Ureide metabolism in response to cadmium treatment in Arabidopsis

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Abstract

Ureides are nitrogenous compounds derived from oxidative degradation of purines. Due to their high nitrogen content, ureides play an important role in nitrogen metabolism, storage, and transport in plants. Among ureides, allantoin has recently been the subject of investigation, not only because it is a nitrogen-rich compound, but also due to its increased concentration in response to different abiotic stresses, and its contribution to stress tolerance in plants. Although a reactive oxygen species (ROS) scavenging property has been proposed for allantoin, the mechanism(s) underlying its protective role is still under debate. This research was carried out to evaluate the effect of cadmium (Cd) on ureide metabolism, clarifying the potential link between allantoin content and Cd tolerance in *Arabidopsis thaliana*. Molecular and biochemical analyses showed that in wild-type (Col-0) Arabidopsis, allantoin accumulates following Cd exposure because of enhanced transcript level of uricase (*UO*) and decreased mRNA abundance and enzyme activity of allantoinase (*ALN*), which are required for allantoin production and degradation, respectively. Interestingly, allantoinase-negative (*aln-3*) mutants, containing increased amount of allantoin, are relatively tolerant to Cd due to decreased ROS accumulation in response to Cd toxicity. Activity of three antioxidant enzymes, superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT), demonstrated higher SOD and APX activity in *aln-3* leaves, together with greater activity of SOD and CAT in roots, support better *aln-3* growth and confer Cd resistance to these plants. In contrast, allantoinase-overexpressed (*ALNox*) lines, containing lower allantoin levels, and respond to Cd in the opposite manner than *aln-3* mutants, showing a Cd-sensitive phenotype. Differential and opposite responses of *aln-3* mutants and *ALNox* lines to Cd treatment suggests that there is a positive correlation between allantoin content and resistance to Cd treatment. Additionally, ABA-insensitive (*abi*) mutants were used to investigate the possible cross-talk between allantoin and ABA signalling pathway, indicating that regulatory function of allantoin may be mediated through both ABA-dependent and -independent signalling pathways. These data contribute to our understanding of ureides regulation and function in response to abiotic stresses, setting the stage for future research on ureide metabolism with the purpose of introducing stress-resistant plants.

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Finally, I would like to dedicate this dissertation to the loving memory of my mother, who is my inspiration to pursue my dreams. This is for you.

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List of Abbreviations

$^1O_2$	singlet oxygen
µl	microliter
µM	micromolar
AAH	allantoate amidohydrolase
aah	allantoate amidohydrolase-negative mutants
AAS	atomic absorption spectroscopy
ABA	Abscisic acid
aba	ABA-deficient mutants
abi	ABA-insensitive
ABRC	Arabidopsis Biological Resource Center
ABRE	ABA responsive element
ACT	actin
AGRIS	Gene Regulatory Information Server
ALA	5-aminolevulinic acid
ALN	allantoinase
Aln	allantoin
aln	allantoinase-negative mutants
ALNox	allantoinase-overexpressed lines
ALNS	allantoin synthase
AMP	adenosine monophosphate
AMPD	AMP deaminase
ANOVA	analysis of variance
AP2	apetala2
APX	ascorbate peroxidase
As	arsenic
AsA	ascorbic acid
Asn	asparagine
At	*Arabidopsis thaliana*
ATP	adenosine triphosphate

BC	backcross
BG1	β-glucosidase 1
bp	base pair
bZIP	basic leucine zipper
°C	degree Celsius
C	carbon
Ca	calcium
CaM	calmodulin
CaMV	cauliflower mosaic virus
CAT	catalase
Cd	cadmium
$CdCl_2$	cadmium chloride
CDF	cation diffusion facilitator
CDPK	calcium-dependent protein kinase
CDS	coding sequence
cDNA	complementary DNA
cm	centimeter
CO_2	carbon dioxide
Col.0	Columbia
Cr	chromium
CSD	Cu/Zn superoxide dismutase
Cu	Copper
DAB	diaminobenzidine
df	degree of freedom
dH_2O	distilled water
DHA	dehydroascorbic acid
DHAR	dehydroascorbate reductase
DMSO	dimethyl sulfoxide
DRE	dehydration-responsive element
EDTA	ethylene diamine tetra acetic acid
EtBr	3,8-diamino-5-ethyl-6-phenylphenanthridinium bromide

ER	endoplasmic reticulum
ETC	electron transport chains
EX	executer
F	filial generation
Fd	ferredoxin
Fe	iron
FSD	Fe superoxide dismutase
F_v/F_m	yield of PSII photochemistry
×g	gravitational force
g	gram
gDNA	genomic DNA
GFP	Green Fluorescent Protein
Gln	glutamine
Gm	*Glycine max*
GR	glutathione reductase
GSH	reduced glutathione
GSSG	oxidized glutathione
GTP	guanosine-triphosphate
GUS	beta glucuronidase
h	hour
H_2O_2	hydrogen peroxide
5-HIU	5-hydroxyisourate
$HClO_4$	perchloric acid
Hg	mercury
HMA	heavy metal ATPases
HNO_3	nitric acid
$HO^{\bullet}$	hydroxyl radical
HPLC	high-performance liquid chromatography
Hyg	Hygromycin
IMP	inosine monophosphate
IRE1	inositol requiring enzyme 1

JA	jasmonic acid
K	potassium
Kan	Kanamycin
kg	kilogram
KOH	potassium hydroxide
LB	Luria-Bertani medium
LEA	late embryogenesis abundant
LHC	light harvesting complexes
Li	lithium
LiCl	lithium chloride
LPE	leaf permease
M	molarity
m	meter
MAPK	mitogen-activated protein kinase
MDHA	monodehydroascorbate
Mg	magnesium
mg	milligram
ml	millilitre
mM	millimolar
mm	millimeter
mRNA	messenger RNA
MS	Murashige-Skoog medium
N	normality
Na	sodium
NaCl	sodium chloride
NAD^+	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NaN_3	sodium azide
NBT	nitroblue tetrazolium
NCED	9-cis-epoxycarotenoid dioxygenase
NH_4	ammonium

Ni	nickel
$NiCl_2$	nickel chloride
nm	nanometer
NO_3	nitrate
NPQ	non-photochemical quenching
O_2	molecular oxygen
O_2^-	superoxide anion
OD	optical density
$OH^\bullet$	hydroxy radical
OHCU	2-oxo-4-hydroxy-4-carboxy-5-ureido-imidazoline
Pb	lead
PC	phytochelatins
Pc	plastocyanin
PCR	polymerase chain reaction
PP2C	type 2C protein phosphatase
PQ	photochemical quenching
PSI	photosystem I
PSII	photosystem II
PTM	post translational modification
PUP1	purine permease1
Pv	*Phaseolus vulgaris*
PYR/PYL	Pyrabactin resistance protein1/PYR-like proteins
Q	plastoquinone
qRT-PCR	quantitative reverse transcriptase PCR
R	resistant
RD	dehydration-responsive genes
Rif	Rifampicin
RNAi	RNA interference
ROS	reactive oxygen species
rpm	Revolutions per minute
RT-PCR	reverse transcriptase PCR

S	sensitive
s	second (time)
SA	salicylic acid
SAG12	senescence-associated gene 12
SAIL	Syngenta Arabidopsis Insertion library
SALK	Institute for biological studies
SDS	sodium dodecyl sulfate
SE	standard error
SOD	superoxide dismutase
SPSS	Statistical Package for the Social Sciences (statistics software)
SnRK2	SNF1-Related Kinase
T_x	Transformed $_{\text{generation}}$
T-DNA	transfer DNA
TF	transcription factor
UAH	ureidoglycolate amidohydrolase
UGlyAH	ureidoglycine aminohydrolase
UO	uricase
UPS	urate permease
UV	ultra violet
v	volume
W	Watt
w	weight
X^2	Chi-Square
XDH	xanthine dehydrogenase
XMP	xanthosine monophosphate
XO	xanthine oxidase
XOR	xanthine oxidoreductase
Y	yield
Zn	Zinc

1. Introduction and Literature Review

1.1. Abiotic stress in plants

Biological stress is defined as a negative effect of an internal or external change on an organism. In plants, this can be further classified as biotic and abiotic stresses, based on their origin. Damage caused by a living factor such as herbivores, pathogens, bacterial and viral infections are considered as biotic stresses. In contrast, environmental changes such as drought, flood, salinity, high and low temperatures are some examples of abiotic stresses (Chaves *et al*., 2003; Madlung and Comai, 2004; Bhatnagar-Mathur *et al*., 2008). Since plants are immobile organisms, adjusting to environmental changes is vital for their survival under stress conditions. Stresses, regardless of their natures, cause a series of molecular and metabolic changes in plants, influencing plant function and growth (Shao *et al*., 2007; Agrawal *et al*., 2010). Stress perception and plant responses to stress are highly dependent on plant species and age (Zamboni *et al*., 2010), plant organ (Dinneny *et al*., 2008), and type and duration of the stress (Tattersall *et al*., 2007; Pinheiro and Chaves, 2011).

Stress perception is the first and most important part of a stress signal, determining the proper stress response in plant cells. Based on which cell compartment is the primary receiver of stress, different sensors/receptors have been described for various stresses. For example, OSCA1 (reduced hyperosmolality-induced calcium increase1) is a putative sensor for osmotic stress in Arabidopsis. OSCA1 is a plasma-membrane protein that detects osmotic stress inducers such as salinity, cold and metals, causing an increase in calcium (Ca^{2+}) content of the cell. It is still unknown how OSCA1 perceives osmotic stress, but it is assumed that its function is related to cell turgor and interaction between the cell wall and plasma membrane, leading to Ca accumulation (Arnadottir and Chalfie, 2010; Hedrich, 2012; Yuan *et al*., 2014). In addition to the cell surface, cellular compartments also sense stress by means of specific receptors. Endoplasmic reticulum (ER) stress is a consequence of various biotic and abiotic stresses that induce protein misfolding/unfolding. Misfolded/unfolded proteins are detected by two ER-membrane stress sensors: ER membrane-associated transcription factors (bZIP17 and bZIP28)

and inositol requiring enzyme 1 (IRE1). These two sensors are associated with transcription factors that regulate the expression of proteins and chaperons required for protein folding and ER stress response (Walter and Ron, 2011; Liu and Howell, 2016). Chloroplasts, due to their electron transport chain, are considered as a stress-initiating cite. Environmental stresses, such as high light, may interfere with chloroplast homeostasis. This chloroplast perturbation is sensed by two proteins located in plastid membranes, EXECUTER1 and 2 (EX1 and EX2). EX 1/EX 2-dependent transduction of signals from the chloroplast to nucleus alter gene expression and mediate programmed cell death (Wagner *et al*., 2004; Lee *et al*., 2007; Mignolet-Spruyt *et al*., 2016).

Once stress is detected, a cascade of signal transducing reactions are initiated in the plant cell, mediated by plant hormones such as abscisic acid (ABA) (Lake and Woodward, 2008), mitogen-activated protein kinase (MAPK) (Wang *et al*., 2007), Ca^{2+} ions (Bai *et al*., 2009), and reactive oxygen species (ROS) (Osakabe *et al*., 2005). A MAPK signalling pathway is usually activated by plasma membrane-mediated stress receptors. It is composed of the consecutive phosphorylation reactions, initiated by MAP kinase kinase kinase kinase (MAP4K) and followed by the activation of down stream kinases (MAP3K, MAP2K and MAPK). Final targets of MAPK signalling pathway are other enzymes (including kinases), proteins, transporters and transcription factors which are phosphorylated and induced by the last kinase, MAPK, and modulate various stress responses (Khokhlatchev *et al*., 1998; Dan *et al*., 2001; Rodriguez *et al*., 2010). A Ca-mediated signalling pathway is responsive to abiotic stresses (such as cold and drought) and is indicated to have cross-talk with the MAPK signalling cascade. Normal cytosolic Ca concentration is in the range of 0.1–1 mM which is regulated by Ca^{2+} transporters and Ca^{2+}/H^{+} antiporters (Trewavas and Malho, 1998; Knight, 2000; Yang and Poovaiah, 2003). A transient increase in Ca content of the cell, due to environmental stresses and developmental processes, activates Ca-binding proteins such as calmodulin (CaM), calcium-dependent protein kinase (CDPK), and calcineurin B-like protein. Integration of Ca and Ca-binding proteins induces a wide range of cellular responses mediated by transcription factors, protein kinases (from the MAPK family), ion transporters and membrane proteins (Yang and Poovaiah, 2003; Tuteja and Mahajan, 2007; Tuteja, 2009; Batistic and Kudla, 2012).

For most stress, perception and signal transduction leads to changes in gene expression patterns with the purpose of managing cellular metabolism, maintaining homeostasis and regulating stress acclimation in plants (Osakabe *et al*., 2013). For example, in plants exposed to water limitation, differential patterns of gene regulation lead to the production of compatible solutes (for osmotic adjustment) and late embryogenesis abundant (LEA) proteins (to avoid protein aggregation) following dehydration (Hoekstra *et al*., 2001; Hirt and Shinozaki, 2004). Similarly, under freezing conditions (below 0° C temperatures), water freezes in plant tissue and the amount of accessible water decreases, causing cellular dehydration. Additionally, growth of ice crystals induces mechanical damage to cell structure and plasma membrane. Therefore, molecular regulation in response to this condition modifies membrane structure, lipid composition, and fluidity to protect plants from freezing damage (Xin and Browse, 2000; Hirt and Shinozaki, 2004; Rehem *et al*., 2012). Flooding imposes hypoxia (shortage of O_2) or anoxia (lack of O_2) on plants. Therefore, prolonged flood stress induces the formation of aerenchyma, which is an air transport system in aquatic plants, in the stems and roots of stressed plants (Peng *et al*., 2005). In response to excess sodium (Na^+) concentrations, gene expression modulates cellular osmotic pressure via producing transporters to sequestrate Na^+ in vacuoles and take up potassium (K^+) to maintain the turgor (Zhu, 2001; Hirt and Shinozaki, 2004).

