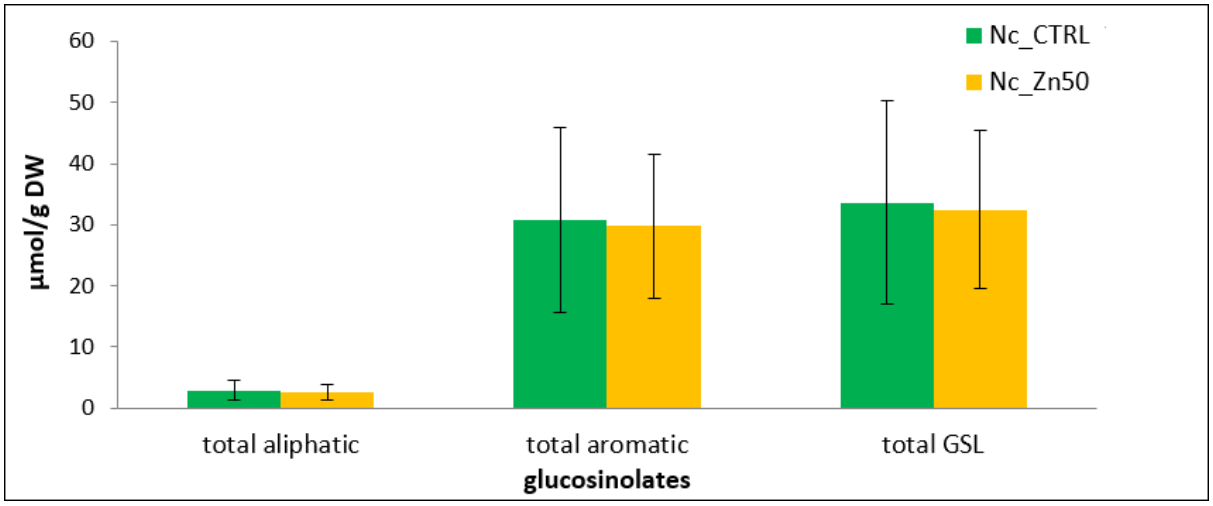


Figure 3.13 Aliphatic, aromatic and total glucosinolate content in leaves of 36 days old *N. caerulescens* Zn treated (50µM) and control (CTRL) plants grown on modified half strength Hoagland's medium under short term (24 hr) condition before harvesting. The glucosinolates content presented here are the average of four replicates and the standard deviation is represented by the error bar. Statistical treatment of data was performed using PC program Sigma plot13. Data were treated by ANOVA, significance ($p<0.05$) was calculated with Tukey test. DW: Dry weight

3.3.4 Glucosinolates content in leaves of *N. caerulescens* under long term Zn exposure (14 days)

It was found that p-OH bz (aromatic GSL) is the most abundant glucosinolate found in Noccaea in both batches as seen in *Figure 3.14*. With the Zn treatment on Noccaea, each glucosinolate content in leaves was found to be decreased on both of the batches when compared to their respective controls but none of them were significant. High error bars in the figure indicate the variation between the replicates used. The glucosinolate levels in control leaves also differed quite substantially between the two batches.

Although not significant, there was a trend towards lower concentrations of total GSL in plants grown with higher Zn treatment as compared to the control plants in both the batches. From the *Figure 3.15*, it can be observed that the total GSL content in the first batch for untreated leaves was around 90 µmol/g DW while, upon the zinc treatment (25µM) GSL content decreased to around 55µmol/g DW. In the second batch, total GSL content decreased from around 50µmol/g DW to 40µmol/g DW upon Zn (15µM) treatment. Here, the total GSL content is mainly due to the contribution of total aromatic GSL.

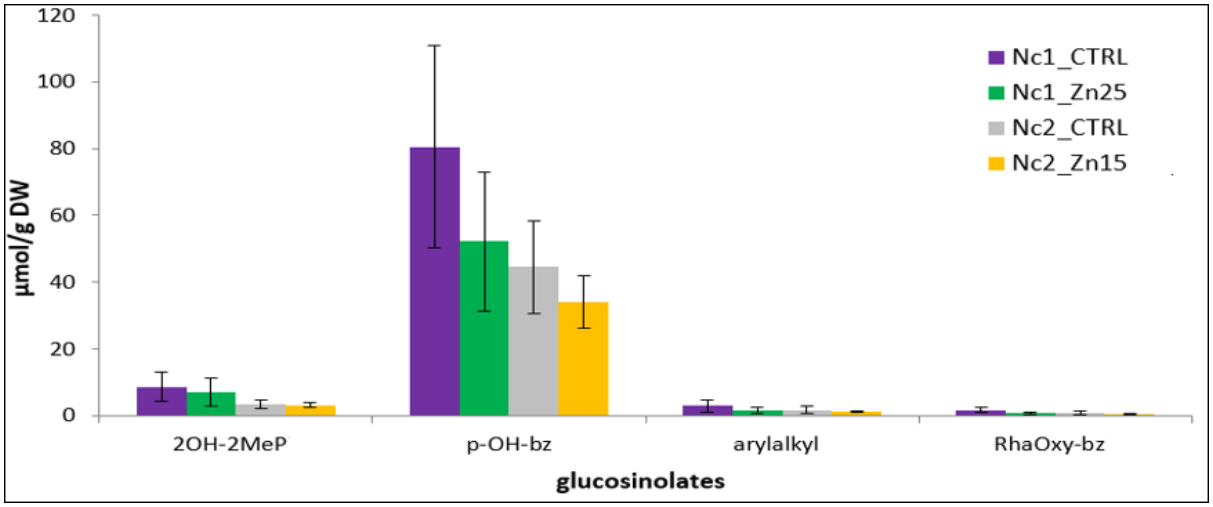


Figure 3.14 Individual glucosinolates profiles of 36 days old *N. caerulescens* Zn treated (25µM and 15µM) and control (CTRL) plants from two batches grown on modified half strength Hoagland's medium under long term (14 days) condition before harvesting. The glucosinolates content presented here are the average of four replicates and the standard deviation is represented by the error bar. Statistical treatment of data was performed using PC program Sigma plot13. Data were treated by ANOVA, significance ($p<0.05$) was calculated with Tukey test. DW: Dry weight, putatively identified glucosinolates: 2OH-2MeP: 2-hydroxy-2-methylpropylglucosinolate (glucoconringiin), p-OH-bz: p-hydroxybenzylglucosinolate (glucosinalbin), arylalkayl (unidentified GSL with UV spectrum indicating an arylalkyl structure), RhaOxy-bz: 4-α-rhamnosyloxybenzyl glucosinolate (glucomoringin).

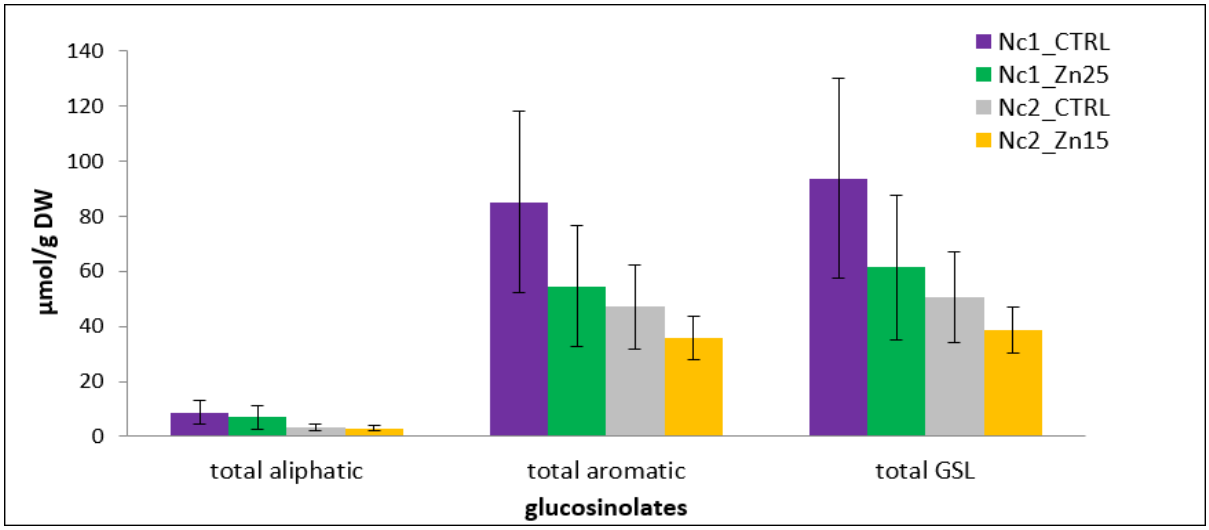


Figure 3.15 Aliphatic, aromatic and total glucosinolates content of 36 days old *N. caerulescens* Zn treated (25µM and 15µM) and control (CTRL) plants grown on modified half strength Hoagland's medium under long term (14 days) condition before harvesting. The glucosinolates content presented here are the average of four replicates and the standard deviation is represented by the error bar. Statistical treatment of data was performed using PC program Sigma plot13. Data were treated by ANOVA, significance ($p<0.05$) was calculated with Tukey test. DW: Dry weight

3.4 Cloning the coding sequences of *HIPP25* gene from *Arabidopsis thaliana* and *Noccaea caerulescens in pCRII-TOPO vector*

3.4.1 Selection of *HIPP25* gene sequences and design of primers

The full-length coding sequence of *Arabidopsis thaliana* HIPP25 (At4g35060; Genebank accession NM_119672) was retrieved from The Arabidopsis Information Resource (TAIR) and was used for sequence similarity searches (BlastN) on sequence data from *Noccaea* sp. (taxid:290573) in public database (Transcriptome Shotgun Assembly, NCBI) to determine the *Noccaea caerulescens HIPP25* gene sequence. The transcript sequences of *HIPP25* gene in *N. caerulescens* from Ganges (Lin et al., 2014) was retrieved by that time (January 2015) because the sequence from La calamine available in the database was not complete. Since the Nc*HIPP25* gene sequence retrieved by Blast from the NCBI TSA database belongs to the Ganges accession (Genbank accession GASZ01015567) while the cDNA from Nc_R2 used in the PCR reactions belongs to the La Calamine accession, some sequence polymorphisms might be expected. The At*HIPP25* full length coding sequence (462 bp) and Nc*HIPP25* transcript sequences (783 bp), are shown in *Appendix D.1, D.2*. Primers to amplify *HIPP25* from Arabidopsis and Noccaea cDNA were then designed using Primer 3 Plus (version 2.4.0) and are listed in *Appendix B.2*. For designing At*HIPP25* primer, restriction sites were added to the ends of that piece of DNA so that the restriction enzymes create compatible ends and it can be easily cloned into a plasmid of interest. Restriction site EcoR I (GAATTC) and Xho I

(CTCGAG) were added to the 5' end of the forward and reverse primer respectively. Many restriction enzymes do not cut DNA efficiently at the end of a linear piece so 2 bases upstream of the restriction site was added to improve cutting efficiency. Nc*HIPP25* transcript sequence when aligned with At*HIPP25* CDS, region from 157-628bp in Noccaea was found to be homologous with Arabidopsis. For Nc*HIPP25*, a longer fragment of Nc was amplified including 5'UTR and 3'UTR to clone full length CDS of Nc*HIPP25*. Since there is presence of another start codon (ATG) ahead of 157 bp and stop codon (TAA) after 628 bp, the primers were designed including the UTR regions *(Appendix D.3)*.

3.4.2 First attempts to amplify *HIPP25* from *Arabidopsis* and *Noccaea* samples

First set of PCR was performed on cDNA from flower and silique tissue of *A. thaliana* Col-0 and from shoot and root tissue from *N. caerulescens* La Calamine as described in *section 2.5.1* to amplify *HIPP25*-encoding sequences. The expected size of the amplicons was 478 bp for At*HIPP25* and 772 bp for Nc*HIPP25*. Bands of the expected size were obtained from Arabidopsis flower (At_F) and siliques(At_S), approximately 500 bp (*Figure 3.16a*), and one of the *Noccaea* root samples Nc_R2, approximately 700bp (*Figure 3.16b)*. From Noccaea, the Nc_S, Nc_R1 samples were discarded from the experiment and only Nc_R2 was used for further study and cloning purpose since it did not produce any bands of the expected size.

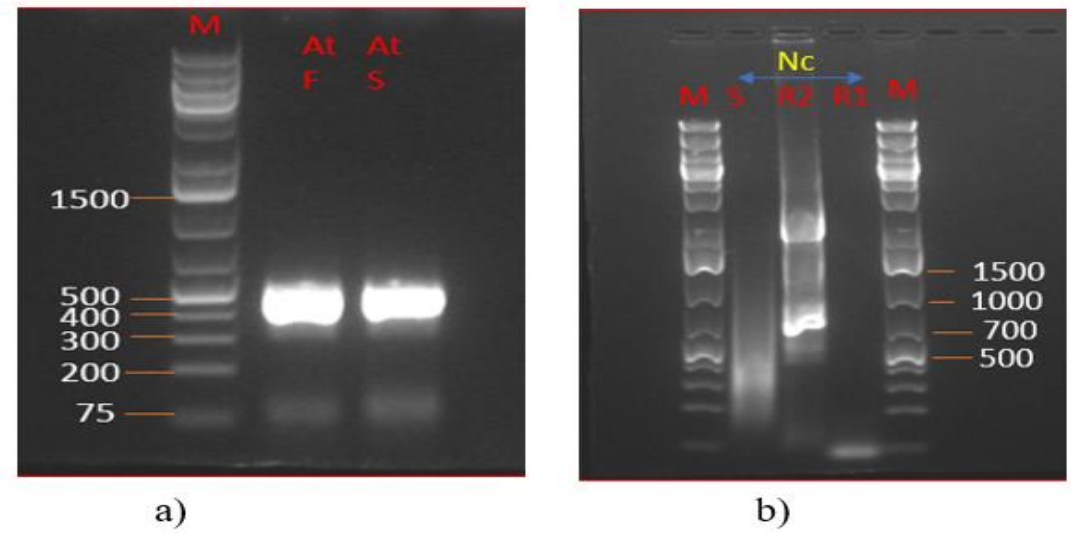


Figure 3.16 a) PCR amplification of cDNA from flowers (At_F) and siliques(At_S) of *A. thaliana* with the first set of At*HIPP25* and Nc*HIPP25* primers on cDNA (b) PCR amplification of cDNA from shoot (Nc_S) and root (Nc_R1and Nc_R2) of *N. caerulescens* with the first set of Nc*HIPP25* primers.

The PCR products described above were purified from the gel and cloned into pCRII-TOPO as described in *section 2.5.2-2.5.5*. The recombinant vectors were transformed into *E. coli* Dh5α and miniprep was performed on six colonies for each of the At*HIPP25* and Nc*HIPP25* constructs (called respectively *At_HIPP25* and *Nc_HIPP25*). The twelve plasmid preparations were submitted to a digestion with the restriction enzyme EcoR I (*section 2.5.8*) to verify the presence and size of the insert. Two bands can be seen in *Figure 3.17*, one band of lower size represents the insert and the higher size band represents the plasmid vector. The expected size of the excised insert was figured out to be around 482 bp for At*_HIPP25* and 790bp for *Nc_HIPP25*. For Arabidopsis, samples y1, y2, y3, y6 produced the bands of expected size. No band was seen for y5 which could be the result of pipetting error (addition of enzyme could have been missed). The other clone, y4 has a longer insert, probably contain an intron indicating that the cDNA used for PCR might contain some gDNA. The samples y1, y2, y3 and y6 were submitted to sequencing (*section 2.5.9*). For Noccaea, all the samples except y2 which produced the expected band size were sent for sequencing.