1.1.1. Reactive oxygen species and antioxidant systems

Reactive oxygen species (ROS) are by-products of normal plant metabolism which are derived from molecular oxygen (O_2) (Sewelam *et al*., 2016). An electron transfer to O_2 prompts the production of different forms of ROS such as superoxide (O_2^{-}), singlet oxygen ($^1O_2$), hydroxyl radical ($HO^{\bullet}$) and hydrogen peroxide (H_2O_2). Photosynthesis and respiration, taking place in the chloroplast and mitochondria, rely on electron transport chains which can be a major source of ROS generation in plant cells (Maxwell *et al*., 1999; Asada, 2006). Additionally, peroxisomes and oxidases located in cell wall and plasma membrane (such as NADPH oxidase and amine oxidase) engage in ROS generation (Foreman *et al*., 2003; Hu *et al*., 2003). A dual function of ROS in plants under stress conditions has been described in many studies, intimating that role of ROS is concentration-dependent. Low concentrations of ROS under normal conditions functions as a messenger, inducing various signalling pathways, whereas

higher concentrations under stress conditions are toxic and deleterious, inducing oxidative stress (Sharma *et al*., 2012). Increased ROS content as a result of stresses such as drought, salinity, high light and pathogen attack can cause serious damage to DNA, proteins, and lipids, influencing gene regulation, enzyme activity, ion/nutrient transport and finally leads to cell death (Buettner *et al*., 1993; Imlay and Linn, 1988; Cabiscol *et al*., 2000; Bethke and Jones, 2001; Sharma *et al*., 2012). It is still unknown how specific ROS and ROS-induced signals are transported to the nucleus to modify gene expression. However, it has been suggested that anion channels may invovlve in O_2^- transmission while aquaporins mediate H_2O_2 transport (Shapiguzov, 2012).

To prevent or attenuate ROS toxicity, plants have enzymatic and non-enzymatic antioxidant systems. Non-enzymatic antioxidants are low molecular weight compounds such as ascorbic acid (AsA), glutathione (GSH), phenolic compounds, and proline. They function to quench free radicals and accumulated ROS. The content of flavonoid compounds in plants is considered as a determining criterion to evaluate antioxidant capacity of a species, due to their engagement in inhibiting lipid peroxidation and scavenging subsequent hydroxyl radicals (Balasundram *et al*., 2006; Ahmad *et al*., 2009; Panda, 2012). Proline is a water soluble amino acid that accumulates in response to dehydration conditions with the purpose of osmotic adjustment. However, its antioxidant properties to diminish stress-induced singlet oxygen generation have been reported in many studies (Smirnoff and Cumbes, 1989; Matysik *et al*., 2002; Verslues and Sharma, 2010; Hayat *et al*., 2012). Enzymatic antioxidants such as superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT) and glutathione reductase (GR) establish the other half of antioxidant machinery, locating in different cellular compartments (Foyer and Noctor, 2005; Halliwel, 2006; Ahmad *et al*., 2008; Jaleel *et al*., 2009; Ahmad *et al*., 2010).

SODs are metalo-enzymes that convert two molecules of superoxide (O_2^-) to hydrogen peroxide and molecular oxygen. Considering that O_2^- generation provokes a series of oxidative reactions, SOD is considered as the first protective system against oxidative damage, playing a crucial role in O_2^- detoxification (Elstner, 1991; Bowler *et al*., 2011). SODs are categorized based on their metal cofactor: copper/zinc (Cu/Zn) (CSD), iron (Fe) (FSD) and manganese (Mn) (MSD). Additionally, they are present in organelles containing electron transport chain

and thus a major source of O_2^- accumulation. Fe-SOD is located in chloroplast, Mn-SOD is mainly found in mitochondrion and peroxisomes and Cu/Zn -SOD is situated in cytosol and chloroplast (Kanematsu and Asada, 1990; Smith and Doolittle, 1992; Alscher *et al*., 2002).

CAT is a heme-containing enzyme and it catalyzes the decomposition of two molecules of H_2O_2 into two water molecules and O_2 (Srivalli *et al*., 2003; Ben Amor *et al*., 2005). The primary site of CAT activity is peroxisomes, but this enzyme has been also detected in cytosol, chloroplast and mitochondria of plant cells (Mhamdi *et al*., 2010). CAT expression and activity have been reported in three different tissues: in the peroxisomes and cytosol of photosynthetic tissues to remove photorespiration-derived H_2O_2, in the peroxisomes and cytosol of plant seeds to decompose H_2O_2 generated from fatty acid degradation, and in the mitochondria of vascular tissues that is presumably related to lignification process (Willekens *et al*., 1994; Das and Roychoudhury, 2014).

APX has the same function as CAT, degrading H_2O_2, although it is mainly located in the chloroplast and cytosol (Das and Roychoudhury, 2014). APX reduces H_2O_2 to two water molecules, employing ascorbate (AsA) as an electron donor, and generating monodehydroascorbate (MDHA). MDHA can either disproportionate to dehydroascorbic acid (DHA) through a non-enzymatic reaction or is reduced back to AsA by the function of monodehydroascorbate reductase (MDAR), requiring NADH oxidation. DHA, which was previously produced from MDHA, is reduced to AsA by the function of dehydroascorbate reductase (DHAR). In this reaction GSH serves as and electron donor and oxidized glutathione (GSSG) is generated. Glutathione reductase (GR) uses NADPH as the final reducing agent to regenerate GSH, while $NADP^+$ is formed in this reaction (Fig. 1.1) (Xiong *et al*., 1992; Tao *et al*., 1998; Foyer *et al*., 2005; Smirnoff, 2005; Zhang *et al*., 2008). The AsA-GSH cycle, mediated by APX, MDAR, DHAR and GR, has an important effect on the content of AsA and GSH and their ratio as two non-enzymatic antioxidants regulating redox homeostasis of plant cell in response to various stress conditions (Das and Roychoudhury, 2014; Sofo *et al*., 2015).

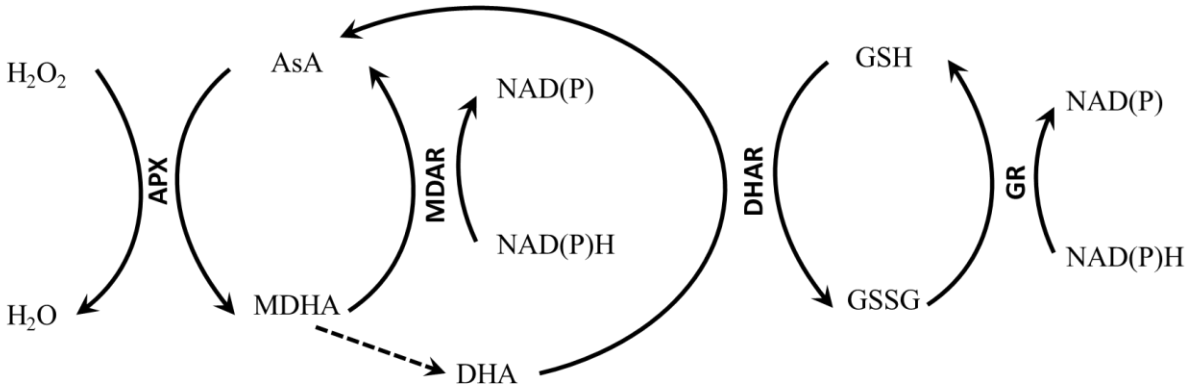

Figure 1.1. Ascorbate-glutathione (AsA-GSH) cycle. Picture adapted from Li *et al*. (2010). APX, Ascorbate peroxidase; AsA, Ascorbic acid (ascorbate); DHA, Dehydroascorbic acid; DHAR, Dehydroascorbate reductase; GR, Glutathione reductase; GSH, Reduced glutathione; GSSG, Oxidized glutathione, MDAR, Monodehydroascorbate reductase; MDHA, Monodehydroascorbate.

1.1.2. ABA signalling pathway

Abscisic acid (ABA) is known as a stress hormone in plants, regulating plant functions in response to different abiotic stresses such as salt (Tao *et al*., 2011), drought (Okamoto *et al*., 2013), low and high temperature (Tuteja, 2007), and high light (Galvez-Valdivieso *et al*., 2009). It is also used for biotic stresses such as bacterial invasions (Melotto *et al*., 2006). Once a plant cell perceives stress, signal transduction cascades are activated to induce the expression of stress-responsive genes in the nucleus. ABA biosynthesis genes are among the first genes activated in response to stress, initiating a second wave of signaling cascades that stimulate additional stress-responsive mechanisms. Therefore, stress-responsive genes can be divided into ABA-dependent or -independent genes based on their reliance on ABA for induction under stress (Swamy and Smith, 1999; Chinnusamy *et al*., 2004; Tuteja, 2007). Stress-responses regulated through ABA signalling pathway require different components such as ABA receptors, phosphatases, and protein kinases to detect accumulated ABA and promote transcription of various ABA-dependent stress-responsive genes. In the absence of ABA, PP2C (Type 2C Protein Phosphatase) binds with SnRK2 (SNF1-Related Kinase) and inhibits its

function via dephosphorylation of its active site (Schweighofer *et al*., 2004; Kuhn *et al*., 2006). It causes the suppression of downstream transcription factors that rely on SnRK2 for their activation (Fujita *et al*., 2009; Umezawa *et al*., 2009). Once ABA is accumulated in a stressed cell, it binds to its specific receptor PYR/PYL (Pyrabactin Resistance Protein1/PYR-Like proteins), resulting in the physical interaction between PYR/PYL and PP2C that separates PP2C from SnRK2 (Park *et al*., 2009; Santiago *et al*., 2009). Therefore, ABA-PYR/PYL complex removes the inhibitory effect of PP2C on SnRK2, leading to phosphorylation and consequently activation of transcription factors that regulate the expression of ABA-responsive genes (Daszkowska-Golec and Szarejko, 2013; Kim, 2014).

To gain a better understanding of the engagement of signalling transduction mediated by ABA, ABA-insensitive (*abi*) mutants have been generated and identified in different studies. *abi* Arabidopsis genotypes carry a mutation (loss-of-function) in one of the components that participate in ABA perception and signalling mechanism. Mutation of each of the two PP2C protein phosphatase homologs results in *abi1* and *abi2* mutants, and mutation in transcription factors activated by SnRK2 generates *abi3*, *abi4* and *abi5* which have been well characterized in Arabidopsis (Finkelstein, 1993 and 1994; Finkelstein *et al*., 1990,1998 and 2000; Leung *et al*., 1997).

1.2. Metal stress

Many metals are toxic elements even at low concentrations (Markert *et al*., 2000; Krauss *et al*., 2001; Rascio and Navari-Izzo, 2011). Metal contamination in the environment is a widespread problem. Soil and water are the main sinks of metal accumulation originating from agricultural pesticides and fertilizers, industrial wastewater, and mining process (Jones and Jarvis, 1981; DeVolder *et al*., 2003; Wuana and Okieimen, 2011). Absorption of metals by plant roots is influenced by root exudates, chemical properties, and the microorganism profile of the soil, playing an important role in metal mobility in soil and uptake by plants (Wenzel *et al*., 2003; Manara, 2012). The cell wall is the first barrier against metal entrance into the cells. Cell wall composition may alter plant sensitivity to metals. For example, callose, mucilage, and hystidyl groups in the cell wall of tobacco roots decreases Mn uptake in tobacco, conferring Mn-resistance to this plant under exposure to a toxic level of Mn^{2+} (150 mM) (Wang *et al*., 1992). The plasma membrane is the second line of defense in roots, regulating metal entrance via

different families of transporters such as ZIP (Vert *et al*., 2002), NRAMP (Thomine *et al*., 2000) and cation diffusion facilitator (CDF) (Williams *et al*., 2000). These transporters are required for uptake of essential metals and nutrients from soil. However, they also participate in metal uptake and are associated with metal response. For example, the ZIP family of transporters participate in the transition of cations across plasma membrane. A characterized ZIP transporter in *A. thaliana*, IRT1, was shown to be specific for Fe^{2+} uptake from soil but it also shows lower selectivity for Mn^{2+}, Zn^{2+}, and Cd^{2+} (Vert *et al*., 2002; Eide *et al*., 1996). It has been also indicated that ZIP transporters play an important role in metal uptake by hyperaccumulators (Kramer *et al*., 2007). NRAMP transporters are described in many organisms (animals, plants, bacteria and fungi) to be involved in the uptake and transport of various cations such as Mn^{2+}, Cu^{2+}, Fe^{2+}, Cd^{2+} and Ni^{2+}. NRAMP1 in *A. thaliana* is an iron-specific transporter, also transports Cd (Nevo and Nelson, 2006; Curie *et al*., 2000). Members of CDF family mediate transport of metals to vacuoles, apoplastic area and ER. Therefore, their overexpression induces metal tolerance and enhanced metal accumulation in plants. AtMTP1 is an example of this transporter in *A. thaliana* that promotes Zn sequestration in vacuole, while overexpressed lines exhibit Zn tolerance (van der Zaal *et al*., 1999; Kramer *et al*., 2007; Peiter et al. 2007).