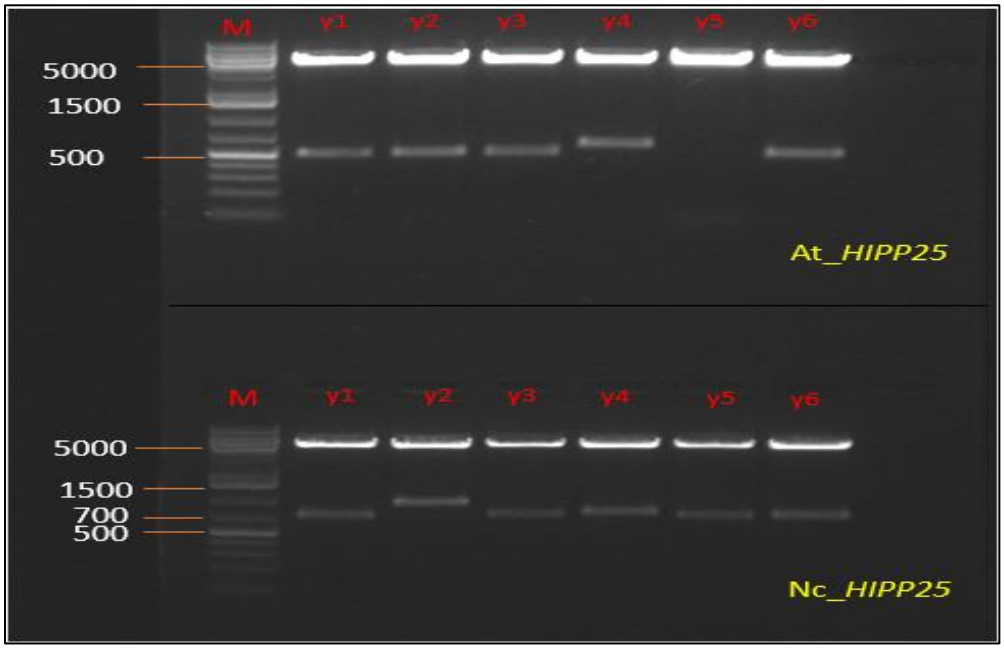


Figure 3.17 Enzyme digestion check by EcoR I on *At_HIPP25*and *Nc_HIPP25* recombinant vector clones to check the presence of insert. Abbreviations: M-Marker, At-*Arabidopsis thaliana*, Nc-*Noccaea caerulescens*, y1-y6: sample 1 to 6

The sequencing results from the four *At_HIPP25* clones were aligned to the reference sequence At*HIPP25_CDS* using MULTIALIN software (version 5.4) as shown in *Figure 3.18*. On comparing to the reference gene (At*HIPP25*_CDS), y2 contains one single nucleotide polymorphism, y3 and y6 contains two nucleotide polymorphisms. Single-

nucleotide polymorphisms (SNPs) could fall within coding sequences of genes, non-coding regions of genes, or simply occur in the regions between genes. These SNPs might change the encoded amino acids (nonsynonymous) or could remain silent due to the degeneracy of the genetic code (synonymous). If SNPs change the amino acid sequence of a corresponding proteins then it may alter the protein function which can have drastic phenotypic consequences (Ng et al., 2006). So, these clones could not be used for further study. Since only y1 with full length coding sequence was identical to the At*HIPP25*_ CDS obtained from TAIR, clone y1 was chosen for further studies like overexpressing At*HIPP25* gene in *A. thaliana* to identify its role in metal tolerance and accumulation.

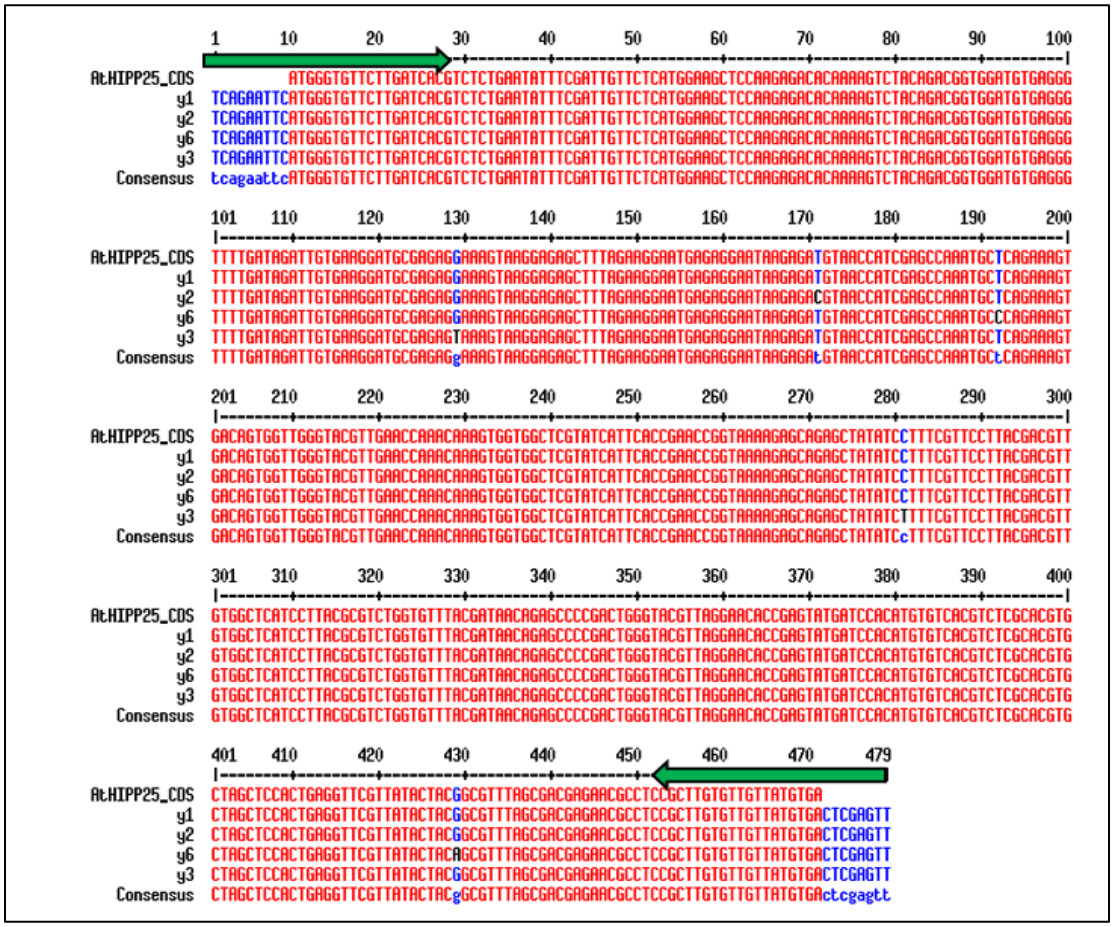


Figure 3.18 Multiple alignment of the At*HIPP25* reference sequence from TAIR (At*HIPP25*_CDS) and the sequences obtained for the four At*HIPP25* clones. A residue that is highly conserved is seen in red color and as an uppercase letter in the consensus line, a residue that is weakly conserved is seen in blue color and as a lowercase letter in the consensus line and the single nucleotide mismatch is seen in black color. Green Arrow represents the annealing site of forward and reverse primer. (Consensus: protein-coding regions that agree at the start codon, stop codon, and splice junctions, and for which the prediction meets quality assurance benchmarks (Pruitt et al., 2009)).

With Nc*HIPP25*, all the clone sequences showed much more variation when aligned to Nc*HIPP25* CDS as shown in (*Appendix Figure 7.19, 7.20).* This seemed that two different genes could have been amplified by the primers. y1 and y5 looks similar and y3, y4 and y6 looks similar. The result suggests that not *HIPP25* but some other gene has been amplified. The sequences of the clones were submitted to BlastN in NCBI which showed sequence similarity with some other genes than *HIPP25*. So, these clones could not be used for further study. Thus, the cloning was not regarded successful and it was repeated for Noccaea sample (Nc_R2) with some process optimization.

3.4.3 Optimization of PCR conditions for the cloning of *NcHIPP25*

Since the first set of PCR condition and sequencing did not produce satisfactory results with Noccaea root, Nc_R2. The process was repeated with the two conditions at the same time:

a. Second set of PCR: For, Nc_R2, primers designed for Arabidopsis *HIPP25* CDS (*Appendix B.2*) and the PCR conditions for Nc*HIPP25* as in the first set were used but the annealing temperature was increased from 50°C to 64°C to increase the primer specificity. Since the *CDS of HIPP25* of both plants are very similar (*Appendix D.3),* there was a possibility that the primers designed for Arabidopsis could work for Noccaea as well.
b. Third Set of PCR: Same Nc*HIPP25* primers as in the first set for Nc_R2 were used but the annealing temperature was increased from 50°C to 55°C to increase the primer specificity

The primers used for second and third set of PCR are mentioned in *Appendix B.2*. The second set of PCR using At*HIPP25* primers at the conditions mentioned in *Table 2.8* produced an amplicon of the expected size 462 bp while the third set of PCR using *NcHIPP25* primers at a higher annealing temperature *(Table 2.8)* than previously did not allow to amplify a product of the expected size 772 bp (*Figure 3.19a)*. Then, the PCR amplified product (Nc_R2 sample amplified with At Primers), as previously were excised and purified from the gel, and an aliquot was verified by agarose gel electrophoresis (*Figure 3.19b).*

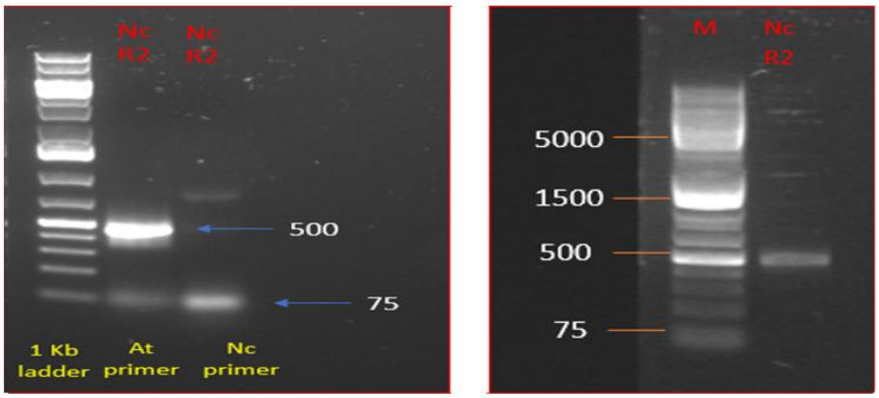


Figure 3.19 a) PCR amplification on Nc_R2 with At*HIPP25* and Nc*HIPP25* primers with the change in PCR conditions b) Presence of only one band for Nc_R2 gel clean DNA

The putative Nc*HIPP25* fragment amplified by At*HIPP25* primers was then cloned into pCRII-TOPO as described in *section 2.5.5*, the recombinant vectors were transformed (*section 2.5.6*) into *E. coli* Dh5α and miniprep (*section 2.5.7*) was performed on eight colonies. These plasmid preparations were submitted to a digestion with the restriction enzyme EcoR I (*section 2.5.8*) to verify the presence and size of the insert. The expected size of the excised insert for Nc_*HIPP25* was around 480 bp. Only three sample y1, y2, y5 produced clear bands around the expected size as seen in *Figure 3.20* and these were verified by sequencing.

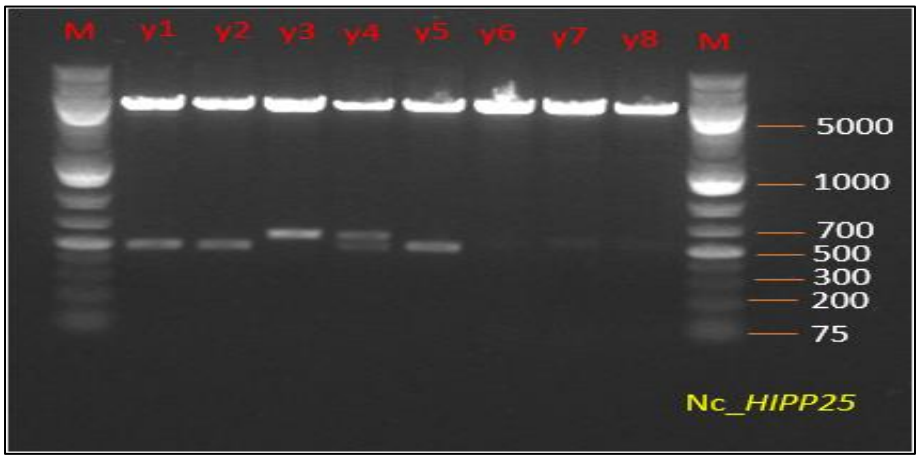


Figure 3.20 Enzyme digestion check carried by Eco*R I* on eight *NcHIPP25* clones (y1 to y8) obtained on Nc_R2 sample. M = size marker.

The Nc*HIPP25* sequences of these three clones were aligned with the Nc*HIPP25* reference sequence from Ganges using MULTIALIN software (version 5.4) as shown in *Figure 3.21*. As mentioned before, Nc*HIPP25* sequence retrieved by Blast from the NCBI TSA database belongs to the Ganges accession while the cDNA from Nc_R2 used in the PCR reactions belongs to the La Calamine accession, so some sequence polymorphisms was expected. y1, y2

and y5 contain few nucleotide polymorphisms when compared with the Ganges accession as seen in black color.

The more longer sequence from La calamine accession was later available in the database (NCBI). In addition to the transcript sequence from Ganges, we also compared the sequences of clones with La calamine sequence as the secondary reference. It was seen that the mismatch is not due to the problem during PCR amplification but due to the difference in ecotype used. y2 had one and y5 had two nucleotide polymorphisms as compared to La calamine accession but y1 had sequence similar as La calamine accession. The presence of nucleotide "G" in place of "A" near to 3' end of the clone sequence was due to the PCR amplification by the primer designed for Arabidopsis (refer to *Appendix D.2*) and not due to nucleotide polymorphism or error introduced during the PCR amplification. Therefore, partial coding sequence from Nc*HIPP25* has been cloned into y1 and it was chosen for further studies like overexpression of Nc*HIPP25* gene in *A. thaliana* to identify its role metal tolerance and accumulation.

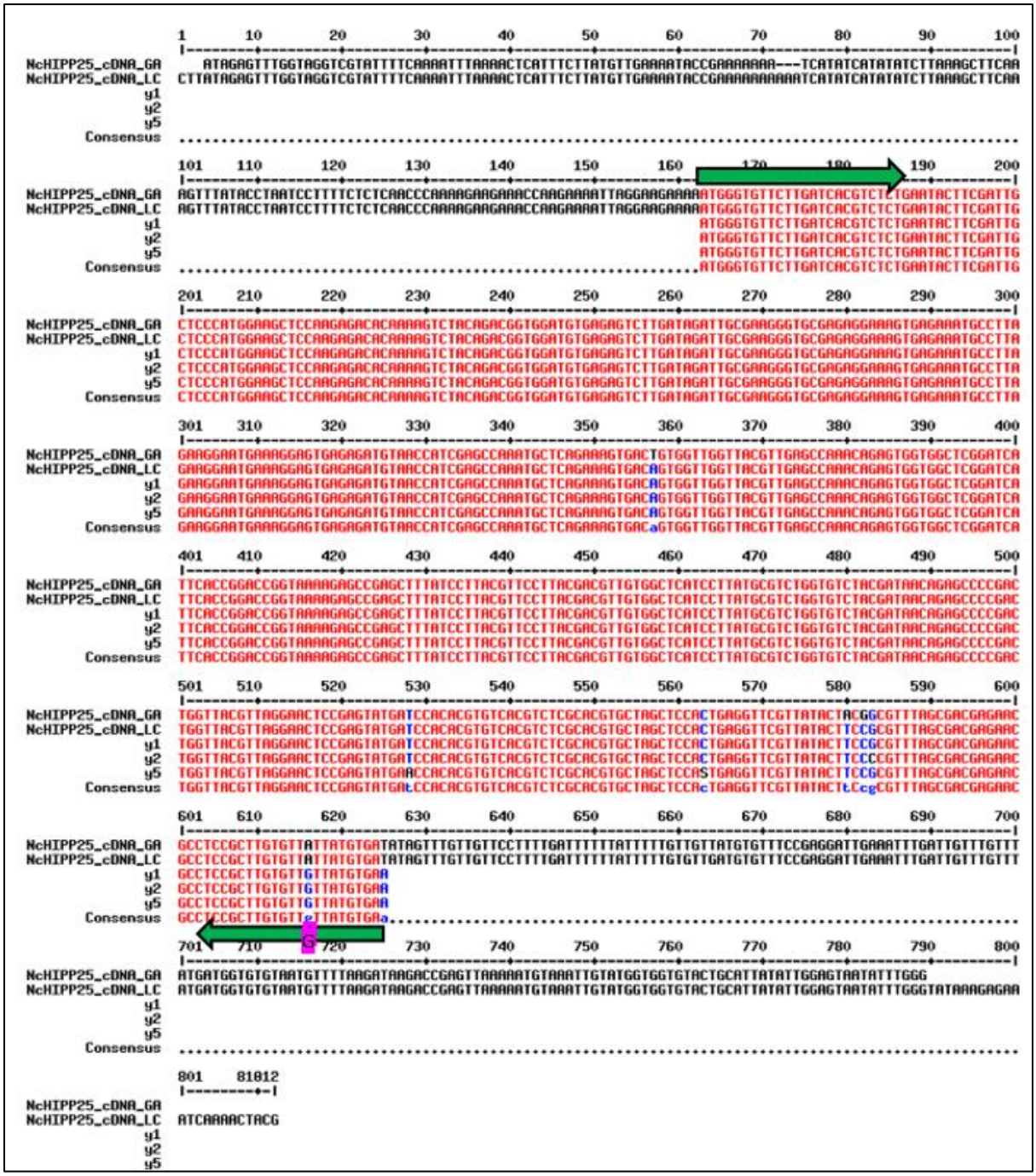


Figure 3.21 Multiple alignment of the Nc*HIPP25_cDNA* reference sequence from Ganges and La calamine and the sequences of three Nc*HIPP25* clones (y1, y2, y5). A residue that is highly conserved is seen in red color and as an uppercase letter in the consensus line, a residue that is weakly conserved is seen in blue color and as a lowercase letter in the consensus line and the single nucleotide mismatch is seen in black color. Green arrow represents the annealing site of forward and reverse primer. The ambiguity" character “S” represent the possibility of bases G and C. Abbreviations: GA: Ganges, LC: La calamine.

4 DISCUSSION

The data obtained from the results are interpreted in three main aspects:

- Change in *HIPP25* along with its phylogenetically closely related *HIPP26*, *HIPP27* gene expression by heavy metal treatments (Ni, Cu, Zn and Cd) in *A. thaliana* and *N. caerulescens* under the long term (14 days) and short term (24 hr) conditions compared to control condition (*section 4.3)*
- HIPP25 protein expression in control condition and heavy metal (Ni, Cu, Zn, Cd) treated conditions under long term (14 days) and short term (24 hr) (*section 4.4*)
- Glucosinolates analysis in both plants in control and heavy metal (Zn) treated conditions under long term (14 days) and short term (24 hr) (*section 4.5*)

4.1 Reason for the selection of plant species and *HIPP25* gene

We now know the importance of hyperaccumulators in phytoremediation and its role in the metal detoxification and accumulation. From the pool of hyperaccumulators, *Noccaea caerulescens* was selected for our experiment as it is regarded as the best model hyperaccumulator and shares the 88% sequence similarity with the coding sequence region of model plant, *A. thaliana* which is a non-hyperaccumulator (Peer et al., 2003) .

Comparing the differentially expressed genes between *A. thaliana* and *N. caerulescens* through transcriptional profiling helps us to identify the important genes involved in metal hypertolerance and hyperaccumulation (van de Mortel et al., 2006; van de Mortel et al., 2008). The involvement of some HIPPs in metal homeostasis and detoxification has been demonstrated as well. Apart from the importance of At*HIPP26*, At*HIPP27* in metal detoxification another gene from the same cluster IV of HIPPs gene family, At*HIPP25*, is still functionally unknown. The involvement of At*HIPP25* in metal tolerance is indicated by a higher gene expression of At*HIPP25* in *N. caerulescens* than in *A. thaliana* which was identified from published cDNA microarray data (van de Mortel et al., 2006). Therefore, to explore the possible function of *HIPP25* gene on different metal tolerance and accumulation, this gene along with its phylogenetically closely related genes *HIPP26*, *HIPP27* was selected for the study.

To our knowledge no data concerning *HIPP25* gene/protein level in different tissues/ organ of *A. thaliana* are available, but from HIPP25p-GUS expression results reported in the literature, one could postulate that in normal conditions HIPP25 would be higher in roots than in leaves,

flower and siliques (Tehseen et al., 2010). On the other hands, *HIPP25* gene expression is found to be higher in flowers than in the leaves and roots as per the gene expression map obtained from TAIR (*Appendix Figure 7.8*). In *N. caerulescens, HIPP25* gene expression is found higher in roots (van de Mortel et al., 2006). Thus, the tissues from root, leaves, flowers and siliques from *A. thaliana* whereas, root and leaves from *N. caerulescens* were selected for the experiment. The flowers and siliques were harvested together as the tissues only from the flowers were not enough to carry out the analysis. It takes around 22 weeks for *N. caerulescens* to produce flowers under the normal condition so, only root and leaves were used for the present study.

4.2 Possible reasons for the poor RNA quality

Compromised RNA quality is suggested to lead to unreliable results in gene expression studies (Vermeulen et al., 2011). Therefore, assessment of RNA integrity and purity is very essential prior to gene expression analysis. The quantity and purity of RNA isolated from both the plants Arabidopsis and Noccaea was assessed in Nanodrop, where samples with A260/A280 ratio above 2 and A260/A230 above 1.5 was obtained and stored at -80°C until use.

Then, the integrity of RNA samples was checked in the denaturing RNA agarose gel for Arabidopsis root, leaves and flower and siliques and Noccaea root and leaves. We have not included positive control or RNA molecular weight marker which would give an exact size of RNA as we just wanted to see the quality of RNA and the difference of heavy metal treatment on RNA quality. As seen from the *Figure 7.1-7.5*, the RNA does not seem to be degraded under long term condition but under short term condition, Cu 25µM treatment might have effect on the RNA quality of *A. thaliana* roots which is predicted from the smearing of bands in all replicates. In *N. caerulescens* roots, again the band intensity was very low for all the replicates treated with Cu 25µM. It could be either due to the problem of the overestimation of the quantity of RNA which means it was thought that 1µg of RNA was loaded in the gel but it was less amount being loaded which might have resulted in the lower intensity of the bands. The next possibility could be the effect of Cu treatment on rRNA quality. As RNA becomes degraded, the quantitative expression levels determined by RT-qPCR also decreases, but this would have been case only if the RNA degradation is homogenous i.e. if the target mRNA is as degraded as the rRNA. In that case, the reference genes should have been affected by the treatments as well. But, the reference genes observed in our case did not seem to have distinct effect upon the Cu treatment.

Little smearing of the bands was seen in few other replicates of metal treated tissues (not in all the replicates), which might indicate some effect in the RNA quality but they are not distinct enough to be specified as degraded RNA. A possible explanation could be the repeated thawing and mishandling of the samples could have caused some change in RNA quality. Use of some other techniques like bioanalyzer which relies more in the quantitative data might be of help in verifying the effects on RNA quality with the metal treatments.

4.3 Expression of *HIPP25, HIPP26, HIPP27 gene* in different tissues of *A. thaliana* and *N. caerulescens* after short term and long metal treatments

From the literature reported, 45 heavy metal-associated isoprenylated plant proteins (HIPPs) are present in *A. thaliana* and only few HIPPs has been studied so far (Abreu-Neto et al., 2013). From another study, it has been found that the transcription of many HIPPs is altered in response to heavy metal stresses, which indicates that HIPPs may be involved in homeostasis of these elements (Abreu-Neto et al., 2013). But, *HIPP25* gene in particular has not been characterized yet and only the putative function is known which is supposed to be involved in metal homeostasis. No paper has been published confirming the function or involvement of *HIPP25* gene in metal homeostasis. Very few papers have been published for its closely related gene, *HIPP26* and *HIP27*, for its involvement in metal tolerance and detoxification. Since, *HIPP25* gene belong to the same cluster IV of the HIPP gene family and have high sequence similarity, the putative function of *HIPP25* gene in our experiment, is studied in conjunction with the information reported in the literature for *HIPP26* and *HIPP27*. The study of gene expression of the main target gene *HIPP25* and its phylogenetic neighboring genes *HIPP26, HIPP27* were analyzed by exposing *A. thaliana* and *N. caerulescens* plants to four heavy metals (Ni, Cu, Zn and Cd) over short term exposure (24 hr) and long-term exposure (14 days) and compared with their respective controls.

4.3.1 Target genes expression in *A. thaliana*

a. Short term treatment

Arabidopsis plants treated with different heavy metals (Ni 100µM, Cu 25µM, Zn 50µM, Cd 30µM) for short term when compared with respective controls, none of the metal treated plants gene expression were found to be significant (*Table 3.1)*. Although, the gene expression level was not significant the three *HIPP* genes showed a different response, as it was found that

HIPP25 and *HIPP26* gene was several folds downregulated in roots with Ni 100µM and Cu 25µM whereas *HIPP27* was upregulated by the same treatments. In leaves as well as flowers and siliques however *HIPP26* seemed to respond differently from *HIPP25* and *HIPP27* to these two heavy metals by showing a tendency to be downregulated.

The Cd 30µM treatment did not have much effect on *HIPP25* gene expressions or reduced slightly after 24h, but induced *HIPP27* expression two-fold in flowers and siliques (although not significant). This is different from the induction of *HIPP26* expression in roots and to lesser extent in shoots of *A. thaliana* exposed to 1mM Cd for 24h reported by Gao et al (Gao et al., 2009). In our study, Cd treatment reduced *HIPP26* whereas induced *HIPP27* gene expression of *A. thaliana* but was not significant enough.

Gao et al. (2009) also reported an induction of *HIPP26* by 1 mM Zn for 24 hr in both root and shoots, while under treatment with 50 µM Zn in our case the expression of *HIPP26* was downregulated in root and somewhat upregulated in flower and siliques. *HIPP25* responded similarly to *HIPP27* where the expression was higher in flower and siliques, slightly induced in leaves and had no change in the roots.

b. Long term treatment

There were two rounds of 14 days' exposure of heavy metal treatments in *A. thaliana* grown in Hoagland's medium where the first round (Cu 25µM, Zn 25µM, Cd 10µM) had the higher concentration of heavy metals than the second (Ni 80µM and 50µM, Cu 15µM, Zn 15µM, Cd 5µM) (*Table 3.2*). Upon Cu 25µM treatment, the root tissues were highly affected and they were not enough for RNA extraction so the data for roots is not available. Only few treatments led to significant changes in expression of target gene *HIPP25*. In leaves of *A. thaliana*, *HIPP25* gene was induced by Cu 25µM and Cd 5µM treatment whereas Ni 80µM treatment reduced the expression.

The effect of the long-term Cu 15µM treatment on the *HIPP25* gene was less obvious but mirrored that of the long term and short-term treatments with Cu 25µM (i.e. upregulated in leaves and downregulated in roots). Also, under the phenotypic observation of plants grown in different metals (*Figure 7.13-7.16*), it was seen that Cu treatment strongly reduced the growth of roots. These results might indicate *HIPP25* gene involvement in Cu homeostasis.

Upon Zn treatment (25µM and 15µM) *HIPP25* gene was upregulated in root and leaves, the lowest concentration having the biggest effect. This is different from the short-term treatment with 50µM Zn where *HIPP25* gene was not affected in roots and only slightly upregulated in

leaves. On phenotypic level of plants treated with Zn, the roots were in very good condition and seems to very little affected by the treatment.

Under 10µM and 5µM Cd exposure, the *HIPP25* gene was slightly downregulated in roots but upregulated in leaves. This is different from the short-term treatment with 30µM Cd where *HIPP25* was slightly downregulated in leaves. *HIPP25* is described as possible being involved in Cd transport and detoxification in the UNIPROT database, but whether this is true or not remains to be proven experimentally.

With Ni treatment, the *HIPP25* gene was downregulated in leaves and roots and upregulated in flower and siliques of plants exposed to the highest concentration (80 µM). While *HIPP25* was not affected in leaves by Ni 100µM under the short-term treatment, it responded similarly in roots and flowers and siliques.

The closely related gene *HIPP26* was downregulated around two-fold by Zn in flower and siliques, and to a lesser extent in the other tissues. The other treatments did not affect *HIPP26* in a consistent manner, when comparing the two applied concentrations and/or the short to the long-term treatment. *HIPP27* expression in roots was induced by Ni and Cu, as was already observed under short term exposure. The other heavy metals had a less consistent effect on *HIPP27* expression.

4.3.2 Target gene expressions in *N. caerulescens*

a. Short term treatment

In the hyperaccumulator Noccaea, treated with different heavy metals (Ni 100µM, Cu 25µM, Zn 50µM, Cd 30µM) for short term (*Table 3.3*) when compared with non-treated controls, the *HIPP25* and *HIPP26* genes responded somewhat differently from their homologs in *A. thaliana* to the different heavy metal treatments. This was especially obvious for *HIPP26* that was found to be significantly induced in *N. caerulescens* roots upon Cu treatment, while *HIPP26* was downregulated in *A. thaliana* roots. On the other hand, *HIPP25* responded to Cu effect in an equivalent way in both species, with a decrease in expression in roots and an increase in leaves. The down regulation in roots of *HIPP25* and *HIPP26* by Ni and of *HIPP26* by Zn seen in *A. thaliana* was not observed in *N. caerulescens*.

b. Long term treatment

There were two rounds of 14 days' exposure of heavy metal treatments in *N. caerulescens* grown in Hoagland's medium where the first round (Ni 80 µM, Cu 25µM, Zn 25µM, Cd 10µM) had the higher concentration of heavy metals than the second (Ni 50 µM, Cu 15µM, Zn 15µM,

Cd 5µM), see *Table 3.4.* The root tissues treated with Cu 25µM were highly affected and they were not enough for RNA extraction so the data for roots is not available. As none of the gene were found to be significantly affected by any metal treatments, except *HIPP26* gene in roots treated with Ni 80µM which was downregulated, the few observed differences must be interpreted with caution. Overall the gene expression of *HIPP25* and *HIPP26* in *N. caerulescens* seemed to be less responsive to heavy metal treatments than the *A. thaliana* homologs.

As observed for the Cu treatments on *A. thaliana* and the short-term Cu treatment on *N. caerulescens*, Cu also led to a reduced *HIPP25* expression in roots of *N. caerulescens* after long term exposure. An increase of *HIPP25* expression in leaves was however not observed under these conditions. The possible reason for leaves to be upregulated in short term is that leaves are less affected by metal treatment within 24 hr while the Cu has more effect on the roots which are in direct contact with the medium. *HIPP26* seems to be differently affected by Cu in roots than *HIPP25* as its expression was induced in the short term with 25µM and long term with 15µM Cu.

4.3.3 Inconsistent result observed in *HIPP25, HIPP26*, *HIPP27* gene expression in both plants

Based on our experiment, the results are not consistent and significant enough under both long term and short-term treatments to make any valid conclusion regarding the expression of *HIPP25, HIPP26, HIPP27* genes. However, some predictions could be performed. Under long term condition with both concentrations, Cd and Cu and Zn treatment led to higher expression and Ni treatment led to lower expression of *HIPP25* gene in leaves of *A. thaliana* indicating that *HIPP25* could involve in metal homeostasis. Zn is possibly involved in the metal tolerance and accumulation as the expression of roots and leaves treated with Zn under long term condition is higher. Also, *HIPP25* seems to be involved in Cu homeostasis as the gene is induced in leaves both under the long term and short term in *A. thaliana,* and reduced in roots (long term and short term) in *A. thaliana.* In *N. caerulescens*, *HIPP25* gene under Cu treatment is reduced in roots (long term and short-term) and induced in leaves under short term but not long term. There are a lot of possible reasons for the difference in results from first batch to second under the long-term conditions.

The most likely reason could be the difference in metal concentration used from first batch to second, first batch having higher concentration than the second. Different concentration of

metals could have effect on the gene expression differently. While making comparison with the literature and information found in the database, a lot of factors might have led to different results between the studies. For example, experimental set up, the conditions the plants were grown, developmental stage of the plant, harvesting time, the medium used for the growth of the plants where in our case is hydroponics while in most of the information found in the database belongs to the plant grown in soil or MS medium, the age of the plants etc. For example, as shown in *Table 3.5*, *A. thaliana* plants grown in MS medium when treated with Ni 80µM and Cu 25µM had different *HIPP25* gene expression as compared to the plants grown in Hoagland's medium.

4.4 Expression of HIPP25 and actin proteins in *A. thaliana* and *N. caerulescens* under different metal treatments

After isolation of RNA; flower and siliques, root tissues were not enough to perform protein extraction. The time limitations opposed us to grow new plants which takes 36 days for harvesting the tissues. Thus, protein extraction was performed with all the available leaf tissues under long term treatments and root, leaf tissues under short term treatments from both *A. thaliana* and *N. caerulescens*.

The qPCR results obtained for *A. thaliana* were not always consistent, e.g. gene expression turned out to be significant in lower concentration of one metal but insignificant in higher concentration. Even though significant changes in *HIPP25* expression could not be seen at the gene level this does not preclude the possibility of change in expression at the protein level. It's quite reasonable to see higher protein level with higher expression of gene level but, the protein level sometime might be affected by many steps in transcription, translation or degradation. For example, post translational modification like phosphorylation has been suggested to change the mechanism that control the cell division and differentiation in the animal cells which will consequently affect the protein level. Though it's in the animal cell, but it has also been found that mRNAs are not equal with regard to translation into proteins (Kendrick, 2014). So, all the metal treated available samples were included for western blot analysis even though it did not turn out significant from the qPCR analysis.

The molecular weight of HIPP25 proteins were predicted to be 17.16 kD and the closely related HIPP26, HIPP27 proteins also have less than 20 kD (TAIR). The Western blot results described in *section 3.2* showed that in most samples a single immunoreactive band with an apparent size above 30 kD was observed with the commercial anti-HIPP25 antibody. Observation on the

band size creates the question whether the proteins detected was HIPP25 or not. In addition, the presence of two protein bands seen in the membrane for some samples increased the suspicion for the presence of HIPP25. However, both bands did not produce the expected band size, therefore both bands seen on the membrane might not be HIPP25 but some other proteins similar to HIPP25 that might had cross reacted with antibody. Also, the reason could have been that HIPP25 protein might be present but it is expressed very low that they were not seen on the blots.

However, the Western blot results will be discussed in this section with the assumption that the ~34 kD protein detected was HIPP25 inspite of higher band size. The proteins were seen very clearly on the membrane for anti-HIPP25 antibody. The general trend seen in the membrane was higher expression of proteins in metal treated tissues than control ones which indicates towards the HIPP proteins. It was more likely that the proteins were HIPP25 although the band size was not same. The lower band seen on the blots is most probably the nonspecific binding.

One of the possible reason for the higher band size seen could be due to the nature of post translational modifications like phosphorylation, acetylation (Guan et al., 2010; Tarrant et al., 2009). As HIPP25 is isoprenylated protein which is also a form of post translational modification, it was speculated that the proteins that run on SDS page still remains isoprenylated and the modifications by isoprenyl group could have increased the band size. This increased band size protein was recognized in both root and leaves of *A. thaliana* and *N. caerulescens*. However, this is just an assumption and supporting literatures could not be found for the increased band size for the isoprenylated protein.