After entering root cells, metals are transported from roots to shoots through xylem. The chemical composition of xylem sap, which includes organic acids, amino acids, and nicotianamine, allows these organic compounds to function as ligands, determining root-to-shoot translocation of metals (Tiffin, 1970; Leea *et al*., 1977; Kramer *et al*., 1996; Pich and Scholz, 1996; Rascio and Navari-Izzo, 2011; Manara, 2012). Once shoot cells take up and accumulate metals, they may be utilized as a micronutrient or cofactor for enzymes (such as Fe, Mn, Cu and Zn), whereas metals with no biological function are restricted and sequestrated by metal-binding peptides and phytochelatins (PC) (Cobbett, 2000; Hall, 2002; Schat *et al*., 2002; Manara, 2012).

One of the mechanisms that is associated with toxicity due to different metals is ROS accumulation, oxidative stress, and altered redox state of cell. Therefore, stress perception takes place via detecting reactive compounds in the cytosol and cellular compartments (chloroplast and mitochondria), and is mediated through diverse signal transduction pathways

that regulate metal-responsive genes (Kacperska, 2004; Jaspers and Kangasjarvi, 2010; Rascio and Navari-Izzo, 2011). Metal-responsive genes are induced in response to toxic amounts of metals that cause stress in plants. They include genes for metals sequestration such as PC synthase that produces PC in cytosol. Once produced, PC binds with metals and is then transferred to vacuoles (Rauser, 1995; Cobbett, 2000). Metal transporter genes, such as CDF and heavy metal ATPases (HMA) transporters, are also activated in response to metal stresses to pump metal ions to apoplast or into the vacuoles and remove it from the cell (Williams *et al*., 2000; Mills *et al*., 2003). Components of defence mechanism, enzymatic and non-enzymatic antioxidants, are also induced in response to metal exposure to minimize stress-induced damage under this condition (Kampfenkel *et al*., 1995; Zhu *et al*., 1999; Romero-Puertas *et al*., 2007).

1.2.1. Cadmium toxicity

Cadmium (Cd) (atomic number: 48; atomic weight: 112.411 g mol^{-1}) is considered a toxic metals. Although Cd is not an essential element and does not have a known biological function in plants, it is readily taken up by plants due to its high solubility in water and high mobility in soil. Cd uptake by plants is the primary way that Cd enters into the food chain, and can then be toxc to animals and humans (DalCorso *et al*., 2010).

Soil properties such as pH and nutrient composition have a considerable effect on Cd uptake. Acidic soil pH lowers Cd absorption in corn (Benavides *et al*., 2005), while excess Zn interferes with Cd uptake in two metal hyperaccumulators, *Thlaspi caerulescens* and *Arabidopsis halleri* (Cosio *et al*., 2004). Roots are the first and main site of Cd entrance to plants (Clemens, 2006). As mentioned above, Cd crosses the cell membrane through transmembrane carriers, channels, and transporters designed for cation uptake. Cd, at high concentrations in soil, competes with other divalent elements (Zn^{2+} Ca^{2+}, Fe^{2+} and Cu^{2+}) to occupy their transporters and enter the root cell. Therefore, plants exposed to high Cd concentrations will have decreased amounts of macro- and micronutrients, potentially resulting in nutrient deficiency and decreased plant growth (Llamas *et al*., 2000; Roth *et al*., 2006; Papoyan *et al*., 2007; Gonçalves *et al*., 2009). Conversely, high levels of nutrients can alleviate Cd toxicity via minimizing Cd uptake, downregulation of transporters, and inhibiting long-distance transportation of Cd in plants (Suzuki, 2005; Song *et al*., 2009; Chou *et al*., 2011).

Upregulation of NRAMP and ABC transporters in response to Cd and enhanced activity of H^+-ATPase in Cd-tolerant plants shows that these transporters are involved in Cd uptake in roots (Obata *et al*., 1996; Williams *et al*., 2000). Upregulation and activation of metal transporters in response to Cd toxicity seems to be a protective mechanism to compensate Cd-induced decrease of nutrient uptake. Moreover, enhanced activity of these transporters contributes to increased proton/ion transfer across the plasma membrane, helping plant to maintain optimum cytosolic pH, maintain ion balance in the cell, and recover redox homeostasis under Cd toxicity (Janicka-Russak *et al*., 2008 and 2012; Takahashi *et al*., 2012).

Signalling transduction pathways initiated by Cd exposure operate as a comprehensive network leading to orchestration of stress-responsive genes. It has been shown that Cd activates different protein kinases participating in MAPK signalling cascades (Jonak *et al*., 2004). Additionally, Cd exposure increases Ca content of plant cells, which in turn induces Ca binding with calmodulin-like proteins (DalCorso *et al*., 2010). Although Cd is not an efficient redox reactive metal, it causes ROS accumulation and oxidative stress by interfering with other essential plant functions (Polle and Schutzendubel, 2003). Chlorosis and decreased photosynthetic yield have been reported in Cd-treated plants due to Cd interference in chlorophyll structure and perturbation in photosynthetic electron transport in chloroplasts. Additionally, enhanced lipid peroxidation and Cd interaction with antioxidant systems are also significant reasons of oxidative damage in response to Cd toxicity (Leon *et al*., 2002; Smeets *et al*., 2005; Martins *et al*., 2011). Reduction of [GSH]:[GSSG] ratio in Cd-treated plants also points to the involvement of glutathione in Cd-stress response (Romero-Puertas *et al*., 2007). Glutathione may engage in Cd stress response via two main routes: i) due to high affinity of Cd for thiol groups it binds with cysteine in glutathione, while glutathione functions as a Cd-chelator; ii) glutathione (GSH) participates in ROS scavenging that is mediated through AsA-GSH cycle, leading to GSH oxidation and GSSG accumulation. Additionally, glutathione as a building block of PC is extensively used by PC synthase to provide more chelating reagents in response to toxic Cd (Rauser, 2001; Cobbett and Goldsbrough, 2002; Mendoza-Cozatl *et al*., 2008; Jozefczak *et al*., 2014).

Stress hormones such as ethylene and salicylic acid (SA) together with stress-induced ROS accumulation are considered as Cd-induced signalling messengers, regulating gene expression

and plant response under Cd treatment (Maksymiec, 2007; Romero-Puertas *et al*., 2007). These signalling mechanisms give rise to molecular modification via inducing transcription factors. Transcription factors that are associated with Cd-toxicity response belong to different families mediating stress response under other biotic and abiotic stresses (Jacoby *et al*., 2002; Singh *et al*., 2002; Wei *et al*., 2008). The transcript profile of Cd-responsive genes in Arabidopsis is classified into three main groups: signal transduction factors (protein kinases and calmodulin-related proteins), proteins with protective function (chaperones and metal binding proteins) and transcription factors (mainly DRE binding protein, DREB) (Suzuki *et al*., 2001). These transcription factors regulate the expression of genes responsible for metal transporters, chelating compounds, and ROS detoxification which can mitigate Cd stress in plants (Thomine *et al*., 2000; Heiss *et al*., 2003; Romero-Puertas *et al*., 2007; Cailliatte *et al*., 2009, DalCorso *et al*., 2010).

1.3. Ureides

1.3.1. General description of ureides and nitrogen fixation

Nitrogen is an essential nutrient for plant growth due to its incorporation in the building blocks of proteins (amino acids), genetic material (nucleic acids) and photosynthetic apparatus (chlorophylls) (Montalbini, 1992). In spite of the high percentage of nitrogen in the atmosphere (~ 78% in comparison with ~ 21% for oxygen), atmospheric nitrogen can not be absorbed and utilized by plants due to lack of the enzyme nitrogenase, required to break the stable triple bond in dinitrogen gas (Nasholm *et al*., 2009; Hoffman *et al*., 2014; Baral *et al*., 2016). Therefore, most plants obtain their required nitrogen through available nitrogenous compounds in soil, such as ammonium (NH_4^+) and nitrate (NO_3). However, legumes (Fabaceae family) have developed a symbiotic relationship with bacteria (Rhizobiaceae family), allowing them to benefit from bacterial nitrogenase that breaks the dinitrogen triple bond and converts atmospheric N_2 to NH_3^+. Plants provide a carbon and energy source for bacteria in the form of dicarboxylic acids. This process (symbiotic nitrogen fixation) takes place in the nodules of infected roots (Schubert, 1986; Udvardi and Day, 1997; Franche *et al*., 2009, Masclaux-Daubresse *et al*., 2010,). Once NH_3^+ is produced inside large organelle like compartments termed bacteroids, it diffuses into the cytosol of nodulated root cells and protonated to NH_4^+ (Swain and Abhijita, 2013; Udvardi and Poole, 2013). In infected cells NH_4^+ is utilized by

glutamine (Gln) synthetase. In tropical legumes (such as bean, soybean and cowpea) the resultant Gln is employed in the purine synthesis pathway in mitochondria and plastids. Purines, adenine (A) and guanine (G), have a heterocyclic structure composing of pyrimidine and imidazole rings. The importance of purines is mainly related to their involvement in nucleotides as a nitrogenous base of DNA and RNA. However, they also function as an energy source, such as adenosine- and guanosine-triphosphate (ATP and GTP), and enzyme cofactors, such as nicotinamide adenine dinucleotide (NAD) (Brown, 1975; Stasolla *et al*., 2003). Once purines are degraded to form xanthine, it is metabolized by the ureide pathway to generate ureide intermediates (Shelp *et al*., 1983; Atkins *et al*., 1997; Chungopast *et al*., 2014).

Ureides are urea-derivative compounds in which hydrogen atoms are replaced by organic groups (Reinbothe and Mothes, 1962). Considering that urea (CH_4N_2O), either applied as an agricultural fertilizer or produced in plant through arginine degradation, is known as a major source of nitrogen for plant growth, their derivatives, ureides, are also considered as an important form of nitrogen for nitrogen storage and transport in plants (Merigout *et al*., 2008; Witte, 2011). Correlation between N_2-fixation and ureide content of xylem sap has been addressed in different studies, showing that ureides are the predominant form of nitrogen in the xylem sap of nodulated soybean (up to 90% of nitrogen content of xylem sap), whereas nitrate is mainly observed in plants fed with soil nitrogen (McClure *et al*., 1980; Pate *et al*., 1980; Patterson and LaRue, 1983; Herridge *et al*., 1988 and 1990). Interestingly, among ureides, allantoin and allantoic acid are shown to be the most abundant transported nitrogen in the xylem sap of many legumes (Reynolds *et al*., 1982). Allantoin content of *Phaseolus vulgaris* leaves was compared between nodulated and non-nodulated samples. Nodulation increases allantoin concentration to around seven times more than that of non-nodulated plants (Pélissier and Tegeder, 2007).

N_2 fixation is not the only way ureides are produced for transport, as studies indicated that non-legume plants synthesize and transport ureides. Uriedes consist 5.6% of transported nitrogen in *Coffea arabica* (Mazzafera and Goncalves, 1999). Additionally, allantoin synthesis has been detected in different plants families including Aceraceae, in *Acer saccharinum* seedlings, (Barnes, 1962), Boraginaceae, in *Symphytum officinale* roots (Castro *et al*., 2001) and Bignoniaceae, in stalks of *Adenocalymma peregrinum* (dos Santos *et al*., 2014). It is well

explained that ureide metabolism derives from purine degradation, while xanthine is known to be first precursor in this pathway. For the purpose of this project, the term "ureides" refers to those compounds derived from purine catabolism, while the process of xanthine degradation leading to the production of ureide intermediates (such as uric acid, allantoin and allantoate) and ending with glyoxylate, carbon dioxide (CO_2) and NH_4^+ generation is considered as the "ureide pathway" (Schubert, 1981; Baral *et al*., 2016).

1.3.2. Purine degradation and ureide metabolism

The earliest reports about the presence of ureides in legumes date back to the studies performed in 1930s (Fosse *et al*., 1930 and Umbreit and Burris, 1938 as cited by Reynolds *et al*., 1982), followed by primary investigations on these compounds carried out by a French group in 1938 and 1947 (Brunel and Echevin, 1938 and Brunel and Capelle in 1947 as cited by Thomas and Schrader, 1981). Besides N_2-fixation, two possible routs for ureides metabolism was proposed in plants.

First, non-enzymatic formation of ureides, allantoin and allantoate, mediated via condensation of glyoxylate with urea was suggested by Steward and Pollard (1957). Presence of this rout has been previously reported in basidiomycetes (Brunei and Brunel-Capelle, 1951 as mentioned in Butler *et al*., 1961). Moreover, radioactivity of allantoic acid was demonstrated in banana leaves after feeding with C^{14}-urea, determining the involvement of urea in the formation of this ureide (Frieberg *et al*., 1957). In contrast, a similar experiment in wheat indicated that administration of C^{14}-glycine into wheat roots and leaves resulted in radioactive allantoin and allantoic acid, whereas no radioactivity was detected upon C^{14}-urea exposure (Krupka and Towers, 1959).