The next probable reason could be that the protein might not have been denatured enough with the denaturation dye. Incomplete unfolding might have influence the electrophoretic mobility that could have increased the band size. Also, the observed MW of a protein might differ from the theoretical value when the protein binds more or less SDS than it should on average due to amino acid composition (Shi et al., 2012).

Three antibodies raised against peptides representing the N-terminal (N), C-terminal (C) and non-terminus (M) regions of HIPP25 were purchased from the company. Although the three antibodies were used in preliminary assays and indicated immunoreactive bands of the same size, the Western blots shown here were all produced using the N terminal antibody. As the exact N-terminal peptide sequences used to raise the antibody are not revealed by the supplier,

the possibility exists that another, larger protein is recognized by this antibody. Further Western blot analyses using the two remaining antibodies might give some answer.

Western blot analysis of an *A. thaliana* mutant which does not produce HIPP25 proteins might also allow us to conclude whether the immunoreactive band is HIPP25. Another possibility could be the use of the antibody in an immunoprecipitation assay to remove out any proteins that stick to HIPP25 antibody and run Mass Spectrometry. Third possibility is to use the amino acid sequence of HIPP25 available from the database and, then produce the recombinant protein in *E. coli*, purify it and run along the plant protein extract in the gel. If both proteins have same band size then it indicated that it is HIPP25 protein. Unfortunately, limited time did not allow us to test any of these approaches.

Although the amount of starting material to be loaded in the well was equalized, but the possible reason for difference in intensity of bands seen for the actin (reference protein) could either be unequal loading of the sample volume in the wells (due to the pipetting error) or the actin proteins could have been affected by metal treatments (especially Cu) in both A. *thaliana* and *N. caerulescens*, see *Figure 3.4- 3.6*. Based on the RT-qPCR analysis, actin 2 gene was not affected by the treatments but the proteins might have been affected at the post transcriptional regulation. Use of another antibody might give some more confirmative results.

4.4.1 Expression of *HIPP25 protein* in leaves of *A. thaliana* under short-term and long-term metal exposure and compared to its transcript level

Under short term metal exposure (*Figure 3.7*), protein expression was higher with Cd(30µM) treatment and lowered upon Ni (100µM) and Cu (25µM) treatment whereas the gene expression was higher upon the Cu treatment but lowered upon the Cd and Ni treatment. The protein expression only fit the RT-qPCR data (*Table 3.1*) from Ni exposure.

Under long term condition *(Figure 3.4)*, HIPP25 protein expression in leaves was higher with Zn 25µM, Zn 15µM and Cd 10µM treatment than under the control treatment, which is in line with RT-qPCR data *(Table 3.2)*. Although gene expression was higher with Cd 5µM, leaves treated with Cd 5µM seems to be expressed slightly lower on protein level than control in the second batch(At2). The Ni treatments seemed to lead to lower HIPP25 protein levels (*Figure 3.4*), which fits the RT-qPCR data (*Table 3.2*) where *HIPP25* gene is downregulated in leaves with Ni treatment. But, for leaves treated with Cu 15µM although not significant, the gene was found to be upregulated. However, protein levels seemed to decrease which indicates that Cu treatment might have some effect in the proteins.

Although it is difficult to conclude the particular effect without significant results, but based on the combined results from RT-qPCR and western blot (*Table 4.1*), *HIPP25* gene is possibly involved in accumulation of Zn in *A. thaliana*, as the expression *of HIPP25* gene and protein in leaves is higher as compared to control plants and *HIPP25* seems to be downregulated by Ni exposure.

Table 4.1 *HIPP25* gene and protein expression in leaves of *A. thaliana* in response to different metal treatments under both long term and short-term condition. Significant change in gene expression are marked by *. Orange color represents the decreased gene and protein expression and green color represents increased gene and protein expression.

Metal treatments	*HIPP25* Gene expression			HIPP25 Protein expression		
	Short term (24 hr)	*Long Term (14 days)*		*Short term (24 hr)*	*Long Term (14 days)*	
Ni	Ni 100µM Decreased	**Ni 80µM Decreased** *	Ni 50µM Decreased	Ni 100µM Decreased	Ni 80µM Decreased	Ni 50µM Decreased
Cu	Cu 25µM Increased	**Cu 25µM Increased** *	Cu 15µM Increased	Cu 25µM Decreased	Cu 25µM No data	Cu 15µM Decreased
Zn	Zn 25µM Increased	Zn 25µM Increased	Zn 15µM Increased	Zn 50µM No data	Zn 25µM Increased	Zn 15µM Slightly Increased
Cd	Cd 30µM Decreased	Cd 10µM Increased	**Cd 5µM Increased** *	Cd 30µM Increased	Cd 10µM Increased	Cd 5µM Slightly decreased

4.4.2 Expression of HIPP25 protein in leaves of N. caerulescens under short- term *and long-term metal exposure and compared to its transcript level*

Although the antibody was produced against peptides of *A. thaliana* HIPP25, an immunoreactive band of an apparent similar size was detected in *N. caerulescens*. Based on the high amino acid sequence similarity between HIPP25 from these two species (*Appendix D.4*), this was expected and we therefore assume that the antibody has similar affinities against HIPP25 from both species and that the immunoreactive band detected in *N. caerulescens* corresponds to HIPP25.

Under short term condition (*Figure 3.7*), HIPP25 protein expression in leaves was higher with Cd treatment, remained almost same with the Ni treatment and slightly reduced with Cu treatment but *HIPP25* gene expression with Cd remained almost same and increased gene expression upon Cu exposure (*Table 3.3*). Only RT-qPCR results by Ni treatment seems to fit the western blot results.

Under long term condition *(Figure 3.5),* HIPP25 expression was higher for Zn and Cd treated leaves than for Ni and control. No HIPP25 bands were seen with Cu treatment, indicating a

decrease in HIPP expression levels, but this might also be due to lower protein loading for Cu-treated samples as shown by the anti-actin antibody. Hence, while the HIPP25 protein in leaves is higher expressed with Cd, Zn and Ni treatment than that of control leaves it can be seen from the RT-qPCR data in *Table 3.4*, that the gene expression ratio is almost close to one indicating that the metal treatments did not have much effect on the *HIPP25* gene expression.

As seen in *Table 4.2*, we can see that western blot results are not in line with RT-qPCR results for *HIPP25*. It could be that due to the post-transcriptional regulation, the proteins expressed despite it remained somewhat unchanged at the gene level.

Table 4.2 *HIPP25* gene and protein expression in leaves of *N. caerulescens* in response to different metal treatments under both long term and short-term condition. Orange color represents the decreased gene and protein expression and green color represents increased gene and protein expression.

Metal treatments	*HIPP25* Gene expression			HIPP25 Protein expression		
	Short term (24 hr)	*Long Term (14 days)*		*Short term (24 hr)*	*Long Term (14 days)*	
Ni	Ni 100µM Almost same	Ni 80µM Almost same	Ni 50µM Slightly increased	Ni 100µM Almost same	Ni 80µM Increased	Ni 50µM Increased
Cu	Cu 25µM Increased	Cu 25µM Almost same	Cu 15µM Almost same	Cu 25µM Slightly decreased	Cu 25µM Decreased	Cu 15µM Decreased
Zn	Zn 50µM Almost same	Zn 25µM Almost same	Zn 15µM Almost same	Zn 50µM No data	Zn 25µM Increased	Zn 15µM Increased
Cd	Cd 30µM Almost same	Cd 10µM Almost same	Cd 5µM Slightly decreased	Cd 30µM Increased	Cd 10µM Increased	Cd 5µM Increased

4.4.3 Comparative expression of HIPP25 proteins in *A. thaliana* and *N. caerulescens* under long-term exposure in leaves and under short-term metal exposure in leaves and roots

A direct comparison of *HIPP25* between *A. thaliana* and *N. caerulescens* was not performed at gene level. The indirect comparison of amplification cycle of *HIPP25* gene from qPCR results showed similar Cq values for both Arabidopsis and Noccaea. However, comparison of HIPP25 protein levels between them indicates that HIPP25 proteins in leaves are more expressed in hyperaccumulator *N. caerulescens* than non-hyperaccumulator *A. thaliana* both in the control condition and metal treated conditions except Cu (*Figure 3.7*). HIPP25 proteins in leaves were found to be expressed very low with Cu treatment for both plants. Comparable

results, were obtained under short term exposure (*Figure 3.8*), where HIPP25 protein expression was higher in *N. caerulescens* than in *A. thaliana* in both root and leaves.

Among all the metals, Zn treatment have been found to increase the protein expression in leaves of both *A. thaliana and N. caerulescens* indicating its possibility in metal accumulation. Possible role of *HIPP25* in metal tolerance is also indicated by a higher gene expression of At*HIPP25* in *N. caerulescens* roots than in *A. thaliana* roots under control and zinc treated condition from published cDNA microarray data (van de Mortel et al., 2006). Comparable result was observed in our experiment, where the HIPP25 protein level in roots was higher in *N. caerulescens* than *A. thaliana* under short term metal (Ni) treatments as well as the control roots (*Figure 3.8*). However, further experiments are required to confirm this hypothesis.

4.5 Effects of Zn treatment on glucosinolates content in *A. thaliana* and *N. caerulescens under long-term and short-term exposure*

Glucosinolates production takes place in almost all *Brassicaceae* species against attack by pathogen and herbivores. *A. thaliana,* a non-hyperaccumulator and *N. caerulescens, a hyperaccumulaor* is no exception to this. Several investigations have shown that the accumulation of high Ni or Zn concentrations can protect the plants against attacks by pathogens and herbivores (Boyd & Martens, 1994; Boyd, Shaw, et al., 1994; Ghaderian et al., 2000; Pollard et al., 1997). In our experiment, the hydroponic medium in which the plants were grown already contains Zn required for plant growth and metabolism. So, the main aim of this investigation was to determine whether the GSL content would be modified by increasing Zn concentration under two conditions: long term exposure (14 days) and short-term exposure (24 hr) for both plants. The amount of root tissue that was harvested was insufficient to carry out the experiment so only leaf samples were used for the analysis. For most of the analysis, retention time and area is the basis of the desulfo-GSLs identification. However, it is often useful to associate retention times with UV spectra or other spectrometric data to ascertain identification (Wathelet et al., 2004).

4.5.1 Complicated effect of Zn on glucosinolates in *A. thaliana*

Preliminary results from glucosinolate analysis of *A. thaliana* plants grown for 21 days on solid half-strength MS medium supplemented with 25µM $ZnSO_4$, performed during a previous master thesis, indicated that Zn increased the glucosinolate content compared to the control treatment. From our study, 7 glucosinolates were identified; four aliphatic and three aromatic. The four aliphatic GSL identified were 3MSP (3-Methylsufinylpropylglucosinolate), 4MSB

(4-methylsulfinylbutylglucosinolate), 7MSH(7-methylsulfinylheptylglucosinolate), 8MSO(8-Methylsufinyloctylglucosinolate), and three aromatic glucosinolates were I3M(Indol-3-ylmethylglucosinolate), 4MeO-I3M(4-methoxyindol-3-ylmethylglucosinolate), 1MeO-I3M: (1-methoxyindol-3-ylmethylglucosinolate).

The most abundant GSL found in *A. thaliana* (Col-0) was 4MSB. While the treatment with 25µM Zn did not much affect the individual GSLs (*Figure 3.10*) or the total profile (*Figure 3.11*), each individual glucosinolates content increased with the 15µM Zn treatment and the total GSL content increased from around 15µmol/g DW to 32µmol/g DW. The different results on two batches makes the interpretation challenging, and further assays will be needed to corroborate these results. The reason for the vast difference in GSL accumulation in control plants for the first batch and the second batch is unknown. Indeed, the short term (24 hr) treatment with 50µM Zn led to an overall decrease in glucosinolate (*Figure 3.8,3.9*).

Not much relevant information regarding the effect of Zn treatment on glucosinolate levels in *A. thaliana* is available. In the study done in *Brassica napus*, Gluconapin (3-butenylglucosinolate) which accounted for nearly 90%, was the only aliphatic GSL which was influenced upon the zinc treatment. It decreased linearly on increasing of the zinc concentration which was highly correlated to Zn toxicity. For the indole GSL, Glucobrassicin (Indol-3-ylmethylglucosinolate) increased and decreased linearly upon the Zn concentration while the aromatic GSL , Gluconasturtin (2-Phenethylglucosinolate), exhibited the quadratic response to increasing Zn (Coolong et al., 2004). On the other hand, studies on *Brassica oleracea* var. *capitata* indicated that the levels of individual glucosinolates as well as the sum of aliphatic GSL, indolic GSL and total GSL increased upon Zn treatment. Increased Zn supply induced an accumulation of total amino acids, the and increased the enzymatic activities involved in sulfur assimilation and synthesis of phenols. As GSL are rich source of sulfur, this led to increase in a foliar accumulation of glucosinolates and phenolic compounds (Barrameda-Medina et al., 2017; Kusznierewicz et al., 2012).

4.5.2 Interactions between Zn and glucosinolates as defense mechanisms in *Noccaea caerulescens*

Relatively high total glucosinolate concentrations have been reported in metal hyperaccumulator species of the Brassicaceae family (Ernst, 1990). In the study done by Mathys (1977), Zn resistant *Thalpsi alpestre* contained twice the amount of benzylglucosinolate as the sensitive one and the glucosinolate was expected to play a role in

Zn tolerance mechanisms. A separate research group demonstrated that GSL production increased in shoot tissue with Zn treatments (Foroughi et al., 2014). In contrary to this, from the study done by Tolra et al. (2001), total GSL content in *Noocaea caerulescens* exposed to Zn, decreased in shoots with increasing Zn concentrations, whereas in roots it increased with increasing Zn concentrations. It was found that Zn hyperaccumulation decreased sinalbin (aromatic GSL; p-OH-bz) concentrations in shoots whereas in root sinalbin concentrations increased with Zn accumulation. Another study confirmed that GSL concentration in shoots decreased when the concentration of Zn in leaves increased (Asad et al., 2015). Thus, both kind of results have been found in the literature.

In our study, four glucosinolates were putatively identified in the leaves of *N. caerulescens*, three indolic and one aliphatic in both long term and short-term conditions. The three aromatic (indolic) glucosinolates identified were p-OH-bz (p-hydroxybenzylglucosinolate), RhaOxy-bz (4-α-rhamnosyloxybenzyl glucosinolate), arylaklayl (based on UV spectra) and one aliphatic glucosinolate identified was 2OH-2Mep (2-hydroxy-2-methylpropylglucosinolate). The investigation under long term exposure was done in two batches where the plants were treated with higher Zn concentration in the first batch (Zn 25µM) than the second (Zn 15µM).

Quantification of individual glucosinolates showed that the p-OH-benzylglucosinolate (sinalbin) which belongs to aromatic class was by far the most abundant glucosinolate present in leaves of *N. caerulescens Figure 3.14* in both batches. Exposure to high Zn concentrations decreased the accumulation of each aromatic and aliphatic glucosinolates identified in shoots as well as the total amount of GSLs, although none of the differences was significant (*Figure 3.15*). Zinc-induced reduction of the total glucosinolate contents in shoots can mainly be attributed to changes in the concentrations of p-OH-benzyl aromatic glucosinolate. Changes in the levels of RhaOxy-bz, arylalkayl- and 2-OH-2MEp caused by Zn hyperaccumulation were quantitatively less important. Like *A. thaliana*, the reason for the vast difference in GSL accumulation in control plants i.e. 15µmol/g DW for the first batch and 8µmol/g DW for the second batch is unknown.

Results reported here, however, do not support a general relation between the potential for metal hyperaccumulation and constitutively high glucosinolate contents. This might be because plants with high foliar concentrations of Zn do not need to synthesize glucosinolates as the production of GSL is metabolically expensive and biosynthesis of GSL involves a series of complex pathways (Halkier et al., 2006). This gives credence to the trade-off hypothesis which states that the plants containing high concentrations of metals do not need to produce secondary

metabolites as defense. However, that other aspects of foliar chemistry might also be linked to GLS content.

The next possible reason for the reduction of GSL content in Zn induced plants might be due to the changes in either or both the availability of amino acid precursors (Cakmak et al., 1989; Domingo et al., 1992) for glucosinolate synthesis or the alteration of one of the substrate specific biosynthetic steps in the synthesis of GSL with different side chains. A specific influence of Zn in glucosinolates was observed in which the levels of the p-OH-benzylglucosinolate (tyrosine-derived) increased in roots, but both the indolylglucosinolates and the methionine-derived alkenylglucosinolate that are synthesized from tryptophane was decreased (Tolra et al., 2001).

In contrast to a decrease in GSLs upon a long-term exposure (14 days) to 15µM and 25µM Zn, GSL analysis performed for short term Zn (50µM) treated and untreated leaves revealed that levels remained unchanged (*Figure 3.12* and *3.13).*

Taken together results from this study, and current data available in the literature indicate that interactions between Zn accumulation and glucosinolates can be much more complex than explained by the trade-off or elemental defense hypothesis.