Second, in 1956, Franke and Hahn, suggested that ureides are formed from the oxidative degradation of purine rings in nucleic acids (as cited by Mothes, 1961). Employing radioactive measurements, Barnes (1959) indicated that if adenine-8-C^{14} (labelled C in position 8) is fed to young leaves of silver maple (*Acer saccharinum* L.), after 24 h radioactive allantoin, allantoic acid and urea would be detected in the ethanolic extract of leaves. This experiment also revealed the reaction sequence of ureide metabolism in higher plants, which was also reported in animals (Hartman and Buchanan, 1959), as shown below:

Adenine → Hypoxanthine → Xanthine → Uric acid → Allantoin → Allantoic acid → ammonia + Glyoxylate

Similarly, generation of radioactive uric acid, allantoin, allantoate and urea from guanine-8-C^{14} or hypoxanthine-8-C^{14} are observed in cell-free extracts of cowpea (*Vigna unguiculata* L.) nodules, providing additional evidence that ureides derive from purines in plants (Woo *et al*., 1980). Nevertheless, purine degradation and ureide metabolism is not restricted to legumes and plants. Degradation products of purines (uric acid, allantoin, allantoic acid, urea and ammonia) have been also identified and reported in animals and microorganisms, in which existence or lack of functional enzymes in this pathway throughout the organisms determine the ultimate produce of purine catabolism (Oda *et al*., 2002; Lee *et al*., 2013).

1.3.2.1. Purine degradation and ureides in microorganisms

Growing *Penicillium chrysogenum* in a growth culture containing xanthine as the sole nitrogen compound reveals the existence of ureides enzymes and illustrated the reaction steps initiated by xanthine dehydrogenase (degrading xanthine to uric acid), followed by uricase (converting uric acid to allantoin), continued with allantoinase (catalysing allantoin to allantic acid) and allantoicase (breaking allantoic acid into glyoxylic acid and urea) in this fungus (Allam and Elzainy, 1969). Interestingly, similar studies with *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Penicillium citreoviride* and *Penicillium noiaium* revealed that conversion of allantoic acid to glyoxylic acid is a two-step reaction in which allantoicase cleaves allantoic acid to urea and ureidoglycolate, while the second enzyme called ureidoglycolase catalyses the complete breakdown of ureidoglycolate to urea and glyoxylic acid (Trijbels and Vogels, 1966).

In *Bacillus subtilis*, purine degradation is activated and serves as an alternative source of nitrogen when primary nitrogenous compounds such as ammonia or glutamate are not accessible. Purine catabolism initiates with adenine as a substrate for adenine deaminase (*adeC*) to form NH_3 and hypoxanthine which is then converted to xanthine by the function of xanthine dehydrogenase (*pucABCDE*). The alternate rout to generate xanthine is mediated by guanine deaminase (*gde*), transforming guanine to NH_3 and xanthine. Xanthine is then converted to uric acid by xanthine dehydrogenase. Uricase (*pucLM*) generates allantoin from uric acid, that is utilised by the enzyme allantoinase (*pucH*) to form allantoic acid. Allantoic acid aminohydrolase (*pucF*) converts allantoic acid to ureidoglycine, followed by further

degradation to ureidoglycolic acid and NH_3. Ureidoglycolase (*pucG*) breaks ureidoglycolic acid to glyoxyate and urea that is fully degraded to NH_3 and CO_2 by urease (*ureABC*) (Nygaard *et al*., 1996 and 2000; Saxild *et al*., 2001; Schultz *et al*., 2001; Beier *et al*., 2002; Goelzer *et al*., 2008; Ma *et al*., 2016).

In *Escherichia coli*, purine catabolism is characterized when allantoin is employed as the only nitrogen source in the growing culture. Bacterial allantoinase cleaves allantoin ring to generate allantoate which is then broken into ureidoglycine, NH_4^+, and CO_2. Further degradation of ureidoglycine leads to the formation of ureidoglycolate and NH_4^+. Resultant ureidoglycolate can be processed via two enzymes: ureidoglycolate hydrolase that produces urea (instead of ammonia) and glyoxylate, whereas oxaluric acid would be the end product if ureidoglycolate is oxidized by ureidoglycolate dehydrogenase. In subsequent reactions glyoxylate undergoes glycerate pathway and oxaluric acid is converted to ATP, NH_4^+, and CO_2, while carbamoyl phosphate serves as an intermediate in this reaction (Tigier and Grisolia, 1965; Ornston and Ornston, 1969; Xu *et al*., 1995; Werner *et al*., 2010). Figure 1.2. shows the metabolic map of allantoin catabolism proposed by Cusa *et al*. (1999).

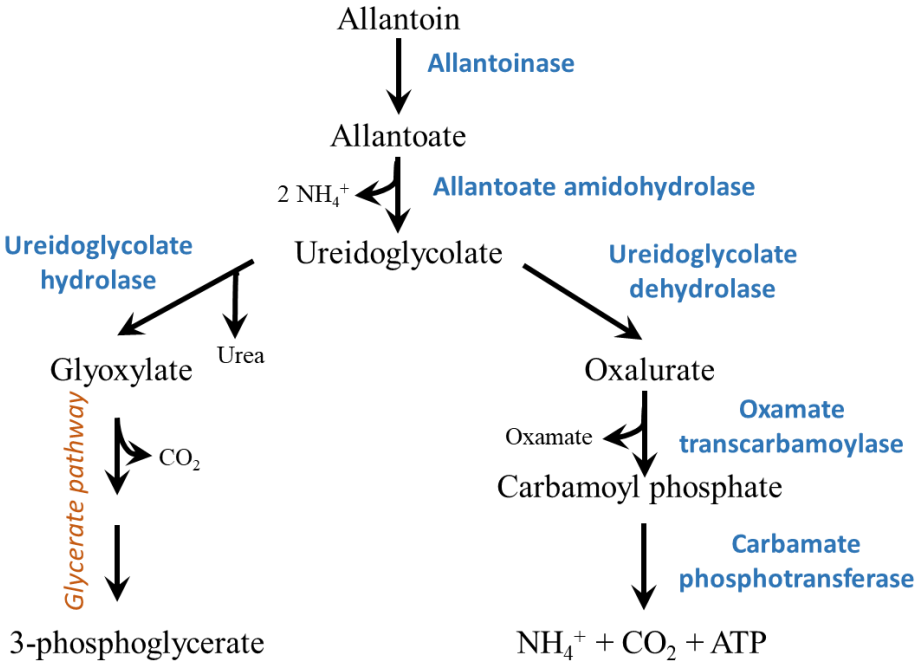

Figure 1.2. Catabolism of allantoin in *Escherichia coli* (Enterobacteriaceae family). Pathway is modified from Cusa *et al*. (1999).

1.3.2.2. Purine degradation and ureides in animals

Purine catabolism in animals leads to the accumulation of a wide range of nitrogenous compounds from primary precursors, such as guanine and xanthine, to last compounds such as urea and ammonia. As described by Vogels and Van der Drift (1976) water availability and "economy" in living organisms determine the predominant nitrogenous waste. For example, in the presence of sufficient water (such as in aquatic invertebrates), the major waste nitrogenous compound is ammonia. Since water is required to quickly remove this toxic compound (Goldstein and Forster, 1965; Balinsky, 1970). Conversely, like in most terrestrial mammals where H_2O may be limiting, urea is most abundant. Urea is less toxic than ammonia and can be stored temporarily before excretion (Forster and Goldstein, 1966; Balinsky, 1970). Birds, reptiles and insects accumulate uric acid (Vogels and Van der Drift, 1976; Oda *et al*., 2002).

Although most mammals have uricase for convertion of uric acid to allantoin (Maiuolo *et al*., 2016), in humans, uric acid is the final metabolite of purine catabolism due to the absence of the enzyme uricase. As a result, the content of uric acid in human serum can be elevated (ten fold higher than in other organisms) and is eventually excreted in urine (Johnson *et al*., 2009). Although uric acid can be also converted to allantoin through a non-enzymatic reaction, this process is relatively slower than the enzymatic oxidation accomplished by uricase. Therefore, if not properly excreted, excess amounts of uric acid in the serum can precipitate in joints, causing gout, or in kidneys, causing kidney stones. External uricase is reported to be clinically exploited to eliminate the pain (Ramazzina *et al*., 2006; Tipton, 2006). It is also worth mentioning that, under extremely alkaline pH, which can occur under stress conditions, uric acid is oxidized to allantoin via a non-enzymatic reaction. Thus, allantoin level of serum is considered as an indicator of stress in human body (Kand'ár and Záková, 2008; Gruber *et al*., 2009).

Interestingly, it has been indicated that conversion of uric acid to allantoin mediated by the enzyme uricase is not the only enzymatic reaction required for uric acid degradation. In the genome of mouse two genes have been identified, encoding two enzymes which are suggested to participate in allantoin generation from uric acid (Ramazzina *et al*., 2006). True oxidation product of uric acid oxidation is reported to be 5-hydroxyisourate (HIU). HIU is a substrate for the enzyme HIU hydrolase resulting in 2-Oxo-4-hydroxy-4-carboxy-5-ureidoimidazoline

(OHCU) formation. Decarboxylation of this compound by OHCU decarboxylase releases CO_2 and yields allantoin as the end product. The function of these two subsequent enzymes, HIU hydrolase and OHCU decarboxylase, is recognized under the name of allantoin synthase. Figure 1.3. presents the schematic pathway for uric acid degradation (Kahn *et al*., 1997; Kahn and Tipton, 1998; Sarma *et al*., 1999; Oda *et al*., 2002; Ramazzina *et al*., 2006; Baral *et al*., 2016).

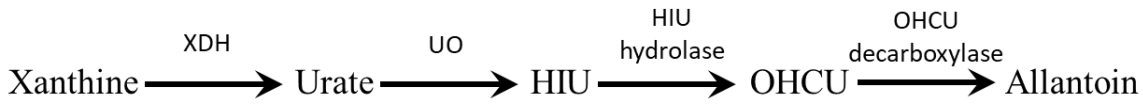

Figure 1.3. Urate degradation and allantoin production as proposed by Ramazzina *et al*. (2006). HIU, 5-hydroxyisourate; OHCU, 2-Oxo-4-hydroxy-4-carboxy-5-ureidoimidazoline; UO, Urate oxidase (uricase); XDH, Xanthine dehydrogenase.

1.3.2.3. Purine degradation and ureides in plants

Purine degradation is an important process for recycling nitrogen (N) in plants. Once AMP deaminase (AMPD) converts adenosine monophosphate (AMP) to inosine monophosphate (IMP), IMP may de-phosphorylate and then hydrolyse to hypoxanthine or be converted to xanthosine through a xanthosine monophosphate (XMP) intermediate. Both hypoxanthine and xanthosine are metabolized to xanthine via xanthine dehydrogenase (XDH) and inosine/guanine nucleosidase, respectively (Schubert and Boland, 1990; Atkins *et al*., 1997; Zrenner *et al*., 2006). Xanthine is often considered as the first compound of the ureide pathway. Ureide metabolism begins with cytosolic XDH cleaving the purine ring of xanthine, generating uric acid. Based on the availability of electron accepters, XDH has an opposing function which has been well characterized in animals and plants. Besides its dehydrogenase activity, XDH can also perform as xanthine oxidase (XO). Therefore, XDH is also known as xanthine dehydrogenase/oxidase or xanthine oxidoreductase (XOR) (Chung *et al*., 1997; Hofmann, 2016; Ma *et al*., 2016). The dual function of XDH makes it a critical enzyme regulating ureide production. In *Arabidopsis thaliana*, this enzyme has a high affinity for NAD^+ as electron acceptor to form uric acid from xanthine. However, in the absence of NAD^+, O_2 serves as an

alternative electron acceptor, generating superoxide radicals along with uric acid (Zarepour *et al*., 2010; Werner and Witte, 2011). There are two copies of *XDH* in the genome of *Arabidopsis thaliana* with 93% identity, *AtXDH1* and *AtXDH2*. However, studies showed that in *xdh1* Arabidopsis mutants, the presence of XDH protein, XDH enzyme activity and accumulation of uric acid are not detectable, intimating that *XDH2* is a pseudogene and does not participate in xanthine degradation, whereas *AtXDH1* is suggested to be the principal gene in ureides pathway due to its response to physiological and environmental variations (Hesberg *et al*., 2004; Yesbergenova *et al*., 2005; Zarepour *et al*., 2010; Hauck *et al*., 2014).

XDH is a key enzyme in plant ureide biosynthesis due to the conversion of hypoxanthine to xanthine and then to uric acid (Tajima *et al*., 2004; Alamillo *et al*., 2010). The resultant uric acid is transported to peroxisomes where its oxidation is catalyzed by urate oxidase (also known as uricase, UO) to form 5-hydroxyisourate, which is then converted to allantoin by allantoin synthase (ALNS) (Bergmann *et al*., 1983; Le *et al*., 1993; Stasolla *et al*., 2003; Lamberto *et al*., 2010; Pessoa *et al*., 2010). Translocation of allantoin to the endoplasmic reticulum (ER) initiates further hydrolysis of allantoin to allantoate mediated by the enzyme allantoin amidohydrolase (also known as allantoinase, ALN) (Yang and Han, 2004; Mullen and Trelease, 2006; Raso *et al*., 2007). The next reaction is catalyzed by a Mn-dependent enzyme, allantoate amidohydrolase (AAH), breaking allantoate into S-ureidoglycine, CO_2 and NH_4^+ (Todd and Polacco, 2006; Werner *et al*., 2008 and 2010; Serventi *et al*., 2010). Further degradation of S-ureidoglycine, catalysed by ureidoglycine aminohydrolase (UGlyAH) and ureidoglycolate amidohydrolase (UAH) releases three more nitrogen as NH_4^+, and yields CO_2 and glyoxylate as final components of this pathway (Werner *et al*., 2010). Fig. 1.4 shows the ureide pathway in Arabidopsis.

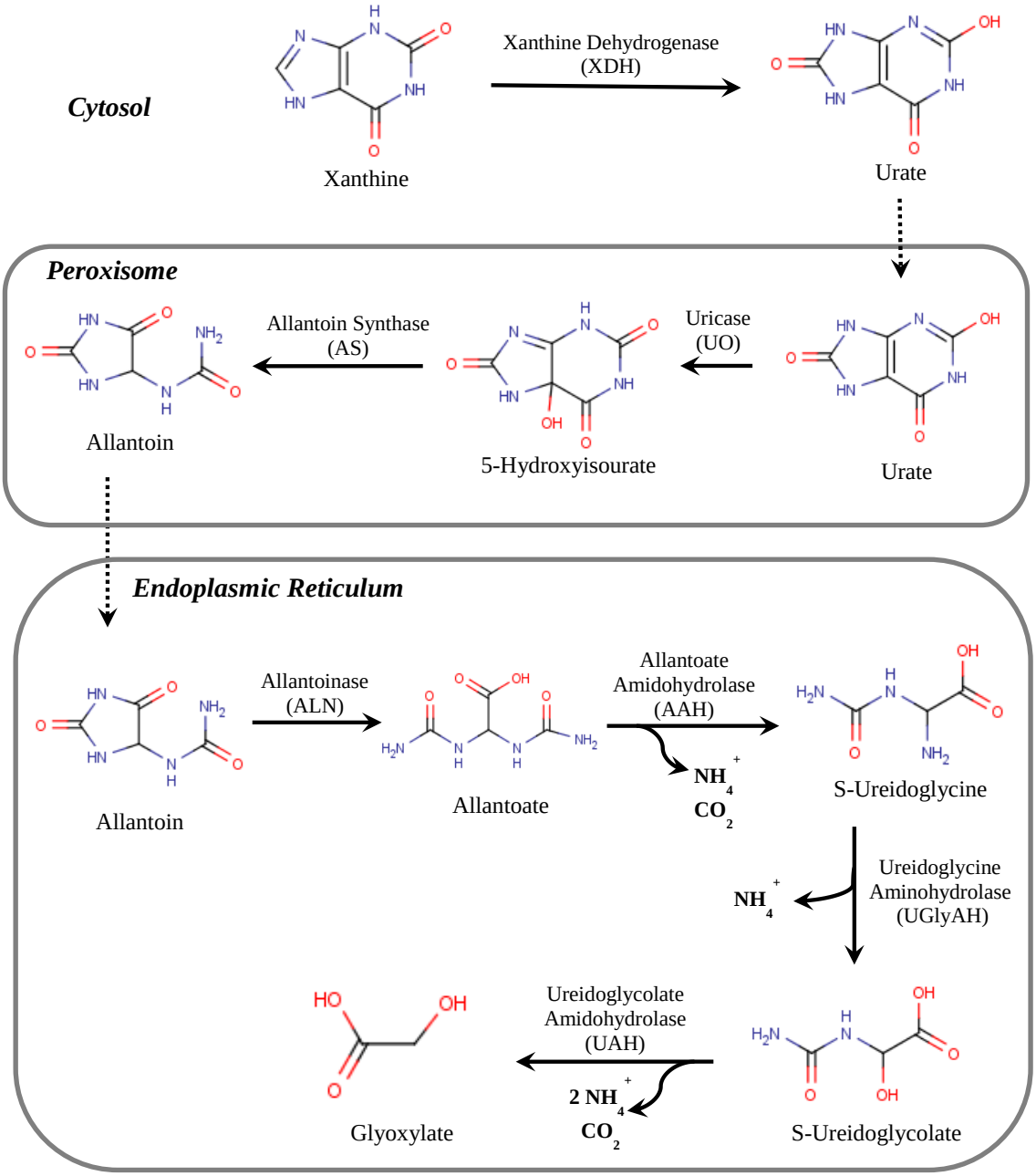

Figure 1.4. Ureide pathway in *Arabidopsis thaliana*. Adapted from Watanabe *et al*. (2014)

1.3.3. Ureide transporters

In legumes ureides are produced in nodules and are loaded into the xylem and translocated to aerial parts of the plant, metabolized in leaves, and organic nitrogen is transported from leaves (source tissue) to growing tissues (sink such as flowers and fruits) through phloem (Tegeder, 2014). Considering that the vascular system in nodules are surrounded by a Casparian band that makes apoplasmic transit impossible, ureides rely on plasma membrane proteins and symplasmic transporter(s) for leaving nodules and entering the xylem (Pélissier *et al*., 2004). Therefore, nitrogen distribution in tissues, cells and organelles requires the function of various transporters to modulate ureide root-to-shoot translocation and nitrogen circulation (Lalonde *et al*., 2003). Urate permease (UPS), belonging to the drug/metabolite transporter (DMT) protein family, is one of the ureide transporters showing variable affinities for ureides in different plants (Desimone *et al*., 2002; Schmidt *et al*., 2004 and 2006). In soybean (*Glycine max*) two proteins, GmUPS1-1 and GmUPS1-2, have been identified that participate in exporting allantoin and allantoate from nodules. RNAi *UPS1* plants demonstrate increased amount of ureides in their nodules, whereas symptoms of nitrogen deficiency are reported in their leaves, exhibiting declined nitrogen transport from nodule to shoot. Interestingly, repression of *UPS1* not only change ureides accumulation and nitrogen partitioning, but also negatively influence nodule development and their N_2-fixation (Collier and Tegeder, 2012).

A similar protein has been characterized in French bean (*Phaseolus vulgaris*). PvUPS1 displays a high affinity for allantoin while molecular analysis demonstrates that there is a positive correlation between *PvUPS1* transcription and allantoin content of different plant organs. Furthermore, expression of *PvUPS1* in the phloem suggest that this transporter in *P. vulgaris* not only participate in xylem loading of allantoin in nodules, but also is important in xylem-to-phloem transport (phloem loading) of this ureide (Pélissier and Tegeder, 2007). Likewise, *Pelissier et al*. characterized *PvUPS1* in an allantoin transport-deficient yeast mutant, indicating that this protein transports allantoin preferentially, although can transport xanthine and uric acid when they are available in excess amounts. *PvUPS1* demonstrates the highest expression level in roots and specifically in nodules (Pelissier *et al*., 2004).

In non-legume plants a nucleobase transporter, purine permease 1 (AtPUP1), has been identified for adenine, guanine and hypoxanthine transport in Arabidopsis, and it is also

suggested to play a role in the transport of purine-oxidation derivatives (Gillissen *et al*., 2000). Additionally, identification of UPS1 in Arabidopsis (AtUPS1) suggests a role for this protein in transporting allantoin, uric acid and xanthine. Expression analysis of *AtUPS1* demonstrates that nitrogen source and availability have a considerable effect on the transcript level of this gene. *AtUPS1* exhibits the highest transcript level when allantoin is applied as the sole nitrogen source in growing media, in comparison with ammonium nitrate-containing plates. Besides allantoin, lack of nitrogen or nitrogen deficiency is suggested to be another inducer of *AtUPS1* expression. In the event of allantoin limitation, AtUPS1 can bind with other purine degradation derivatives (Desimone *et al*., 2002). Similarly, it is reported that in maize (*Zea mays*) uric acid and xanthine are transported by leaf permease 1 (LPE1), also showing affinity for ascorbate at high concentrations (> 30 mM). LPE1 is demonstrated to be a plasma membrane protein and is involved in the development and function of chloroplast in maize (Argyrou *et al*., 2001).

1.3.4. Role of ureides

1.3.4.1. Nitrogen recycling

The importance of plant ureides is due to their nitrogen-rich structure. Once symbiotic bacteria fix atmospheric N_2, resultant organic nitrogen is transported from nodules to above-ground tissues in the form of ureides, allantoin or allantoate, or amides, asparagine (Asn) or glutamine (Gln) (Schubert, 1981 and 1986; Stebbins and Polacco, 1995). Therefore, based on which nitrogenous compound they transport, legumes are called either ureide or amide exporters (Schubert, 1986; Todd *et al*., 2006). Amides are mainly found in the temperate species (vetches, pea, lupine etc.), whereas ureides are the main form of transported nitrogen in tropical species (soybean, common bean, cowpea etc.) (Reinolds *et al*., 1982; Schubert, 1986; Troitskaya *et al*., 2000). Once formed, these nitrogen-rich compounds are translocated to the aerial tissues through xylem sap where their nitrogen is released. Studies have shown that ureides are more efficient for plants than amides. In plants, nitrogen is added to a C skeleton for assimilation and transport. Ureides show lower C:N ratio, implying that they require less photosynthate C for N transportin comparison with amides (1.4 g C per g fixed N in ureides in respect with 3.9 g C per g fixed N in amides). The C:N ratio in transported ureides, allantoin and allantoate, is 1:1 indicating that for each N in their structure one C is required. The ATP cost for carbon used in allantoin is estimated as 5 ATP per molecule. In comparison, the C:N

ratio in amides is half as much, or less than in ureides with 2:1 in Asn and 2.5:1 in Gln. Accordingly, the C-dependent cost of amide generation is reported as 12 ATP for Asn production. Therefore, ATP spent for ureide generation is half that of amides (Atkins, 1991; Schubert, 1986; Smith and Atkins, 2002; Todd *et al*., 2006), making ureides a cost-effective way of transporting nitrogen. Ureide degradation in both legumes and non-legumes results in releasing four molecules of ammonia (NH_4^+) mediated by the function of three enzymes, AAH, UGlyAH and UAH as described above (Piedras *et al*., 2000; Raymond *et al*., 2005; Serventi *et al*., 2010).

Seed germination is a process in which the balance between purine synthesis and salvage plays a significant role. Germinated seeds require adequate purines (and pyrimidines) for nucleic acid synthesis (both DNA and RNA), while they also need to recover stored nutrients (including nitrogen) to support a successful germination of embryo (Deltour, 1985). During the early phase of seed germination, incorporation of purines into ureide is considerably limited, likely related to water limitation and hydration status of seed at the beginning of imbibition stage (Ashihara, 1983; Stasolla *et al*., 2001). However, a few days after germination of black gram (*Phaseolus mungo*) seeds, 60% of applied radioactive hypoxanthine is detected in ureides (Ashihara and Nobusawa, 1981). Similarly, in white spruce (*Picea glauca*) an increase in purine salvage and ureide generation is indicated in day four after germination (Stasolla *et al*., 2001 and 2003). Ashihara also monitored purine catabolism and ureides production during seed germination and in cotyledon and embryonic axes of black gram. This study shows that adenine, adenosine and guanine breakdown initiate in cotyledon at 12-48 h of seed germination while ureide production is detected after 48 h in cotyledons and at later stages in the axis (Ashihara, 1983). These observations show the importance of purine degradation in recovery of stored nitrogen in seeds and providing nitrogen requirements of embryo at early stages of germination (Stasolla *et al*., 2003).

Purine catabolism and ureides are also associated with plant senescence with the purpose of N recovery and remobilization in these tissues. Senesce is an oxidative process that appears due to natural plant aging or as a consequence of environmental stresses such as darkness, drought and salinity. This process is coupled with metabolite turnover and nutrient transition from senescing tissues (source) to sink organs (such as flowers and seeds). Ureides accumulate in

senescing cotyledons in legumes (Stebbins and Polacco, 1995; Baral *et al*., 2016). Studies on *G. max* indicate that plant age induces ureide accumulation in these plants (Osborne and Riedell, 2011). One of the enzymes of ureide metabolism that is responsive to leaf senescence is XDH1. Under prolonged dark treatment and leaf aging ureide metabolism genes are induced an activated in Arabidopsis. In Col-0 samples, senescence induces *XDH1* and *UO* transcription, causing enhanced allantoin and allantoate generation in old leaves. Adversely, compromising *XDH1* (*Atxdh1*) results in early senescence and xanthine accretion in these mutants. This process is accompanied by premature yellowing and higher expression of senescence-associated gene 12 (*SAG12*), leading to chlorophyll degradation and cell death (Brychkova *et al*., 2008).

1.3.4.2. Ureides and abiotic stress responses in plants

In addition to the important role ureides play in nitrogen remobilization, they are suggested to be involved in abiotic stress responses. Different studies have shown the engagement of ureides and ureide metabolism genes and enzymes in stress response and tolerance. For example, irradiation level induces ureides accumulation via regulating ureide metabolism at transcription level. Exposing *Eutrema salsugineum* (*Thellungiella salsuginea*) to high light condition (750 μmol photon m^{-2} s^{-1}) causes a remarkable increase in total ureides concentration in comparison with moderate light levels (250 μmol photon m^{-2} s^{-1}) treatment. This rise in ureide content is related to a slight increase in transcription of *ALN* and *XDH2*, as well as noticeable decreased expression in two other genes, *AAH* and *UGlyAH* (Malik *et al*., 2016). Water limitation induces allantoate accumulation in roots, shoots and leaves of common bean (*Phaseolus vulgaris*). Elevated allantoate production is contributed to drought stress-induced gene regulation. Molecular analysis shows that drought treatment induces *ALN* expression, while decreases the expression of *AAH*, catalyzing allantoate degradation (Alamillo *et al*., 2010). Interestingly, quantification of allantoin content in 15 Chinese rice (*Oryza sativa*) cultivars revealed that there is a positive correlation between allantoin concentration and stress tolerance in rice grains. Cultivars that contain higher allantoin concentrations are more resistant and show increased survival under low temperature and water limitation conditions (Wang *et al*., 2007b).