4.6 Cloning *HIPP25* gene from *A. thaliana* and *N. caerulescens*

We could clone a full-length coding region (CDS) of *HIPP25* gene from *Arabidopsis thaliana* and partial CDS from *Noccaea caerulescens* into pCRII_TOPO vector. The cloning was performed also to overexpress At*HIPP25* and Nc*HIPP25* genes in *A. thaliana* to verify the metal mechanism they are involved and to compare their metal tolerance and accumulation capacity. But the later objective could not be fulfilled due to the time limitations. However, the objective was also to learn the techniques related to cloning and the gene once successfully cloned, further study on *HIPP25* gene could be continued later by others.

4.6.1 Possible reasons *HIPP25* gene not to be amplified by *Noccaea* primers

The gene of interest, *HIIPP25*, had to be amplified from cDNA by PCR before it can be cloned into a cloning vector. For this, specific *HIPP25* primers was designed for both *Arabidopsis thaliana* and *Noccaea caerulescens* There are a lot of things that need to be considered during the PCR amplification and cloning. These includes the size of the region we are trying to amplify (which is 462bp for At*HIPP25*, 772 bp for Nc*HIPP25*), annealing and extension time and temperature, GC content. The sequence variability of primer regions also plays a big part.

In the first attempt of cloning At*HIPP25* and Nc*HIPP25* gene into pCRII-Topo vector, only At*HIPP25* primers could amplify the *HIPP25* gene present in flower and siliques of *Arabidopsis thaliana*. With Nc*HIPP25,* all the clone sequences showed much more variation when aligned to Nc*HIPP5* CDS as shown in *Appendix Figure 7.19 and 7.20*. Though Nc*HIPP25* primers designed were correct and the primers should have worked for amplifying the correct Nc*HIPP25* gene, based on the results, and clone sequence upon the submission to BlastN in NCBI, showed sequence similarity with some other genes than *HIPP25*. Therefore, some other genes might have been amplified. This cloning process was not regarded successful and it was repeated with some process optimization.

The primers for Nc*HIPP25* in the first attempt were designed in such a way that it includes 5'UTR and 3'UTR part too. There could be several reasons for unsuccessful cloning of Nc*HIPP25* gene. One of the reason could be the primers designed for amplifying *HIPP25* gene in Noccaea are not specific enough and might have caused nonspecific annealing to the template. Although the band produced in the gel in the first attempt was around the expected size (700 bp), but the bands were not very distinct and consists more than one bands in the gel. Based on the cloning results, it was not *HIPP25* but some other closely related gene had been amplified and cloned. In another attempt, the same Nc*HIPP25* primers from the first attempt were used but annealing temperature was increased from 50 to 55°C to increase the specificity of the primers. Despite of increasing the annealing temperature, expected band size was not observed but with the same cDNA of Noccaea roots (Nc_R2) as the template when amplified by primers designed for Arabidopsis, the expected band size was observed and was cloned successfully.

One of the plausible explanation is that the cDNA of Nc_R2 that was reverse synthesized from 3'to 5'end of RNA could be incomplete (due to lower incubation time) or may not contain 5'UTR. Since the primers designed for Noccaea not only includes CDS but also includes 5'UTR and 3'UTR, in such case, the forward primer might not have specific annealing to the template and does not allow for correct gene amplification. Cloning the sequence using the genomic DNA as the template might help in cloning the complete Nc*HIPP25* sequence using the primers designed for *Noccaea*.

4.6.2 Process optimization with *Arabidopsis* primers could clone Nc*HIPP25* gene

To clone *HIPP25* gene from roots of *N. caerulescens*, the cloning process was optimized as mentioned in *section 3.4.3*. At*HIPP25* primers mentioned in second set in *Table B.2* were used

with the change in annealing temperature from 50°C to 64° and the bands produced an amplicon of the expected size (478 bp). So, further cloning process was continued with At*HIPP25* primers on Noccaea roots (Nc_R2).

Primers designed for Arabidopsis with process optimization could amplify *HIPP25* gene from *N. caerulescens* since they have high sequence similarity and the primers were designed within the CDS of *HIPP25* gene that do not include the untranslated region (*Figure 3.21*).

4.7 Future Work

HIPP25 gene which has been predicted to have a role in metal homeostasis in *A. thaliana* and *N. caerulescens*, have been cloned in pCRII-TOPO vector. The At*HIPP25* and Nc*HIPP25* clones generated can now further be studied to verify its role in metal mechanisms like tolerance and accumulation by overexpressing them in *A. thaliana* and confirm its expression at RNA level and at protein level under different metal treatments. The metal concentration in transgenic roots and shoots can then be determined by ICP-MS (Inductively coupled plasma mass spectrometry).

To examine the regulation of *HIPP25* gene expression, At*HIPP25* or Nc*HIPP25* promoter region (with partial 5´UTR) can be fused to a β-glucuronidase (GUS) reporter gene and transform them separately to *A. thaliana*. The GUS activity under different metal treatments can then be determined by staining methods and in a quantitative way. A bioinformatics analysis regarding to both promoter could be performed to find out possible metal binding elements.

Only the subcellular localization of HIPP26 protein has been known from the literature. To characterize the subcellular localization of HIPP25 protein, CaMV35S promoter-driven chimeric genes encoding a C-terminal GFP fusion of AtHIPP25 or NcHIPP25 proteins could be constructed and transiently expressed in *Nicotianamine benthamiana*. The subcellular GFP localization of protoplast can then be observed by florescence microscope and confocal laser scanning microscope.

5 CONCLUSION

A successful hydroponic system was established that allows both *A. thaliana* and *N. caerulescens* to grow in liquid medium with the manipulation over the nutrient profile. Different tissues of plants grown in hydroponic medium with and without metal treatments was used for *HIPP25, HIPP26* and *HIPP27* gene expression analysis, Western blot (HIPP25 protein) and glucosinolate analysis under long term (14 days) and short-term exposure (24 hr).

Gene and protein expression analyses reported in this study, point towards a possible role of *HIPP25* in metal homeostasis. *HIPP25* gene which was expected to response upon Cd treatment (hypothesis 3) in *A. thaliana,* increased the gene expression in leaves under long term condition but not under the short term. In addition, under both long term and short-term condition, in leaves of *A. thaliana,* Zn treatment led to higher gene expression and Ni treatment led to lower expression of gene and protein indicating that *HIPP25* could possibly involve in Zn accumulation and Ni homeostasis (*hypothesis 3*). Two other phylogenetically closely related genes, *HIPP26* and *HIPP27* expression which was expected to induce by Cd in roots (*hypothesis 2*), did not change much under short term (Cd 30μM) but slightly induced under long term condition with the lower concentration (Cd 5μM) while, not with higher concentration (Cd 10μM).

In *N. caerulescens*, under short term and long-term conditions, the expression of *HIPP25* genes was not significantly affected by most of the metal treatments, except Cu which decreased *HIPP25* gene expression in roots. At the protein level, under the long-term condition, the metal treated leaves had higher HIPP25 protein expression than the untreated leaves except Cu. *HIPP26* gene in leaves reduced upon higher concentration of Ni (80μM) whereas induced upon the lower concentration (Ni 50μM) under long term condition.

Though, one of our expected hypothesis (hypothesis 1), *HIPP25* gene expression did not turned out to be significantly affected by the heavy metal treatments in Noccaea but on the comparative basis, HIPP25 protein expression seemed to be higher in Noccaea plants treated with metal treatments than control plants. Under the experimental conditions we performed, *HIPP25* might be involved in the accumulation of Zn as the expression of HIPP25 (at both gene and protein level) is higher in leaves of *A. thaliana* and *N. caerulescens* (at protein level) when compared to the control plants. As no literature has been published for *HIPP25* gene characterization and function, more studies are required to compare the role of *HIPP25 i*n metal homeostasis in a hyperaccumulator and a non-hyperaccumulator.

In addition to the study of *HIPP25* gene, glucosinolates present in leaves from both *A. thaliana* and *N. caerulescens* under Zn exposure were extracted and compared with their respective controls. Being a hyperaccumulator, glucosinolates content decreased under long term metal exposure in *N. caerulescens* supporting trade off hypothesis *(hypothesis 5)*. Our next hypothesis, where GSL in *A. thaliana* was expected to increase upon long term Zn treatment (*hypothesis 4*), was confirmed under lower zinc concentration (15µm) but not under the higher concentration (25µM). Under short term exposure (24 hours) with Zn (50µm) the GSL content decreased in *A. thaliana* and remains unchanged in *N. caerulescens*. Repeating HPLC analysis with the new batch of plants under the appropriate conditions would hopefully wipe out our confusions regarding the possibility of manual error during the growth of plants or the possibility of variable growth room conditions at different time period.

Being a hyperaccumulator, understanding the function of *HIPP25* gene in *N. caerulescens* by cloning, overexpressing and analysis of promoter activities will pave an important way for further research. These research findings are expected to identify the way the plants can be engineered to decontaminate metals in the environment.

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7 APPENDIX

A. Medium and buffers

A.1 **LB Broth (Luria-Bertani broth)**

Components	**Weight(gm)**
1.Peptone	10
2.Yeast Extract	5
3. NaCl	10
4. H_2O added to make 1 litre	
5.Autoclave 121°C for 20 mi ns	
For 1 litre LB agar- 1,2,3 + 1.5% agar , 4,5	

A.2 **Components of Hoagland's medium**

Macronutrients	**Stock(g/L)**	**Stock(M)**	**Element**
KNO_3	101.10	1.00	N/K
$Ca(NO_3)_2.4H_20$	236.15	1.00	Ca
$NH_4H_2PO_4$	115.03	1.00	P
$MgSO_4.7H_20$	246.47	1.00	Mg/S
Micronutrients(1X)	**Stock(g/L)**	**Stock(mM)**	**Element**
KCl	0.075	1.00	Cl
H_3BO_3	1.546	25.00	B
$MgSO_4.4H_2O$	0.446	2.00	Mn
$ZnSO_4.7H_2O$	0.575	2.00	Zn
$CuSO_4.5H_2O$	0.025	0.10	Cu
$(NH_4)_6Mo_7O_{24}.4H_2O$	0.018	0.10	Mo
Other	**Stock(g/L)**	**Stock(mM)**	**Element**
Fe(Na)EDTA	7.342	20	Fe/Na

A.3 **Hoagland's medium with different strengths**

Strength	**0.10X**	**0.25X**	**0.50X**	**1.00X**
Stocks: (For 1 lite)	**(ml)**	**(ml)**	**(ml)**	**(ml)**
KNO_3	0.6	1.5	**3.0**	6.0
$Ca(NO_3)_2.4H_20$	0.4	1.0	**2.0**	4.0
$NH4H_2.PO_4$	0.2	0.5	**1.0**	2.0
$MgSO_4.7H_20$	0.1	0.25	**0.5**	1.0
Fe(Na)EDTA	0.2	0.5	**1.0**	2.0
1X Micronutrients	0.2	0.5	**1.0**	2.0

A.4 **10X FA gel buffer**

Components	**Quantity**
200 mM 3-(N-morpholino) propanesulfonic acid (MOPS) free acid	4.185 g
50 mM Sodium acetate	4.10 g
10 mM EDTA	20 ml
pH adjusted to 7.0 with NaOH and filtered with vacuum	

A.5 **RNA agarose gel**

Components	**Quantity For middle size gel**	**Quantity For large size gel**
Agarose	1.2 g	3.6 g
10X FA Buffer	10 ml	30 ml
MQ water	90 ml	270 ml
Total	100 ml	300 ml
Microwaved and cooled to 65°C		
37% Formaldehyde	1.8 ml	5.4 ml
ethidium bromide	1µl	3µl

A.6 **RNA running buffer(1X)**

components	**Volume(ml) For Medium size gel**	**Volume(ml) For Large size gel**
10X FA gel Buffer	70	200
37%formaldehyde	14	40
MQ water	616	1760
Total	700	2000

A.7 **5X RNA loading dye**

Components	**Volume**
Saturated aqueous bromophenolblue solution	16 µl
500 mM EDTA, pH 8.0	80µl
37% Formaldehyde	720µl
60% Glycerol	2 ml
Formamide	3.084 ml
10X FA gel buffer	4 ml
RNAse free water to 10 ml	
stability: approx. 3 months at 4°C	

A.8 **Protein extraction buffer**

Components	**Volume**
1M Tris HCl pH8.0	0.5 ml
1M NaCl	150 µl
500mM EDTA	20µl
H_2O	9.33 ml
Total	10 ml
Add protease inhibitor tablet (half tablet)	

A.9 **Running gel (15%) and stacking gel**

Type of gel	**Components**	**Volume (ml)**
Running Gel (15%)	H_20	9.2
	1.5M Tris pH 8.8	10
	10% SDS	0.4
	30 % Acrylamide mix	20
	10% APS*	0.4
	TEMED*	0.016
	Total	40
Stacking Gel	H_20	11
	1.0 M Tris pH 6.8	2
	30% Acrylamide Mix	2.6
	10%SDS	0.16
	10% APS *	0.16
	TEMED *	0.016
	Total	16
* APS and TEMED added just before use		

A.10 **Buffers for SDS PAGE and western blot**

Type of Buffer	**Components**	**Quantity**
SDS PAGE Buffer	Tris	3g
	Glycine	14.4 g
	SDS	1g
	H_2O added to make 1 litre	
Transfer Buffer	Tris	3.505 g
	Glycine	14.4g
	H_2O added to make 795 ml	
	Methanol	200 ml
	SDS 10%	5 ml
1X TBST	1M Tris pH 7.8	10 ml
	NaCl	8.7 g
	Tween 20	0.5 ml
	H_2O added to make 1 litre	

A.11 **Protein denaturation loading dye**

Components	**5X**	**Quantity for 5X**
1M Tris-Hcl pH 6.8	250 mM	1250 µl
SDS	10%	100 mg
Glycerol	50%	588 µl
Bromophenol blue	0.5%	25 mg
Dithiothreitol (DTT)	500 mM	385 mg
H_2O added to make 5 ml		

B. Primers

B.1 **Primers used for RT-qPCR**

Genes	Gene Id	Primer	Primer sequence (5'-3')	Product size
Reference Genes	At4g34270	Tip41like_For	GTGAAAACTGTTGGAGAGAAGCAA	61bp
		Tip41like_Rev	TCAACTGGATACCCTTTCGCA	
	At3g25800	PP2A_For	TGGCTCCAGTCTTGGGTAAG	83bp
		PP2A_Rev	ATCCGGGAACTCATCTTTCA	
	At3g18780	Actin2_For	GCCATCCAAGCTGTTCTCTC	157bp
		Actin2_Rev	CAGTAAGGTCACGTCCAGCA	
Target Genes	At4g35060	HIPP25_For	GATTGTTCTCATGGAAGCTCC	70bp
		HIPP25_Rev	AATCTATCAAAACCCTCACATCC	
	At4g38580	HIPP26_For	CTCATGGTCACAAGATTAAAAAGC	137bp
		HIPP26_Rev	CTCCAACGTCACAGAAGACAC	
	At5g66110	HIPP27_For	CATTTGTTACCGGAAACATCAC	138bp
		HIPP27_Rev	ACGGTGACTTTGCTGACTCC	

B.2 **Primers used for PCR**

Set	samples	Primer	Primer sequence (5'-3')	Product size
First	At_F, At_S	At_HIPP25_EcorI_For At_HIPP25_XhoI_Rev	CAGAATTCATGGGTGTTCTTGATCACG AACTCGAGTCACATAACAACACAAGCGG	478bp
	Nc_S, Nc_R1, Nc_R2	Nc_HIPP25_For Nc_HIPP25_Rev	ATAGAGTTTGGTAGGTCGTA CTCCAATATAATGCAGTACACC	772bp
Second	Nc_R2_ HIPP25	At_HIPP25_For At_HIPP25_Rev	ATGGGTGTTCTTGATCACGTCTCTG TCACATAACAACACAAGCGGAGG	462bp
Third	Nc_R2_ HIPP25	Nc_HIPP25_For Nc_HIPP25_Rev (same primers as in first set)	ATAGAGTTTGGTAGGTCGTA CTCCAATATAATGCAGTACACC	772bp

B.3 **Primers used for sequencing**

Primer	Primer sequence (5'-3')
M13_For	GTAAAACGACGGCCAG
M13_Rev	CAGGAAACAGCTATGAC