Dark treatment of Arabidopsis seedlings stimulates the expression and enzyme activity of *AtXDH1*, leading to accumulation of allantoin and allantoate under this condition. Interestingly, a comparison between wild-type and *XDH1*-compromised mutants (*Atxdh1*) under dark treatment demonstrates that this mutation results in more yellowing of leaf tissue, lowered chlorophyll content and increased O_2^- production in respect with Col-0 samples under the same treatment. Exogenous application of allantoin and allantoate to Col-0 leaf discs can protect the leaf tissue from stress damage through decreasing ROS accumulation (Brychkova *et al*., 2008). Likewise, RNA interference-mediated silencing of *XDH* in Arabidopsis, not only impinges on plant growth and fertility, but also imposes susceptibility to drought treatment on these mutants in respect with wild-type samples. Suppression of *XDH* leads to decreased chlorophyll, declined biomass, higher cell death and enhanced H_2O_2 content in response to drought stress that is related to diminished uric acid generation and consequently impaired ureides metabolism in these mutants (Nakagawa *et al*., 2007; Watanabe *et al*., 2010).

Involvement of allantoin in plant stress responses and tolerance has also been reported by Lescano *et al*. (2016). Arabidopsis plants exposed to increasing concentration of NaCl show allantoin accumulation that is most likely due to a decrease in *AtALN* transcription under NaCl treatment. The promoter of *AtALN* is susceptible to salt stress, showing lower activity in response to increasing NaCl concentrations. Moreover, the ureide transporter, AtUPS5, is a significant determinant of allantoin accumulation and distribution in salt-treated plants. UPS5 is shown to incorporate in long distance (root-to-shoot) transportation of allantoin, while its knockout causes a salt-sensitive phenotype in *ups5* mutants and causes decreased allantoin content of shoot and altered root:shoot allantoin content (Lescano *et al*., 2016). T-DNA insertion in the *ALN* gene, leading to constitutive accumulation of allantoin, confers stress tolerance to these mutants when exposed to drought-shock and mannitol-induced osmotic stress. Moreover, abscisic acid (ABA) accumulation is shown as a result of *ALN*-knock out mutation and exogenous application of allantoin. It has been reported that enhanced concentration of allantoin increases the transcription of 9-cisepoxycarotenoid dioxygenase 3 (*NCED3*), regulating ABA biosynthesis, and activates β-glucosidase (*BG1*) which is responsible for deconjugation of glucose-ABA. These two allantoin-derived transcriptional and post-translational gene modification lead to elevated amount of ABA in *aln* mutants or in

allantoin-treated plants causing the stimulation of ABA signalling pathway and consequent induction of ABA-responsive genes under stress (Watanabe *et al*., 2014).

The protective effect of allantoin in response to stress has been also shown by Irani and Todd (2016) indicating allantoin accumulation in response to drought, NaCl treatment and mannitol-derived osmotic stress as well as more stress resistance of *aln* mutants grown under these conditions when compared with Col-0 plants. Importantly, their results demonstrated that increased content of allantoin in *aln* mutants protects plants from negative effects of stress via minimizing the concentration of ROS and as a result restricting oxidative damage in these plants (Irani and Todd, 2016).

1.4. Research hypotheses

Based on the above literature review and studies carried out on the ureide pathway in the Todd lab, it has been demonstrated that ureide metabolism is associated with abiotic stresses such as drought, high light, osmotic stress and salinity in plants. Furthermore, it has been indicated that intermediates of ureide metabolism, allantoin and allantoate, accumulate in response to different stress conditions, implying their potential role in plant stress response. Complementary studies using ureide mutants, in which allantoin production is suppressed, demonstrate that the presence and accumulation of allantoin is required for the proper growth of plant at stress conditions. Interestingly, external application of ureides (mainly allantoin and allantoate) and generating *aln* mutants (containing increased amount of allantoin) provided evidence that concentrated amounts of allantoin improves plant growth following stress exposure, while a ROS scavenging mechanism has been proposed for the function of this ureide in response to stress. However, it is still not clear whether involvement of ureides in abiotic stresses is a general approach adopted by plants to confront stress, how allantoin functions in stressed plants and protects them from the stress.

Considering this background information, this study was designed to address the questions concerning the response and regulation of ureide metabolism at stress conditions as well as the mechanism(s) mediated by ureides in stressed plants. Employing physiological, molecular and biochemical assays I evaluated the effect of Cd, as an abiotic stressor inducing ROS generation, on ureides accumulation in *Arabidopsis thaliana*. In this regard, this project relies on the following hypotheses to evaluate the possible link between ureides and plant response to Cd

toxicity. I started this project with the primary focus on characterizing ureide pathway in response to Cd treatment. Therefore, my first two hypotheses point to the metabolic and molecular regulation of this pathway following Cd treatment in Arabidopsis:

i) I hypothesize that the ureide pathway is responsive to Cd treatment as a general stress adaptive response. I predict that Cd will alter ureide gene expression and enzyme activity, resulting in the accumulation of ureides intermediate(s). To determine the effect of different Cd concentrations on ureide metabolism in Col-0 Arabidopsis ureide concentrations (uric acid, allantoin and allantoate), ureides gene expression and related enzyme activity have been assessed and compared in control and Cd-treated samples.

ii) I also hypothesize that mutation of the gene allantoinase (*ALN*), required for allantoin degradation, will lead to a different phenotype and stress response in these plants when compared with wild-type Col-0 Arabidopsis because elevated allantoin, positively influences plant growth and stress tolerance following Cd treatment. To compare *aln*-negative (*aln-3*) mutants with Col-0 Arabidopsis in response to Cd treatment, their phenotypic features (plant growth, root elongation and biomass production), allantoin content, gene regulation and internal Cd content have been monitored in respect with wild-type samples. Following this hypothesis, I also predict that external allantoin confers Cd-resistance to Col-0 Arabidopsis. To assess the effect of exogenous allantoin on Col-0 Arabidopsis, wild-type Arabidopsis has been grown in the presence of exogenous allantoin and the protective effect of allantoin on Col-0 Arabidopsis at different stages (seedling growth, seed germination and seeds) were assessed.

Results obtained from the first section led me to question about the potential function(s) of allantoin in response to stress. To this end, next four hypotheses have been proposed with the purpose of describing regulatory mechanism(s) underlying allantoin-derived stress tolerance in plants:

iii) I hypothesize that positive effect of allantoin on stress response is related to its impact on stress-induced oxidative damage. I predict that Cd-treated *aln-3* mutants contain lower amounts of ROS, resulting in less oxidative damage in these plants when compared with Col-0 samples. To evaluate the possible effect of allantoin content on ROS accumulation and antioxidant capacity, *aln-3* mutants and Col-0 Arabidopsis have

been tested for their ROS content and the activity of antioxidant enzymes in response to different Cd concentrations. Moreover, to analyze the differences between shoot and root of Col-0 and *aln-3* Arabidopsis, roots of both genotypes were compared with shoot in terms of allantoin content, gene expression pattern, internal Cd concentration, ROS accumulation and antioxidant activity.

iv) Testing *aln-3* mutants, raised the question whether overexpression of *ALN*, decreasing allantoin content, influence plant stress responses by imposing a stress-susceptible phenotype on plants due to decreased/lack of allantoin. I predict that there is a direct correlation between allantoin content and stress tolerance in plants. To confirm the association of allantoin with Cd-tolerance, *ALNox* lines were generated and exposed to different Cd concentrations and their growth was compared with Col-0 Arabidopsis.

v) Allntoin-induced ABA accumulation reported by Watanabe *et al*. (2014) raised the question whether the protective effect of allantoin is mediated through ABA-dependent or -independent mechanisms. I hypothesize that stress resistance induced by allantoin is related to ABA signalling as an intermediate. Therefore, I predict that allantoin can not confer Cd-tolerance to ABA-insensitive (*abi*) mutants due to impaired ABA sensing. To illustrate the possible link between allantoin and ABA signalling pathway, *abi* mutants have been employed to study the correlation between protective role of allantoin and signal transduction mediated by ABA. *abi* mutants have been grown in the presence of external allantoin and transcript level of antioxidant genes have been quantified in these mutants and compared with wild-type samples.

vi) I hypothesize that ureide pathway is responsive to metal-induced oxidative stress. I predict other metals (such as Ni and Li) induce allantoin accumulation in Col-0 Arabidopsis, while *aln-3* mutants will show greater tolerance than wild-type samples. To test the effect Ni and Li on ureide pathway, allantoin accumulation have been quantified in Col-0 Arabidopsis and the difference between plant growth of Col-0 and *aln-3* mutants was evaluated under these conditions.

Testing these hypotheses together with answering upcoming questions will provide significant insight into the mechanisms modulating plant responses to stress. Knowledge gained from studying the ureide pathway coupled with information on ureide mutants and their performance under stress conditions will be informative to describe plant defence strategies and set the stage

for a long-term research in this field with the purpose of generating stress tolerant plants and improving plant yield under unfavourable growth conditions.

2. Material and Methods

2.1. Plant material and growth condition

2.1.1. *Arabidopsis thaliana* genotypes and mutant screening

Arabidopsis thaliana (ecotype Columbia-0) seeds were used in all experiments as controls. Mutant Arabidopsis seeds were previously purchased from the Arabidopsis Biological Resource Center (ABRC, Columbus, OH, USA; https://abrc.osu.edu), Homozygous allantoinase-negative (*aln-3*) mutants were back crossed to Col-0 Arabidopsis, performed by Irani and Todd (2016) and *aln-3* mutants used in this study were directly obtained from the BC_2F_3 generation.

Two additional mutants, *Ataln* (SAIL-810-E12) and *Ataah* (SALK-112631), were also used in this work. To screen for T-DNA inserts, genomic DNA (gDNA) extraction was performed following the protocol described by Edwards *et al*. (1991). Two leaves of Col-0 and mutant seedlings were homogenized in DNA extraction buffer containing 0.2 M Tris-HCl (pH 7.5), 0.25 M NaCl, 25 mM EDTA and 0.5% (w/v) SDS. After 5 min centrifuge at maximum speed, 350 µl of supernatant was mixed with the same volume of isopropanol and followed by 10 min centrifuge at maximum speed. Supernatant was discarded, and pellet were allowed to dry. 200 µl of TE buffer (10 mM Tris-Cl pH 7.5 and 1 mM EDTA) was used to resuspend the pellet and extracted DNA was stored at -20° C freezer. Using three-primer PCR, gDNA of SAIL-810-E12 and SALK-112631 lines were tested for T-DNA insertion in *ALN* (At4g04955) and *AAH* (At4g20070). Primers used to identify T-DNA insertions and agarose gel obtained from three-primer PCR gels are shown as Table A1 and Fig. A1 in appendix A. PCR program for three-primer PCR was as followed: 96° C for 3 min, 30 cycles of (94° C for 30 s, 55° C for 55 s, 72° C for 1 min), followed by final extension at 72° C for 10 min. Lack of *ALN* and *AAH* transcription in homozygous SAIL-810-E12 and SALK-112631 lines were confirmed by RT-PCR and results are reported in Fig. A2 (please see sections 2.3.1 and 2.3.2 for detailed information about RNA extraction and gene expression analysis). In addition to ureides mutants, homozygous ABA-insensitive (*abi*) lines were also obtained from ABRC. Three *abi* lines and their corresponding wild-type backgrounds are listed in Table A2.

2.1.2. Plant growth media preparation

0.5X Murashige and Skoog Basal Salt Mixture (MS) (PhytoTechnology Laboratories, Shawnee Mission, KS, USA) containing 1% (w/v) sucrose, 0.8% or 1.2% (w/v) agar (for horizontal and vertical plates, respectively) were used as growth media. pH 5.7 was achieved by using potassium hydroxide (KOH). After autoclaving, metals were added to the mixture for desired concentrations: cadmium (Cd) as 0, 25, 50, 100 and 200 µM $CdCl_2$, lithium (Li) as 0, 10 and 15 mM LiCl, nickel (Ni) as 0, 50 and 100 µM $NiCl_2$. Similarly, MS plates containing allantoin (MS+Aln) were prepared by adding allantoin obtained from Sigma-Aldrich (Oakville, ON, Canada) to the 0.5X MS media to obtain a final concentration of 10 mM.

2.1.3. Seed sterilization and seedling growth on plate

In all experiments seeds were surface sterilized for 10 min in 10% (v/v) bleach (Sodium hypochlorite), followed by washing ten times with sterile dH_2O. Sterilized seeds were plated, kept at 4°C in the dark for 48 h and then transferred to a growth chamber with an 8 h light/16 h dark photoperiod, 70 µmol photons m^{-2} s^{-1}, constant 22° C and 65% relative humidity. After two weeks growing in the chamber, seedlings were harvested, fixed in liquid nitrogen and kept at -80° C for further experiments.

2.1.4. Allantoin treatment of seeds

Sterilized Col-0 Arabidopsis seeds were soaked in filter-sterilized 10 mM allantoin solution (Sigma-Aldrich), incubated at 4° C for 48 h in the dark and either directly used for antioxidant assays or transferred to 0.5X MS plates with or without 10 mM allantoin and different $CdCl_2$ concentrations. Growth condition for plated seeds were as described in section 2.1.3.

2.1.5. Seed germination and root length measurement

In all experiments the emergence of the radical from the seed coat was considered as the indication of seed germination. Two-week old seedlings were photographed, and root length was measured for all seedlings of a petri dish (20 seedlings per plate) using ImageJ software (version 1.46r). The average root length of all seedlings on a single plate was considered as a single replicate.