C. Gallery

C.1 **Results from RNA quality check**

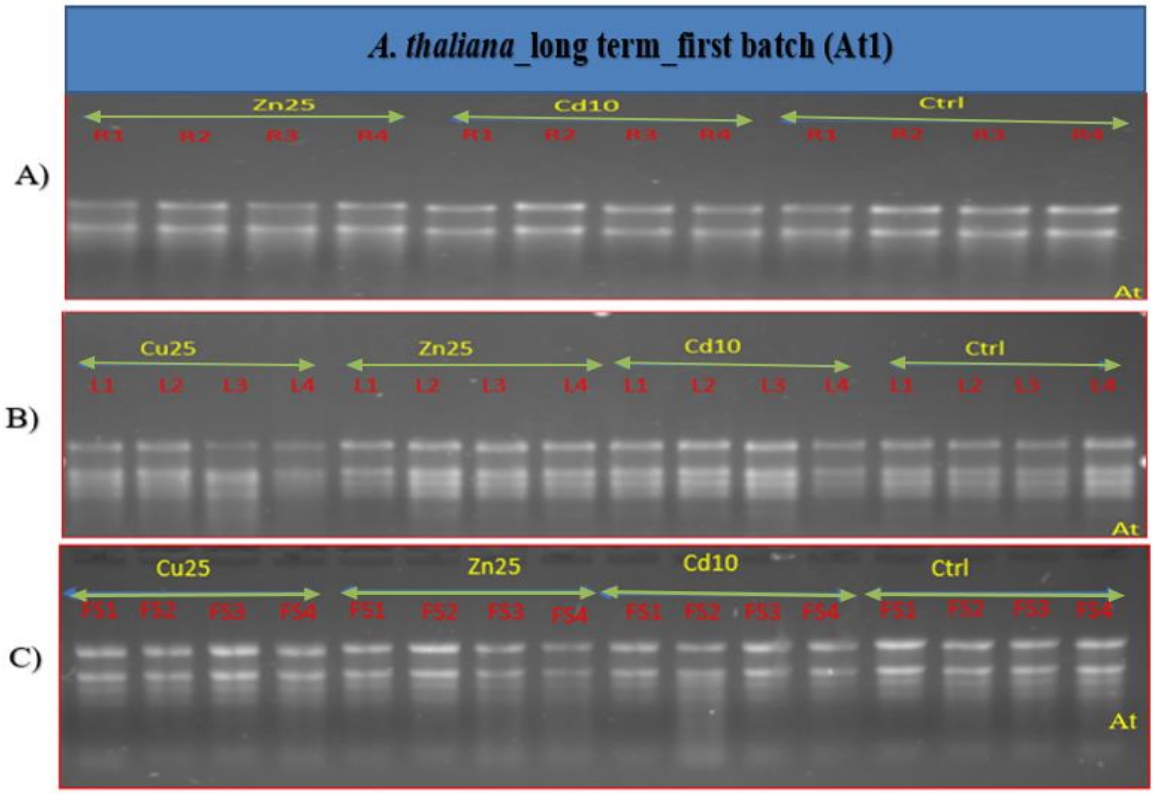


Figure 7.1 Total RNA isolated from leaves of *A. thaliana* first batch exposed to different heavy metals (Cu 25µM, Zn 25µM, Cd 10µM) and without any metal treatment (control) for long term (14 days) with four replicates on each. A) R1-R4 represents root samples b) L1-L4 represents leaves sample c) FS1-FS4 represents flower and siliques. All RNA showed distinct 25S and 18S rRNA bands and additional bands on leaf samples represents chloroplast ribosomal subunits

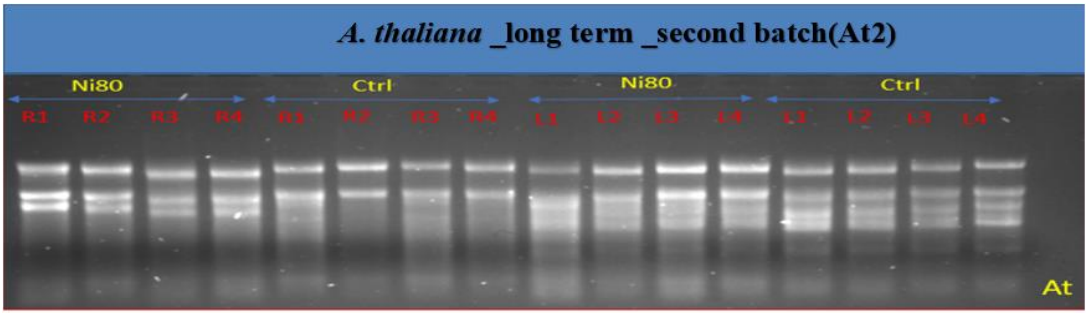


Figure 7.2 Total RNA isolated from root and leaves of *A. thaliana* second batch exposed to Ni80µM and without any metal treatment (control) for long term (14 days) with four replicates on each. R1-R4 represents root samples and L1-L4 represents leaves sample. All RNA showed distinct 25S and 18S rRNA bands and additional bands on leaf samples represents chloroplast ribosomal subunits

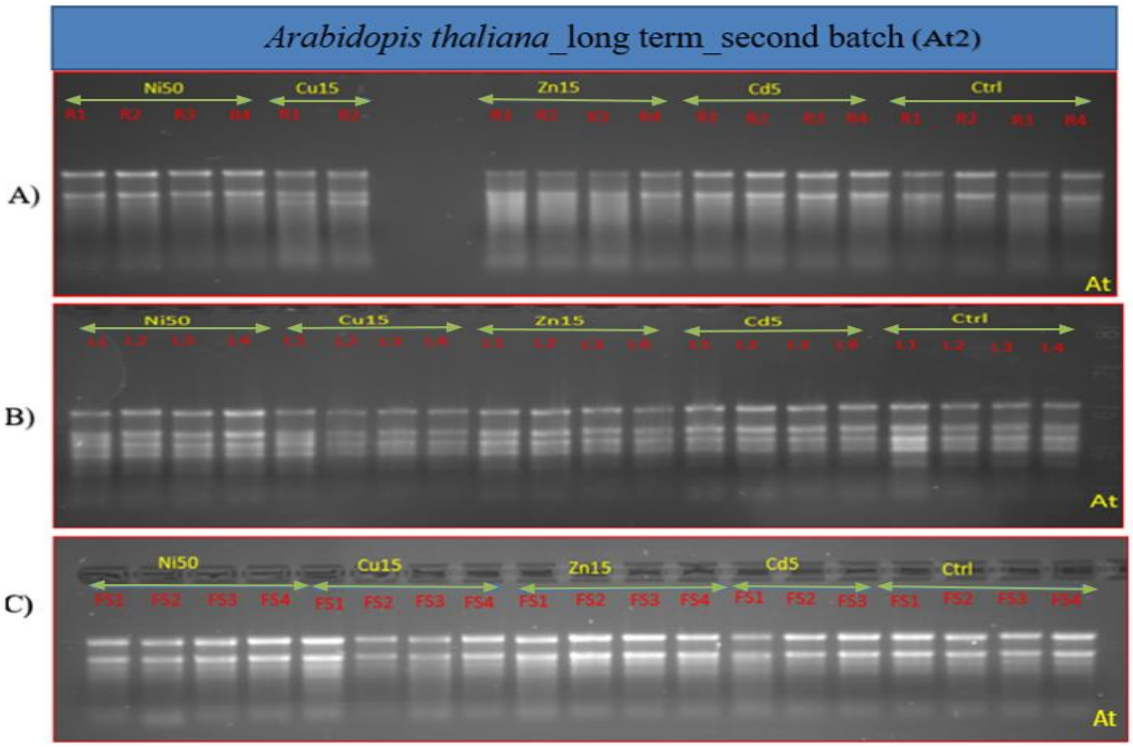


Figure 7.3 Total RNA isolated from root, leaves and flower+siliques of *Arabidopsis thaliana* second batch exposed to different heavy metals (Ni 50µM, Cu 15µM, Zn 15µM, Cd 5µM) and without any metal treatment (control) for long term (14 days. A) R1-R4 represents roots sample in four replicates. B) L1-L4 represents leaf samples in four replicates. C) FS1-FS4 represents flower and silique samples in four replicates. Upper band represents 25s rRNA, lower band represents 18s rRNA and additional bands below 18s rRNA in leaves represents chloroplast subunits.

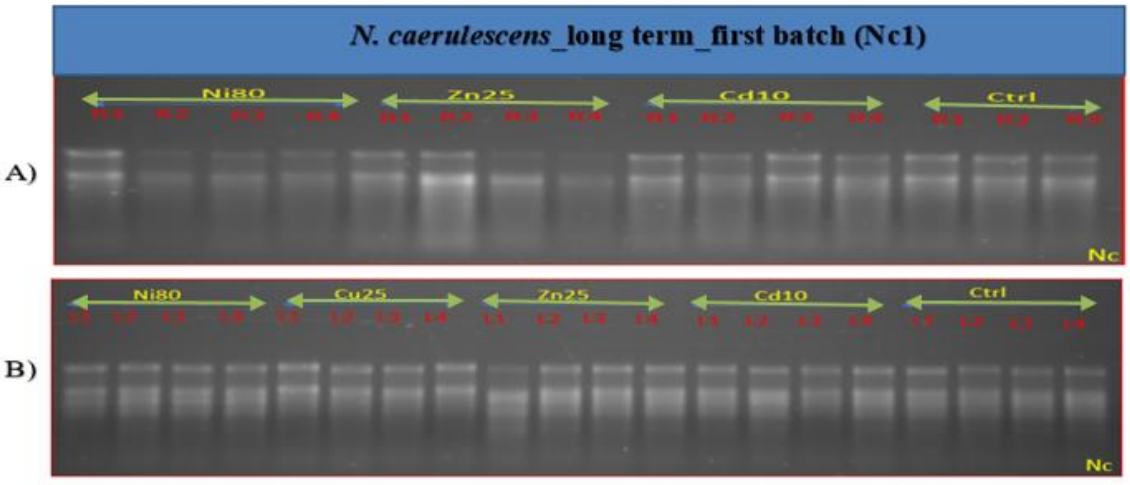


Figure 7.4 Total RNA isolated from root and leaves of *Noccaea caerulescens* first batch exposed to different heavy metals (Ni 80µM, Cu 25µM, Zn 25µM, Cd 10µM) and without any metal treatment (control) for long term (14 days). (A) R1-R4 represents root samples in four replicates (B) L1-L4 represents leaves sample in four replicates. All RNA showed distinct 25S and 18S rRNA bands and additional bands on leaf samples represents chloroplast ribosomal subunits.

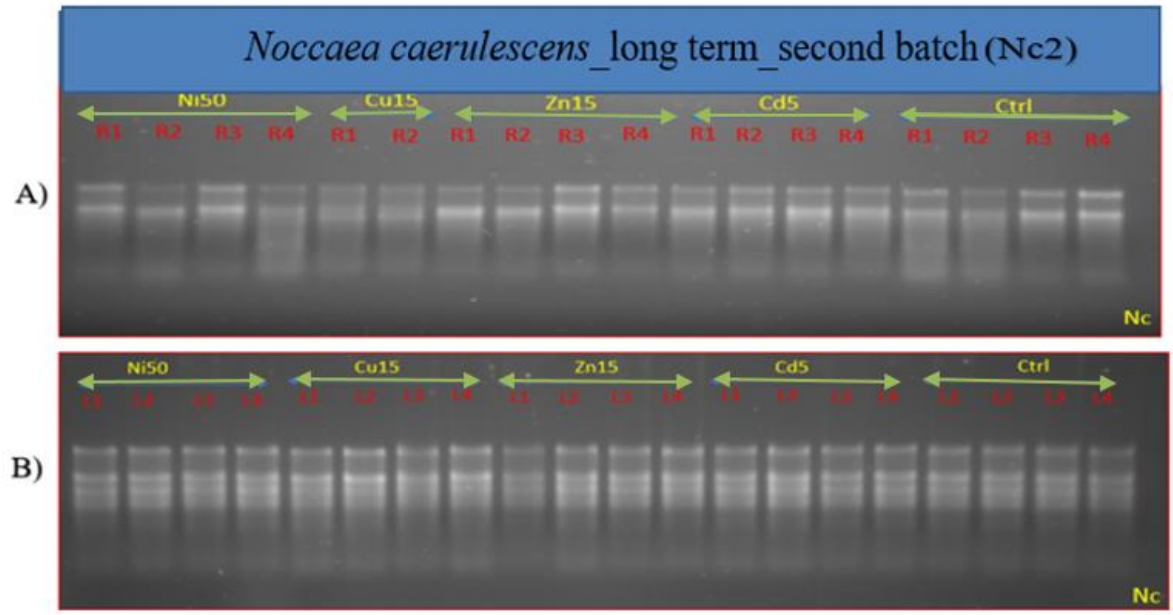


Figure 7.5 Total RNA isolated from root and leaves of *Noccaea caerulescens* second batch exposed to different heavy metals (Ni 50µM, Cu 15µM, Zn 15µM, Cd 5µM) and without any metal treatment (control) for long term (14 days) with four replicates on each. A) R1-R4 represents root samples in four replicates B) L1-L4 represents leaf samples in four replicates. Upper band represents 25s rRNA, lower band represents 18s rRNA, the additional bands below 18s rRNA in leaves represents chloroplast subunits.

C.2 **Melting curve of Nc*HIPP27* gene**

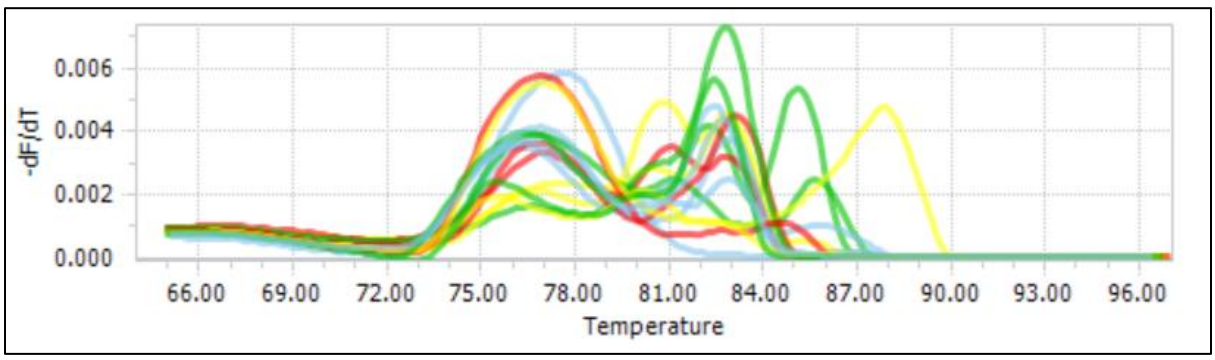


Figure 7.6 Melting peak representing the presence of more than one peak for *HIPP27* gene of *Noccaea caerulescens* for all the roots (metal treated and control) under long term condition. Blue peak=nickel treated roots, yellow peak=Zn treated roots, Green peak=Cd treated roots and Red peak=control roots.

C.3 PCRII_TOPO vector

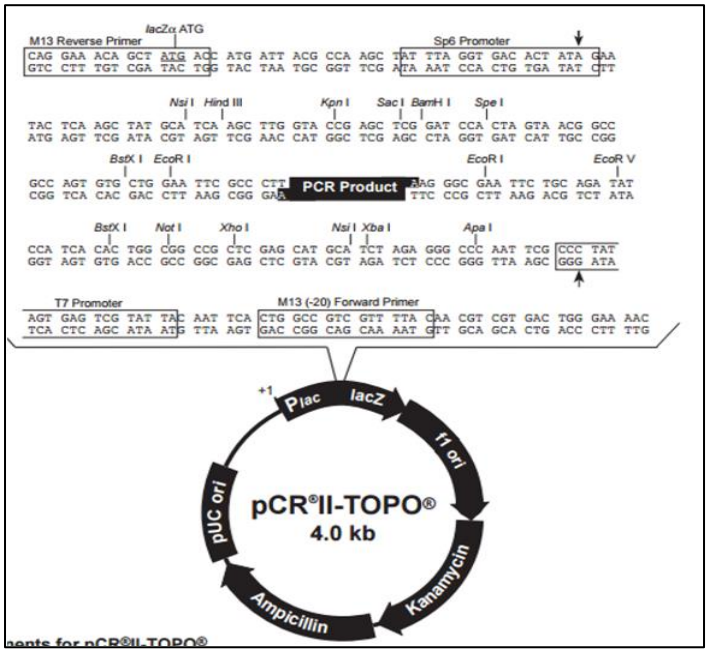


Figure 7.7 The map showing the feature of the PCRII_TOPO vector and the sequence surrounding the TOPO cloning site. Restriction sites are labelled to indicate the actual cleavage site.

C.4 Expression of *HIPP25* in *A. thaliana*

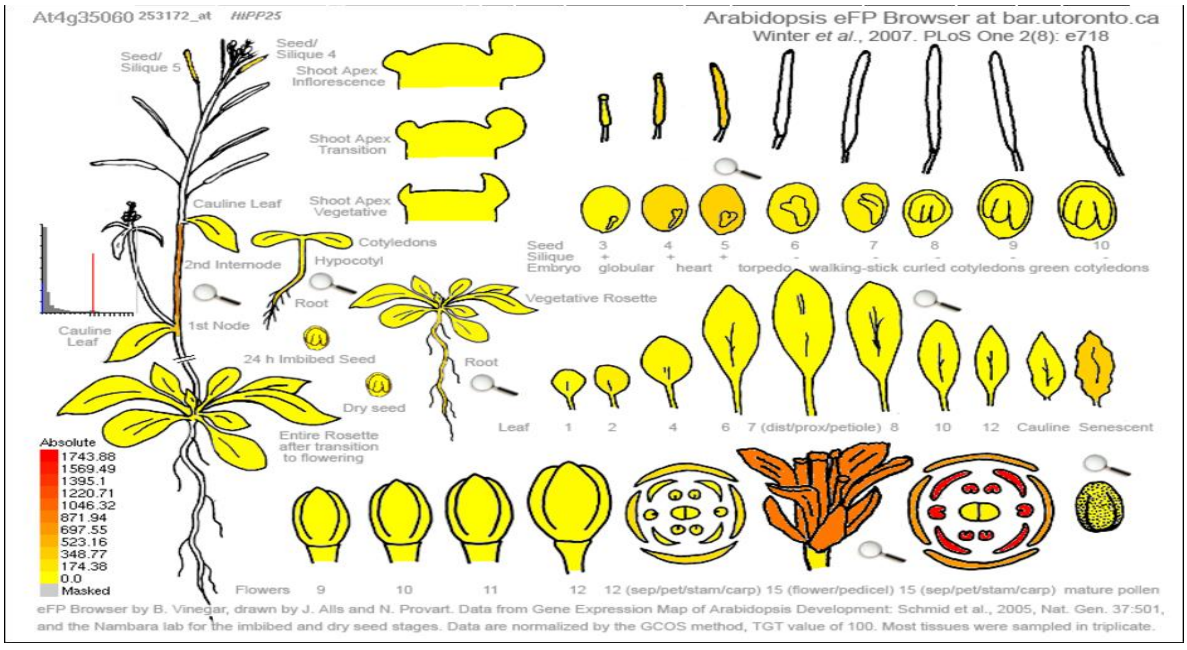


Figure 7.8 Expression of *HIPP25* gene in *A. thaliana* Col-O obtained from developmental map in eFP browser from TAIR. Red color represents higher gene expression which can be seen for flowers. The gene expression is decreased in leaves and then roots as the color decreases from red to yellow and white as shown in the color gradient in the left.

C.5 *A. thaliana* and *N. caerulescens* grown under different conditions

Figure 7.9 *Arabidopsis* and *Noccaea* germinated seedlings grown on small boxes in growth room (120µmol/m^2/sec light intensity, 22°C, 16hour photoperiod)

Figure 7.10 *Arabidopsis thaliana (Col-0) (36 days old)* grown in modified 0.5X Hoagland's solution treated with heavy metal for 24 hour

Figure 7.11 *Arabidopsis thaliana* grown on modified 0.5X Hoagland's solution in growth room (control plants) for 36 days

Figure 7.12 *Noccaea caerulescens* plant grown on modified 0.5X Hoagland's sloution in growth room (control plants) for 36 days

Figure 7.13 Arabidopsis plants (first batch) grown under metal treatment for 14 days

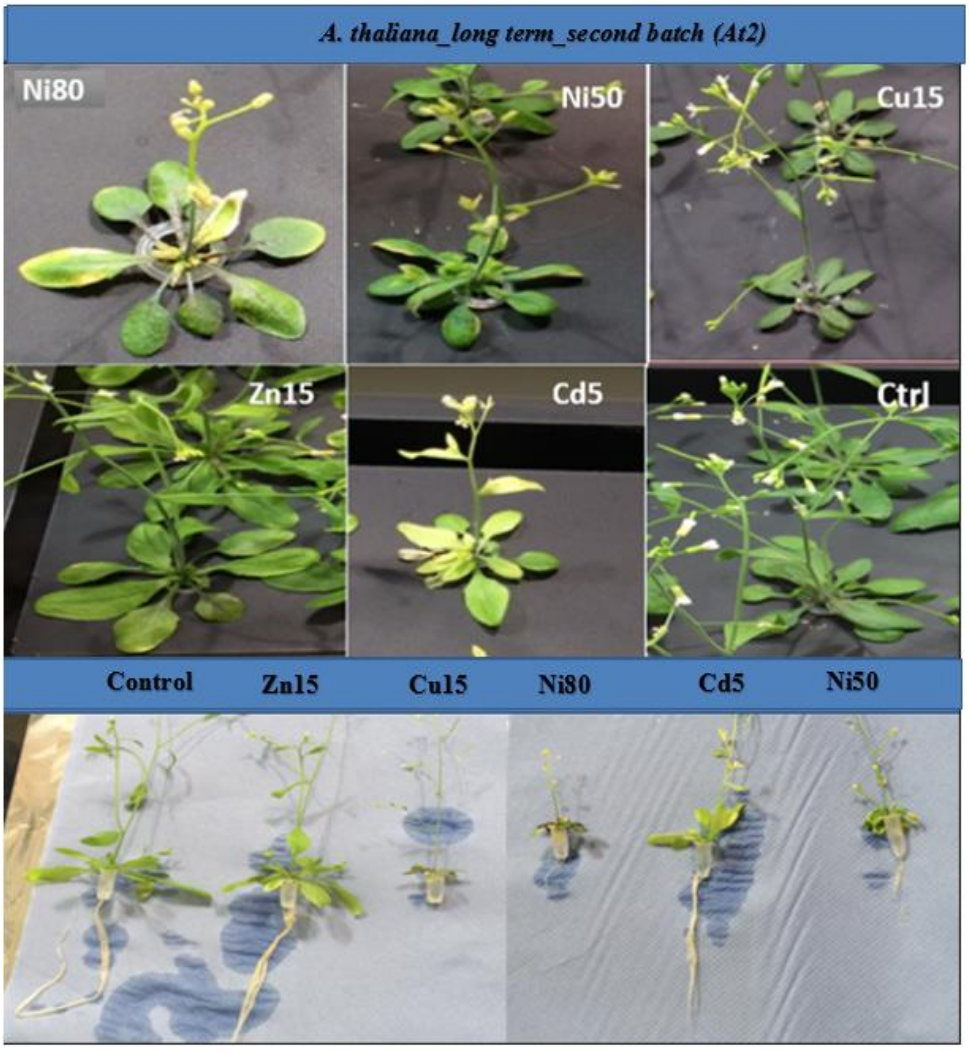


Figure 7.14 Arabidopsis plants (second batch) grown under metal treatment (14 days)

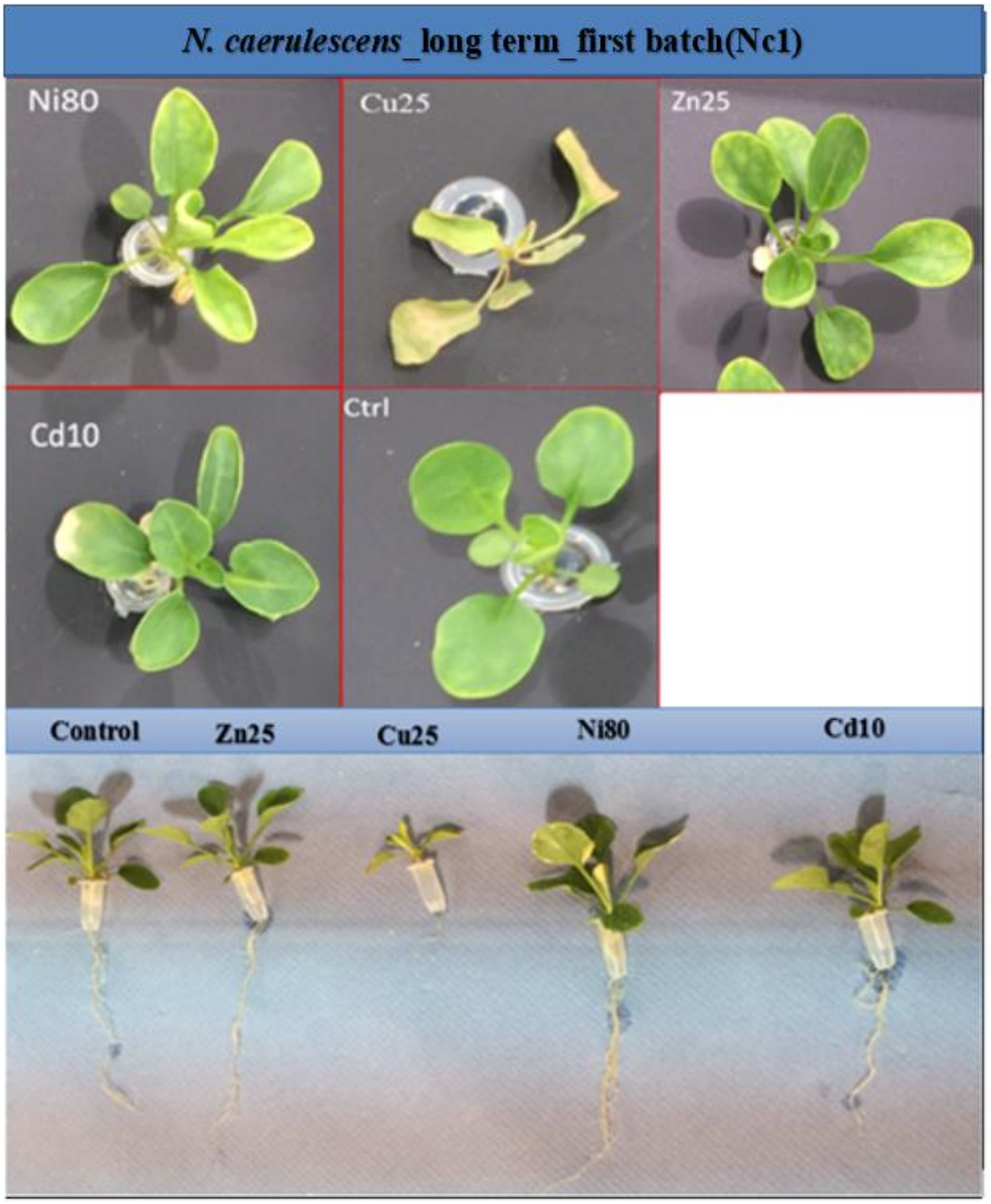


Figure 7.15 Noccaea plants (first batch) grown under metal treatment (14 days)

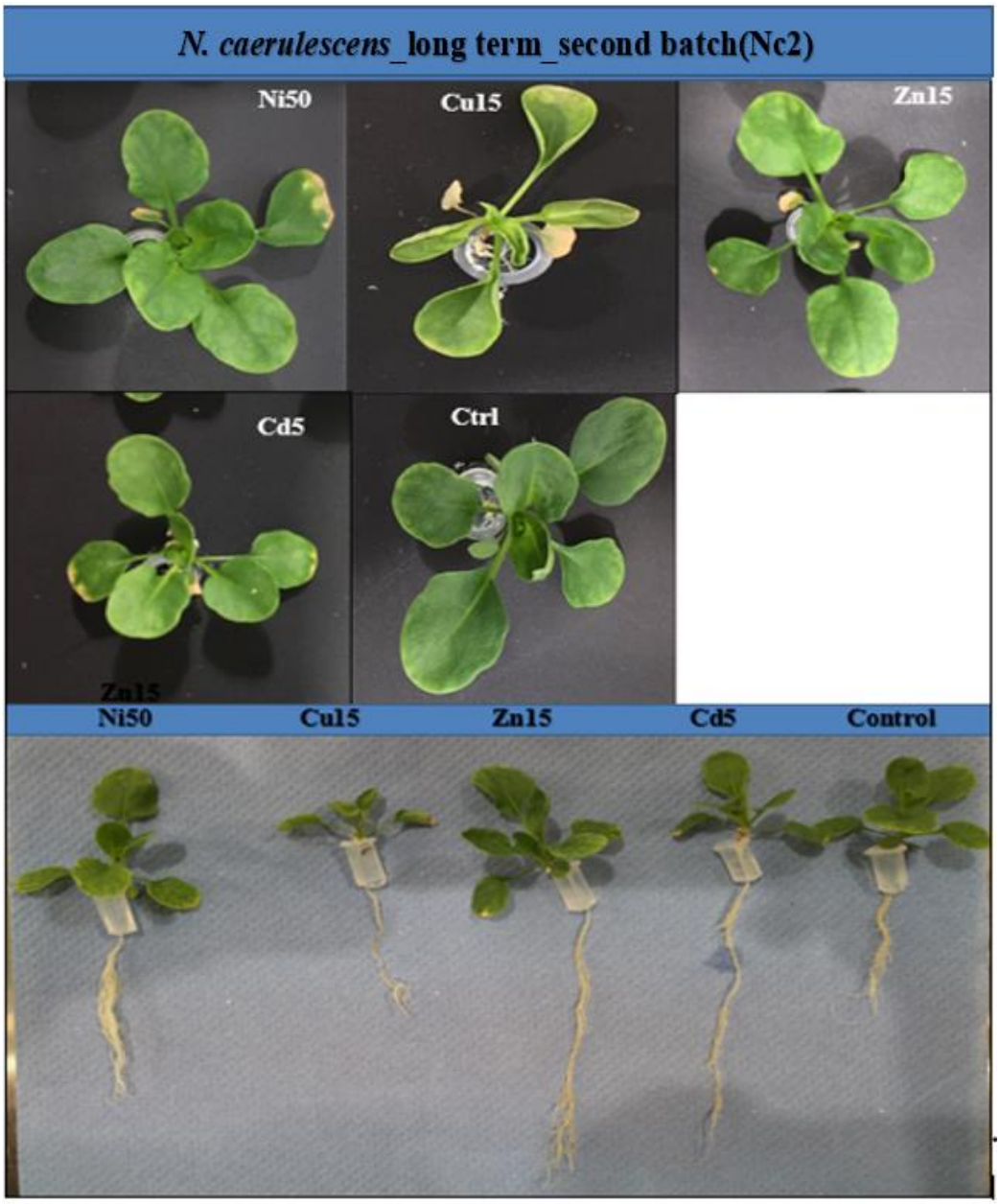
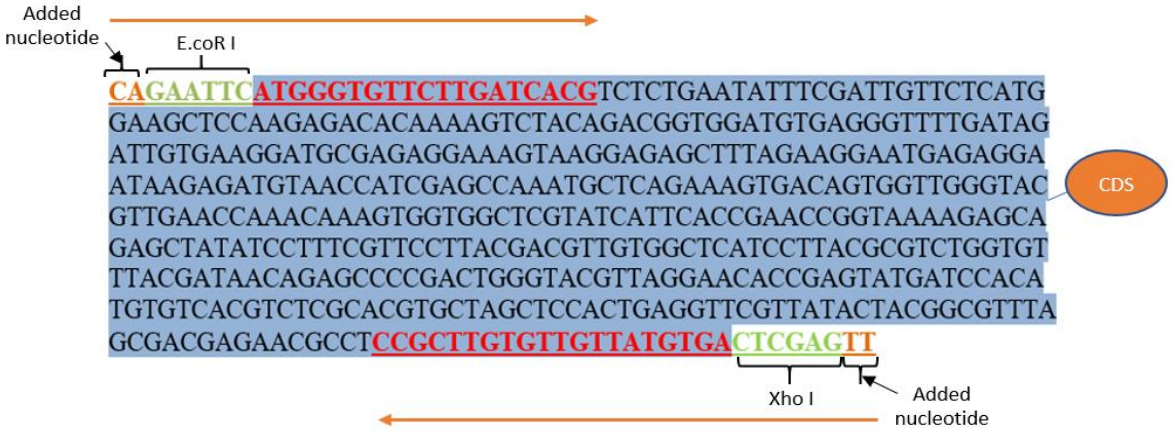


Figure 7.16 Noccaea plants (second batch) grown under metal treatment (14 days)

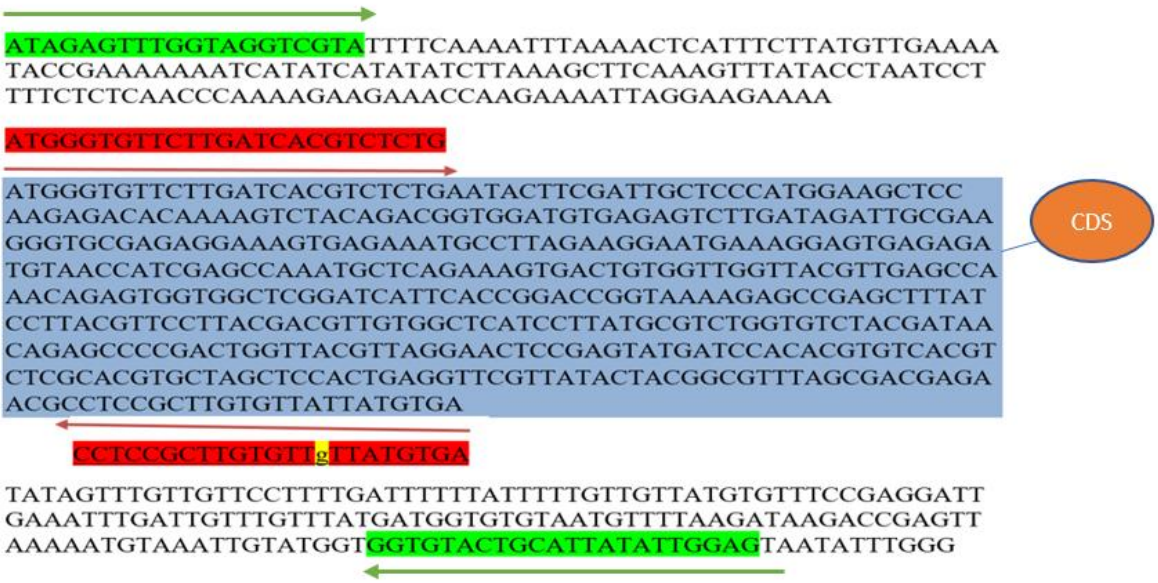
D. Sequences and alignment

D.1 At*HIPP25* CDS: 462 bp (From TAIR)



* The underlined sequences represent the Arabidopsis forward and reverse primer, blue highlighted part represents CDS, nucleotide and restriction site (EcoR I and Xho I) added are labelled in figure

D.2 Nc*HIPP25* cDNA: 783 bp (From NCBI – Gen Bank GASZ01015567)



* Overall sequence represents Nc*HIPP25* cDNA obtained from NCBI. The green part represents the forward and reverse primer designed for Noccaea used for first and third set and the red part indicates forward and reverse primer used for the second set, blue part represents Nc*HIPP25*_CDS from Ganges that is obtained after alignment with At*HIPP25*_CDS as shown in Figure D.3, yellow color represents the different nucleotide "G" in the reverse primer compared to nucleotide "A" in Nc*HIPP25*_CDS (since the primers designed for *Arabidopsis* had been used).