2.1.6. Arabidopsis growth condition in soil

Sterilized seeds were allowed to grow on 0.5X MS plates for ten days and then transferred to 5 cm × 5 cm pots filled with Sunshine Mix 1 soil obtained from SunGro Horticulture (Vancouver, BC, Canada). Pots were irrigated with tap water twice a week. Hoagland solution (Hoagland and Arnon, 1950) was used as a fertilizer once a week. Four-week old seedlings were irrigated with water containing 0, 500, 1000 and 1500 µM $CdCl_2$ for three weeks. Leaves and roots were separated, washed, frozen in liquid nitrogen and stored at -80° C for different measurements.

2.2. Constructing *ALNox* lines

2.2.1. Cloning into *Escherichia coli* (DH5α)

Cloning of *ALN* coding sequences (CDS) (1603 bp) into a TOPO vector was accomplished using the Zero Blunt TOPO PCR Cloning Kit (Invitrogen, CA, USA) and was done previously by Dr. Solmaz Irani. The presence of *ALN-CDS* in TOPO vector was confirmed by NcoI / BstE II digestion and sequencing. Appendix A, Table A3 lists the primers designed to add NcoI and BstEII restriction sites to *ALN-CDS*.

The TOPO vector containing *ALN-CDS* were digested using NcoI and BstEII. Digested *ALN-CDS* was separated from the vector on a 1% agarose gel and purified using the E.Z.N.A. Gel Extraction Kit (Omega Bio-tek, Norcross, GA, US) following the manufacturer's instruction (Appendix A, Fig. A3). *ALN-CDS* was then ligated to the NcoI/BstEII-digested vector pCAMBIA 1303 using T4 DNA ligase (NEB, Ipswich, MA, US). pCAMBIA 1303 containing CaMV 35S promoter as well as restriction sites for NcoI and BstEII is shown in Fig. A4 (Appendix A). Digestion of pCAMBIA and TOPO-*ALN-CDS* by NcoI and BstEII allows *ALN-CDS* to substitute for the removed sequence in pCAMBIA that is located downstream of CaMV 35S promoter, leading to the expression of *ALN* under the control of this promoter.

Following the ligation of pCAMBIA and *ALN-CDS*, *E. coli* (DH5α) were transformed with the ligation mixture using heat-shock method (Froger and Hall, 2007). 50 µl of competent *E. coli* were mixed with 5 µl of ligation mix and incubated in ice for 30 min. After 30 s incubation in 42° C water bath, tubes were immediately transferred on ice. 950 µl of LB was added to *E. coli* suspension and incubated at 37° C for 1 h while shaking (200 rpm). 100 µl of the mix was transferred into LB plates containing 50 µg ml^{-1} Kanamycin (Kan). Plates were incubated at 37°

C overnight and positive colonies were detected for further analysis. Positive colonies were tested for the presence of pCAMBIA+*ALN-CDS* performing colony PCR. 5 µl of a Kan-resistant colony suspended in dH_2O was used as DNA template for PCR using primers in Table A3. Once proper transformation of *ALN+CDS* was confirmed (Appendix A, Fig. A5.A), Kan-resistant colonies were suspended in liquid LB + Kan culture and incubated at 37° C overnight while shaking (200 rpm). E.Z.N.A. Plasmid Mini Kit (Omega Bio-tek., Norcross, GA, US) was employed to isolate pCAMBIA+*ALN-CDS* from bacterial culture following manufacture's protocol. Extracted pCAMBIA+*ALN-CDS* was sequenced (Eurofin Genomics) before transforming *Agrobacterium tumefaciens***.**

2.2.2. Transformation of *Agrobacterium tumefaciens* (GV1301)

Agrobacterium transformation was performed using heat-shock method described in section 2.2.1 with the following modifications. 250 µl of competent Agrobacterium was mixed with 10 µl of plasmid and stored in ice for 30 min. After 5 min heat shock at 37° C, tubes were transferred on ice and kept for 5 min. Bacterial suspension was mixed with 1 ml LB and incubated at 28° C for 3-4 h while shaking (200 rpm). 200 µl of suspension was transferred to LB plates containing 50 µg ml^{-1} Kan and 50 µg ml^{-1} Rifampicin (Rif) and incubated at 28° C for 48 h. After caring out colony PCR for Kan-resistant colonies (Appendix A, Fig. A5.B), positive colonies were suspended in 5 ml liquid LB + Kan + Rif and incubated at 28° C overnight while shaking (200 rpm). 2 ml of this bacterial culture was used for the inoculation of 200 ml liquid LB + Kan + Rif, incubated at the same condition overnight.

2.2.3. Arabidopsis transformation

Arabidopsis transformation was performed following floral-dip method explained by Zhang *et al*. (2006). Bacterial culture obtained from section 2.2.2 was centrifuged at 18° C for 10 min. Pellets were resuspended in 200 ml of 5% (w/v) sucrose solution. Once sucrose solution showed OD600 ~ 0.8, Silwet L-77 was added to yield the final concentration of 500 µl L^{-1}. Flowers of Col-0 Arabidopsis were dipped into the solution and agitated for 2-3 s. Plants were wrapped with plastic wrap and kept in dark for 24 h and then transferred to a growth chamber. Pots were irrigated normally until mature seeds appeared and were collected.

Transformed seeds were identified by growing on MS plates containing 25 µg ml^{-1} Hygromycin (Hyg). Two-week old Hyg-resistant seedlings (Appendix A, Fig. A6) were transferred to pots containing soil and allowed to grow to maturity and seeds were collected (T1 seeds). T1 seeds were screened for Hyg resistance and transformed seedlings were transplanted in soil and grown to maturity to collect T2 seeds. The same procedure was performed to obtain T3 seeds. Number of Hyg-resistant (R) and Hyg-sensitive (S) lines were recorded in each generation. Based on Mendel's segregation law, the 3:1 ratio was expected for R:S lines. Considering total number of tested seeds, observed R:S lines and expected R:S lines, Chi-Square (X^2) was calculated as followed (Rana and Singhal, 2015):

$$X^2 = \sum \frac{(\text{Observed value} - \text{Expected value})^2}{(\text{Expected value})}$$

List of generated lines together with their calculated X^2 for each line are shown in Appendix A, Table A4. Chi-Square significance table (Appendix A, Table A5) was used to confirm the observed segregation (R:S) ratio statistically. 0.05 probability level from Table A5 was considered as a valid 3:1 segregation and true transformed line. Based on these data, line T3-111-1 was selected to carry out experiments regarding the response of *allantoinase-overexpressed* lines (*ALNox* lines) to Cd treatment.

2.3. Gene expression analysis

2.3.1. RNA Extraction and cDNA synthesis

Total RNA extraction was carried out using E.Z.N.A. Plant RNA Kit (Omega Bio-tek., Norcross, GA, US) following the manufacturer's instruction. Quality and quantity of extracted RNA was confirmed using 1% agarose gel and NanoDrop 2000 Spectrophotometer (Thermo Scientific), respectively. A minimum of 50 ng RNA was used to make complementary DNA (cDNA) employing QuantiTect Reverse Transcriptase Kit (Qiagen, Toronto, ON, Canada) according to the manufacturer's instructions. This cDNA was used as template DNA for RT- and qRT-PCR analysis.

2.3.2. Reverse transcription-PCR (RT-PCR)

PCR analysis of transcripts was performed using gene-specific primers designed by Irani and Todd (2016) and list of RT-PCR primers are included in Appendix A, Table A6. PCR program consisted

of 94° C for 3 min, 30 cycles of 94° C for 30 s, 55° C for 30 s (except 60°C for 40 s in At4g04955 amplification), 72° C for 60 s. After the last cycle, a final extension was performed at 72° C for 10 min. PCR products were separated on 1% agarose gels stained with Ethidium bromide (EtBr) and visualized using UV light. Expression of genes of interest was compared with the expression of *Actin7* (At5g09810) as a reference gene.

2.3.3. Quantitative reverse transcription-PCR (qRT-PCR)

Transcript level of genes of interest were quantified using primers designed by Irani and Todd (2016) and Brychkova *et al*. (2008) (Appendix A, Table A7). PCR was performed as described by Brychkova *et al*. (2007) using an iCycler iQ5 System (Bio-Rad) and Evagreen dye (Biotium). Following the method described by Livak and Schmittgen (2001) relative gene expression was calculated and results were normalized with *ACTIN2* (At3g18780) as an internal reference.

2.4. Metabolite measurement

2.4.1. Ureide quantification

An Agilent Technologies 1200-series High Performance Liquid Chromatography (HPLC) system equipped with an organic acid analysis column (Bio-Rad Aminex HPX-87H Ion Exchange Column, 300 mm × 7.8 mm) was used to separate and quantify specific ureides (uric acid, allantoin, and allantoate). 60 mg fresh tissue was ground in 800 µl of dH_2O, centrifuged at 18000×g for 25 min at 4° C. Supernatant was passed through a 13 mm PTFE 0.2 µm syringe filter (VWR International). The mobile phase consisted of 0.0025 N H_2SO_4, flowing at 0.5 ml min^{-1}. Ureides were detected at 191 nm. Concentration of detected ureides were calculated based on peak area using standard curves obtained from the pure ureides. A typical chromatogram obtained from HPLC demonstrating allantoin, uric acid and allantoate peaks is shown in Appendix A, Fig. A7.

2.4.2. Proline assay

Proline content of plant samples were quantified using spectrophotometric method described by Bates *et al*. (1973) with minor modifications in volume. Fresh tissue was ground in 3% aqueous sulfosalicylic acid and passed through Whatman #2 filter paper. Equal volumes of filtrate, acid ninhydrin (0.5 g ninhydrin dissolved in 12 ml glacial acetic acid and 8 ml phosphoric acid) and glacial acetic acid were mixed in a test tube and incubated in the boiling water bath for 1 h. After transferring to an ice water bath, toluene was added to the mixture and vortexed for 20 s causing

the separation of chromophore and aqueous phase. Proline content of sample was detected at 520 nm and calculated based on a proline standard curve.

2.4.3. Protein assay

Weighed amount of plant tissue was ground using liquid nitrogen and homogenized with 50 mM Tricine, 2 mM $MnSO_4$. Samples were centrifuged for 20 min at 4° C and the supernatant was transferred to a fresh tube and kept on ice for protein/enzyme assay. Quantification of total extracted protein was carried out employing the BCA Protein Assay Kit (Novagen, Darmstadt, Germany) according to manufacturer's instructions. A standard curve was generated using Bovine Serum Albumin (Novagen, Darmstadt, Germany).

2.5. Enzyme activity assay

2.5.1. Allantoinase assay

The assay for allantoinase enzyme activity was performed following the protocol described by Duran and Todd (2012) and calculated according to total allantoate evolved. 500 µl of plant extract were mixed with the same volume of 50 mM Tricine, 2 mM $MnSO_4$, 35 mM β-Mercaptoethanol, 2 mM allantoin followed by 30 min incubation in a 30° C water bath. A duplicate tube was kept on ice to measure endogenous allantoate. After incubation, 250 µl of each sample was added to 250 µl of 0.15N HCl, followed by 250 µl 0.33% (w/v) phenylhydrazine hydrochloride, and 1.25 ml dH_2O. Samples were boiled for 2 min and allowed cool in an ice water bath. 1 ml concentrated HCl and 250 µl of 1.67% (w/v) K-ferricyanide were added to each tube, which were mixed thoroughly and absorbance at 520 nm was measured after 10 min incubation at room temperature. In this assay allantoate is generated as a result of allantoinase function, which is then degraded to urea and glyoxylate due to heat- and acid treatments. Resultant glyoxylate undergoes a reaction with phenylhydrazine hydrochloride resulting in glyoxylate phenylhydrazone formation which interacts with K-ferricyanide to produce glyoxylate diphenylformazan. Absorbance of the latter compound at 520 nm is detected and compared with glyoxylate standard curve (Romanov *et al*., 1999). Results were reported based on allantoate evolved by the enzyme allantoinase.

2.5.2. Superoxide dismutase assay

The SOD assay was carried out as described by Elavarthi and Martin (2010). This method is based on the ability of SOD to inhibit the reduction of nitroblue tetrazolium (NBT) in the presence of

superoxide anions, provided by riboflavin reduction in light, while methionine serves as an electron donor in this reaction (Das *et al*., 2000). 200 mg of plant tissue was homogenized in 0.2 M potassium phosphate buffer (pH 7.8) containing 0.1 mM EDTA and centrifuged for 20 min at 4° C. The supernatant was collected and used for SOD, APX, and CAT enzyme assays.

2 ml SOD reaction mixture composed of 50mM phosphate buffer (pH 7.8), 2 mM EDTA, 9.9 mM L-methionine, 55 µM NBT, and 0.025% Triton-X100, was mixed with 40 µl leaf extract sample and 20 µl of 1 mM riboflavin and placed in a box lined with aluminum foil. After a10 min exposure to a light source (15 W incandescent bulb at a distance of 12 cm from the box) the absorbance was read at 560 nm (Elavarthi and Martin, 2010).

2.5.3. Catalase assay

CAT activity was measured by monitoring the change in absorbance at 240 nm due to H_2O_2 breakdown by CAT. 3 ml 50 mM potassium phosphate buffer (pH 7.0) containing 10 mM H_2O_2 was mixed with 10 µl leaf extract. Decomposition of H_2O_2 was recorded as absorbance at time zero and after 1 min. CAT activity was calculated using the extinction coefficient of H_2O_2 (40 mM^{-1} cm^{-1} at 240 nm) (Aebi and Lester, 1984; Elavarthi and Martin, 2010).