D.3 Alignment of nucleotide sequence of *HIPP25* CDS from *A. thaliana* and *HIPP25* cDNA from *N. caerulescens* from Ganges and primers used to amplify At*HIPP25* and Nc*HIPP25*

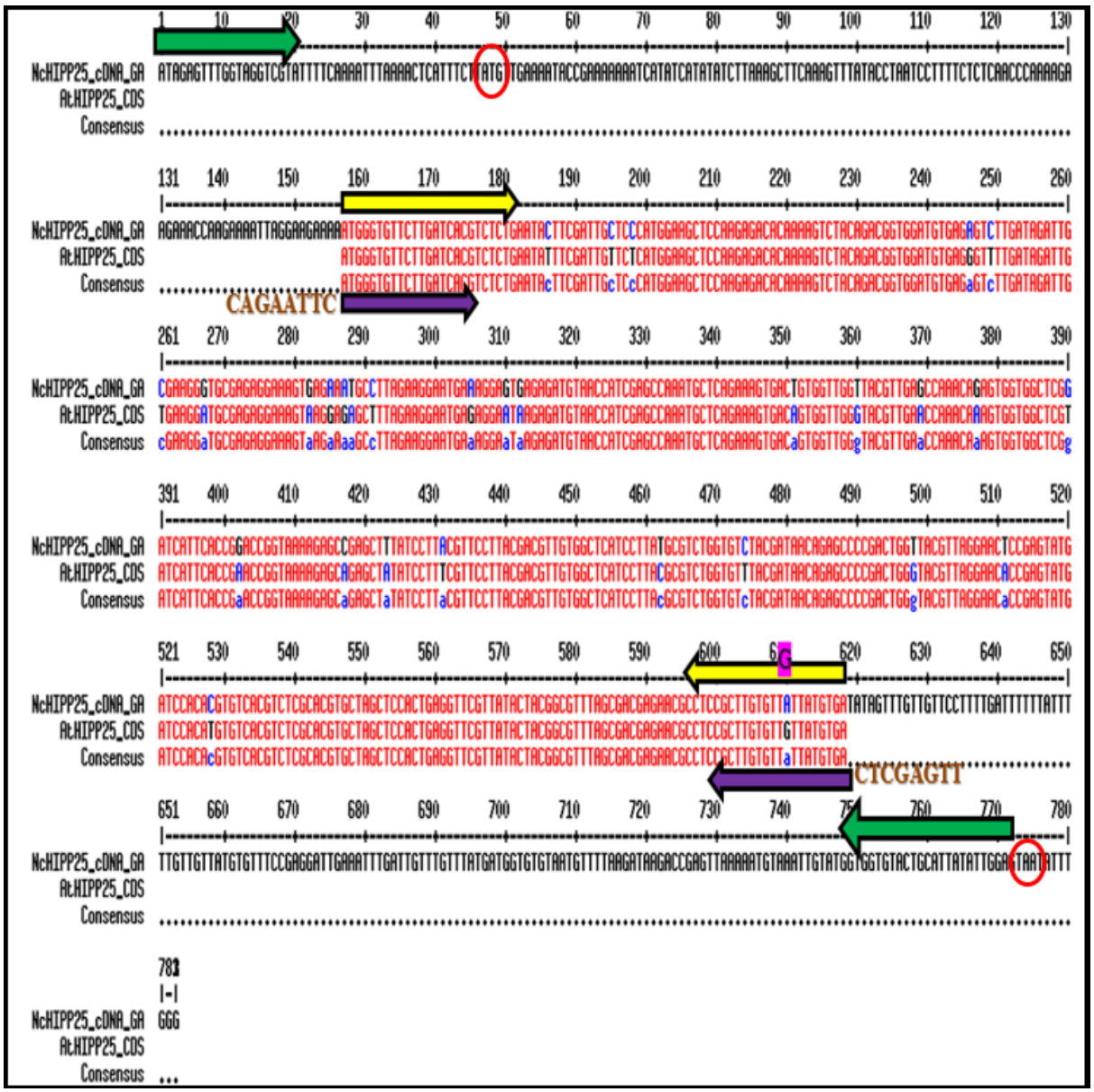


Figure 7.17 Multiple alignment of the At*HIPP25_CDS* obtained from TAIR with the Nc*HIPP25* transcript sequence from Ganges (accession GASZ01015567) obtained from NCBI. A residue that is highly conserved is seen in red color and as an uppercase letter in the consensus line, a residue that is weakly conserved is seen in blue color and as a lowercase letter in the consensus line and the single nucleotide mismatch is seen in black color. Green arrow: First set of forward and reverse Primers to amplify Nc*HIPP25*, Yellow arrow: Second set of forward and reverse Primer to amplify Nc*HIPP25* with a change in one nucleotide "G" in place of "A" in reverse primer, Purple Arrow: First set of forward and reverse Primers to amplify At*HIPP25* with the restriction site and two added nucleotides. Presence of another ATG (start codon) and TAA (stop codon) is encircled in red. Abbreviations: GA: Ganges, LC: La calamine

D.4 Alignment of HIPP25 amino acid sequence from *A. thaliana* and *N. caerulescens*

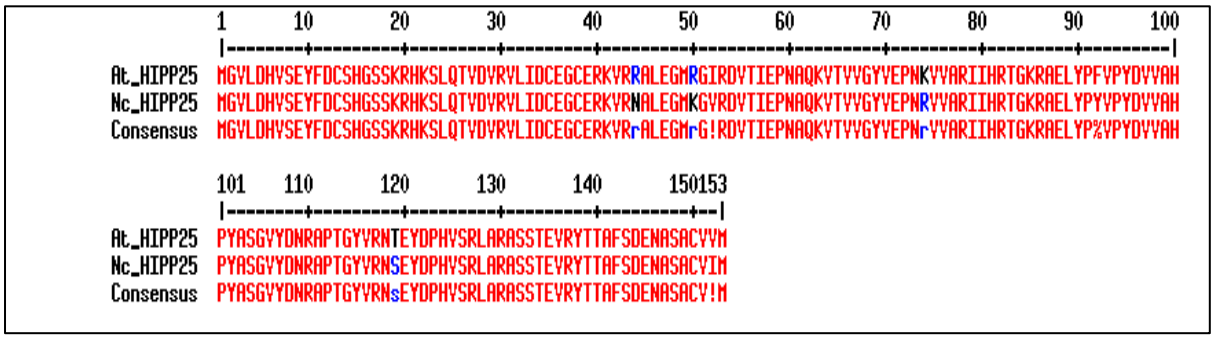


Figure 7.18 Multiple alignment of the At_HIPP25 amino acid sequence obtained from TAIR with the Nc_HIPP25 amino acid sequence in reading frame 1 (nucleotide sequence from Ganges obtained from NCBI translated to amino acid sequence by expasy.org). Black color represents the mismatch. A residue that is highly conserved is seen in red color and as an uppercase letter in the consensus line, a residue that is weakly conserved is seen in blue color and as a lowercase letter in the consensus line and the single codon mismatch is seen in black color.

- alanine - ala - A
- arginine - arg - R
- asparagine - asn - N
- aspartic acid - asp - D
- cysteine - cys - C
- glutamine - gln - Q
- glutamic acid - glu - E
- glycine - gly - G
- histidine - his - H
- isoleucine - ile - I
- leucine - leu - L
- lysine - lys - K
- methionine - met - M
- phenylalanine - phe - F
- proline - pro - P
- serine - ser - S
- threonine - thr - T
- tryptophan - trp - W
- tyrosine - tyr - Y
- valine - val - V

D.5 First Set of Sequencing result of Nc*HIPP25*

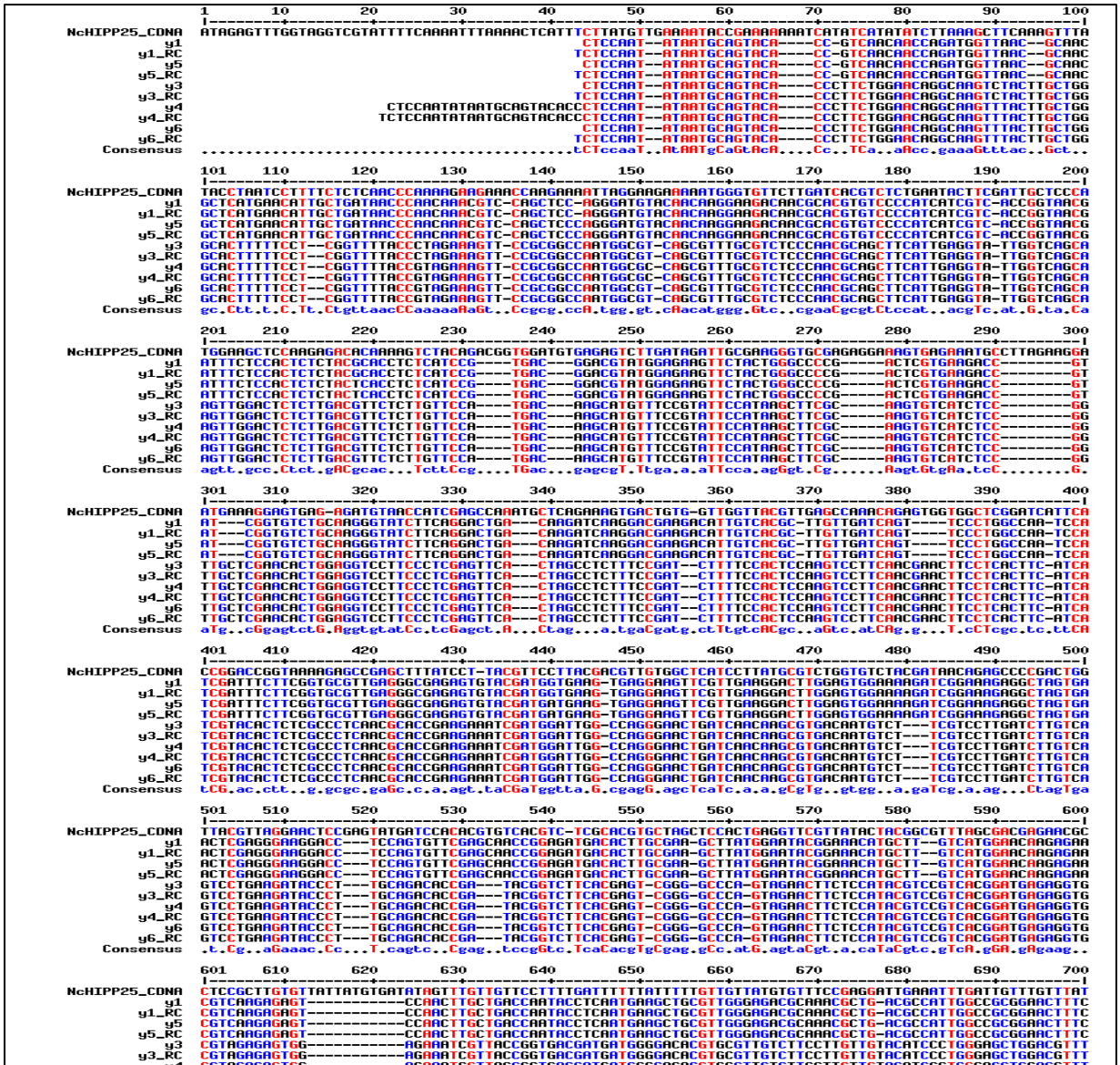


Figure 7.19 Multiple alignment of the Nc*HIPP25* reference sequence with the sequences from the y1, y3, y4, y5, y6 clones obtained during the first PCR attempt to clone Nc*HIPP25*. None of the insert has the sequence similar to Nc*HIPP25*_cDNA reference sequence. A residue that is highly conserved is seen in red color and as an uppercase letter in the consensus line, a residue that is weakly conserved is seen in blue color and as a lowercase letter in the consensus line and the single nucleotide mismatch is seen in black color.

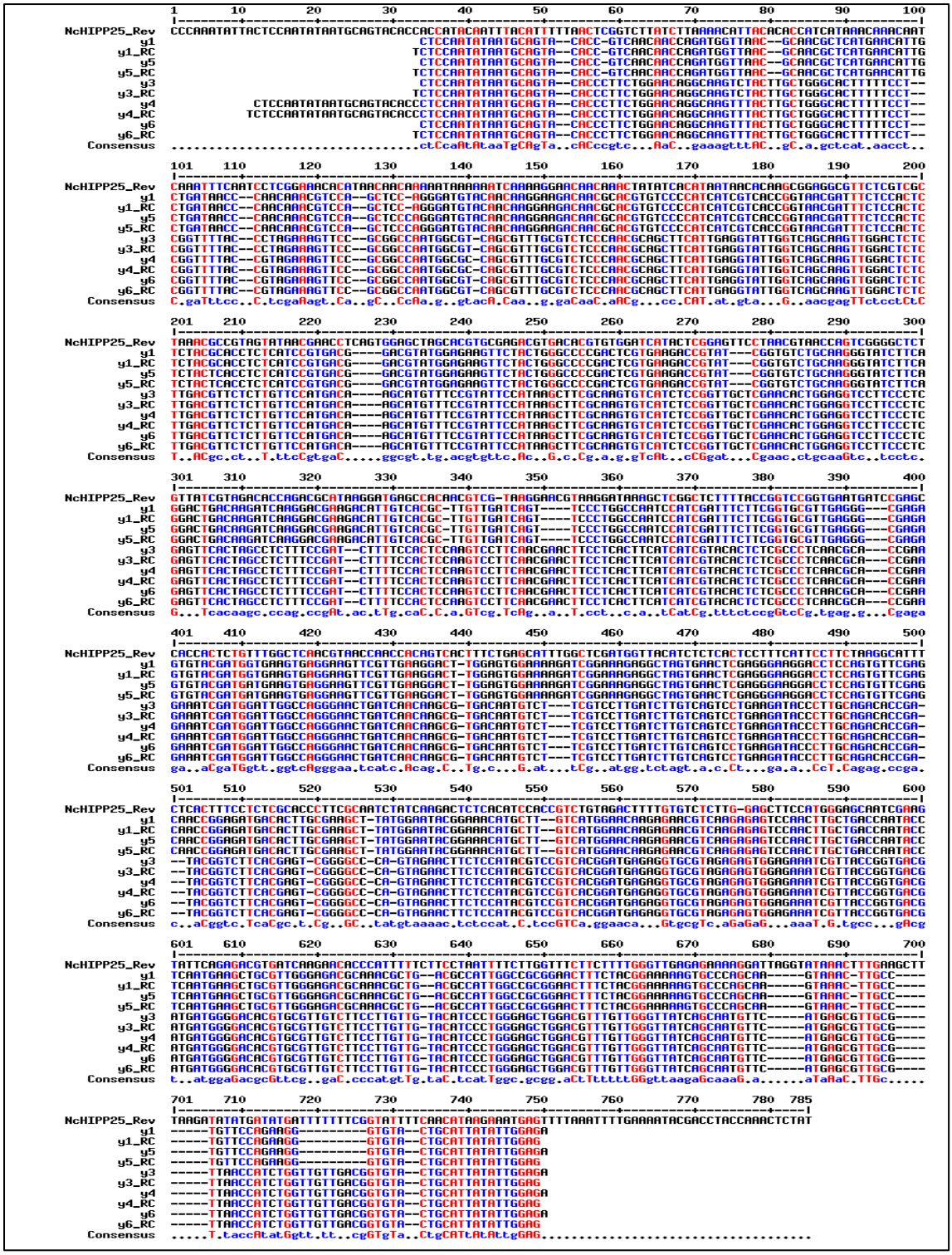


Figure 7.20 Multiple alignment of the reverse complement Nc*HIPP25* reference sequence with the sequences from the y1, y3, y4, y5, y6 clones obtained during the first PCR attempt to clone NcHIPP25. None of the insert has the sequence similar to NcHIPP25_reverse_cDNA reference sequence. A residue that is highly conserved is seen in red color and as an uppercase letter in the consensus line, a residue that is weakly conserved is seen in blue color and as a lowercase letter in the consensus line and the single nucleotide mismatch is seen in black color.