2.5.4. Ascorbate peroxidase assay

APX activity was assayed as described by Nakano and Asada (1981). The APX reaction solution was a mixture of 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbate and 10 µL leaf extract. The reaction was initiated by adding H_2O_2 (at final concentration of 0.5 mM) to 1 ml APX mixture. The change in the absorbance of ascorbate at 290 nm was recorded for 3 min and APX activity was calculated using extinction coefficient of ascorbate (2.8 mM^{-1} cm^{-1}) (Elavarthi and Martin, 2010).

2.6. Reactive oxygen species (ROS) assay

2.6.1. Hydrogen peroxide quantification

Hydrogen peroxide (H_2O_2) measurement was performed according to a colorimetric protocol described by Ramel *et al*. (2009). Weighed plant tissue was immersed in 1 ml of DAB (3,3'-diaminobenzidine tetrahydrochloride) solution containing 1.25 mg DAB dissolved in 1 ml dH_2O at pH 3.8 (pH was adjusted using KOH). After vacuum infiltration for 90 min, plants were

homogenized in 0.2M $HClO_4$ and centrifuged for 10 min at room temperature. The absorbance of the supernatant was measured at 450 nm. H_2O_2 concentration was calculated according to the standard curve generated using 0.4 - 40 mM pure H_2O_2.

2.6.2. Superoxide radical quantification

Following the protocol of Ramel *et al*. (2009), plant tissue was immersed in 1 ml nitroblue tetrazolium (NBT) solution, composed of 3.5 mg NBT in 1 ml 10 mM potassium phosphate buffer (pH 7.8) containing 1 mM NaN_3. Samples were infiltrated under vacuum for 90 min and then ground in 2 M KOH-DMSO (1:1.16 v/v). After centrifuging for 10 min, the absorbance was recorded at 630 nm. The superoxide radical content of plant samples was quantified according to a standard graph obtained from different dilutions of NBT solution in potassium phosphate buffer-NaN_3 (0.43 – 43 µM NBT).

2.7. Cadmium determination in plants

2.7.1. Sample preparation

A wet digestion described by Pequerul *et al*. (1993) was employed to prepare plant tissues for atomic absorption spectroscopy. Plant samples were allowed to dry in oven at 88°C for 48 h. Weighed dried tissue was ground to a fine powder, homogenized in 5 ml HNO_3 and incubated at room temperature for 16 h. Homogenized samples were heated to release NO_2 fumes. After cooling to room temperature, 4 ml 70% $HCLO_4$ was added to the sample and allowed to heat. Once the mixture evaporated to a small volume, it was diluted in 25 ml distilled water and kept at 4° C for Cd measurement.

2.7.2. Atomic absorption spectroscopy (AAS)

A graphite-furnace AAS (Analyst 800) were employed to measure Cd concentrations of plant tissue as described by Kwong *et al*. (2011). Known Cd standards were employed to confirm the accuracy of element analysis and determine the detection limit prior to Cd quantifications in plant samples.

2.8. Statistical analysis

Values in this thesis are generally presented as the mean of at least three independent replicates ± the standard error of the mean (SEM). The significance within groups (genotypes or treatments)

was tested by one factor or two factor ANOVA, using Excel Microsoft Office, followed by a Tukey-Kramer post-hoc test (SPSS statistical program v.22.0, www.ibm.com) to determine the significant differences at $P \leq 0.05$. Once statistically significant interaction between these factors were detected a Student's t-test was computed using Microsoft Excel to test each pair of means. Statistically significant differences within the group was shown as different letters and the difference between each pair of values are displayed using asterisks as $*P \leq 0.05$, $**P \leq 0.01$ and *** $P \leq 0.001$.

3. Results[1]

3.1. Cd treatment of Col-0 Arabidopsis seedlings

3.1.1. Cadmium induces allantoin accumulation in two-week old seedlings.

Wild-type (Col-0) Arabidopsis seeds were allowed to germinate and grow on MS media containing 25, 50, 100 and 200 µM $CdCl_2$ for two weeks. As shown in Fig. 3.1, 25 µM $CdCl_2$ caused discoloration of Col-0 leaves. Seedlings exhibited decreased growth and leaf expansion at higher Cd concentrations (50 and 100 µM $CdCl_2$), and no seedling growth was observed at 200 µM $CdCl_2$ treatment.

HPLC analysis of seedling tissue showed that allantoin content of Col-0 seedlings increased in response to 50 and 100 µM $CdCl_2$ treatment (Fig. 3.2). Uric acid content showed a decreasing trend in response to increasing Cd concentration, and the lowest uric acid amount was measured at 100 µM $CdCl_2$. Measurements of 200 µM $CdCl_2$-treated samples were not performed due to lack of plant tissue.

3.1.2. Cadmium decreases transcript level of allantoinase (*ALN*).

Using RT-PCR, the expression of ureide metabolic genes was determined and compared with a reference gene, *ACTIN7* (*ACT7*). For five tested genes (*XDH1*, Xanthine Dehydrogenase 1; *ALNS*, Allantoin Synthase; *UO*, Uricase; *ALN*, Allantoinase; *AAH* and Allantoate Amidohydrolase), the expression of *XDH1*, *ALNS*, *UO* and *AAH* did not change noticeably in response to the applied Cd concentrations while *ALN* transcript levels decreased at 50 and 100 µM $CdCl_2$ compared to the no Cd control (Fig. 3.3). Quantification of mRNA abundance using qRT-PCR showed that Cd treatment did not decrease the transcript levels of *ALN* and *AAH* significantly. However, Cd

[1] Information presented in this chapter has been published previously: Nourimand and Todd (2016) Allantoin increases cadmium tolerance in Arabidopsis via activation of antioxidant mechanisms. *Plant and Cell Physiology*, 1: 1-12. Nourimand and Todd (2017) Allantoin contributes to the stress response in cadmium-treated Arabidopsis roots. *Plant Physiology and Biochemistry*, 119: 103-109.

treatment significantly increased the expression of *UO* in two-week old seedlings at both 50 µM and 100 µM $CdCl_2$ (Fig. 3.4).

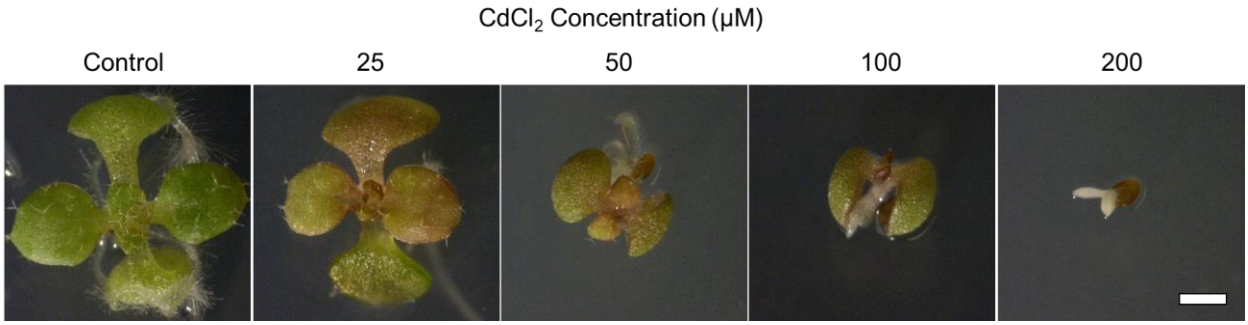

Figure 3.1. Cd treatment of Col-0 Arabidopsis. Image shows the effect of different Cd concentrations on Col-0 seedling growth. Picture is representative of three independent experiments. Scale bar = 1 mm.

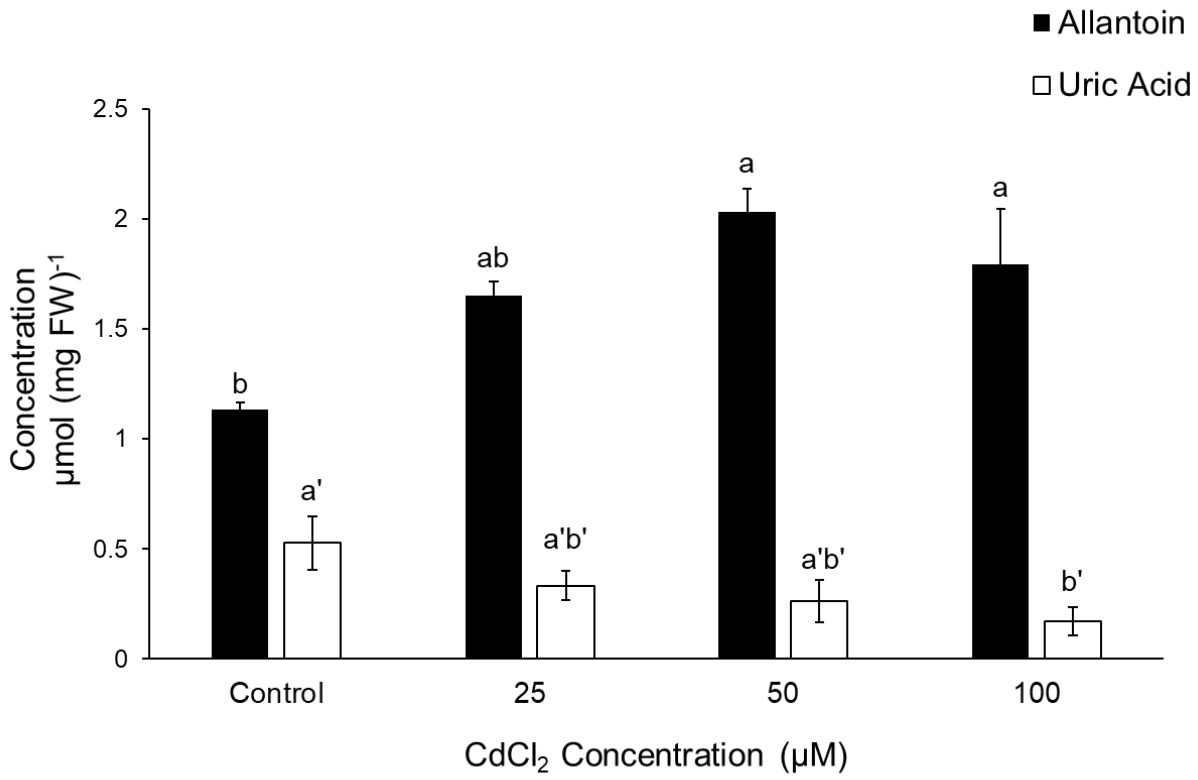

Figure 3.2. Quantification of the ureides, allantoin and uric acid, in Col-0 seedlings in response to Cd treatment. Values shown are the mean of ten independent replicates ± SE. Different letters (a,b,c for allantoin and a',b',c' for uric acid) show significant differences in each group ($P \leq 0.05$).

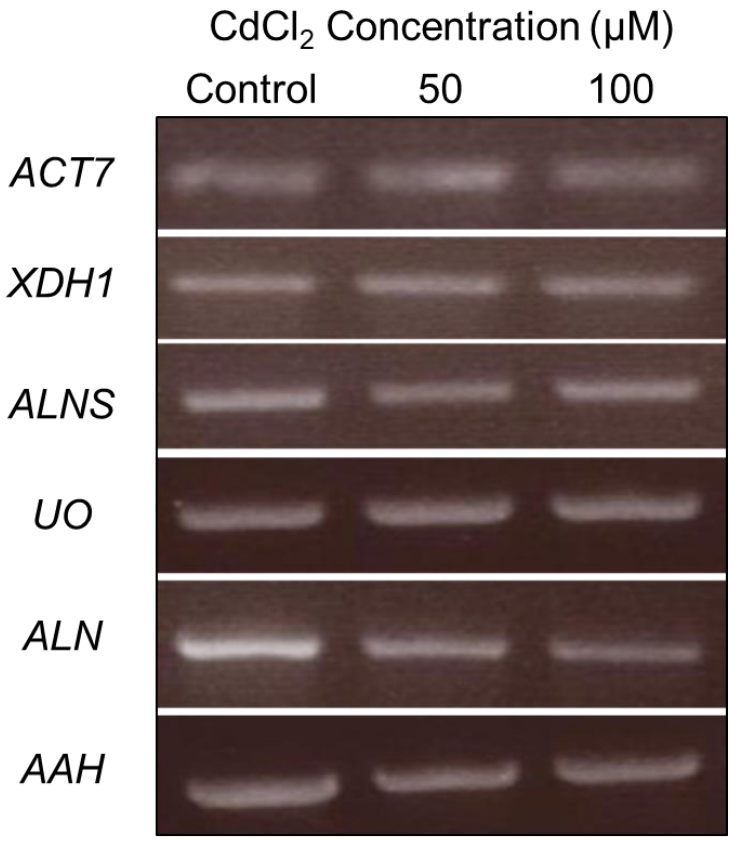

Figure 3.3. RT-PCR analysis of ureides genes in Col-0 Arabidopsis in response to Cd treatment. Picture is representative of three independent experiments. *AAH*, allantoate amidohydrolase; *ACT7*, actin7 (reference gene); *ALN*, allantoinase; *ALNS*, allantoin synthase; *UO*, uricase*; XDH1*, xanthine dehydrogenase 1.

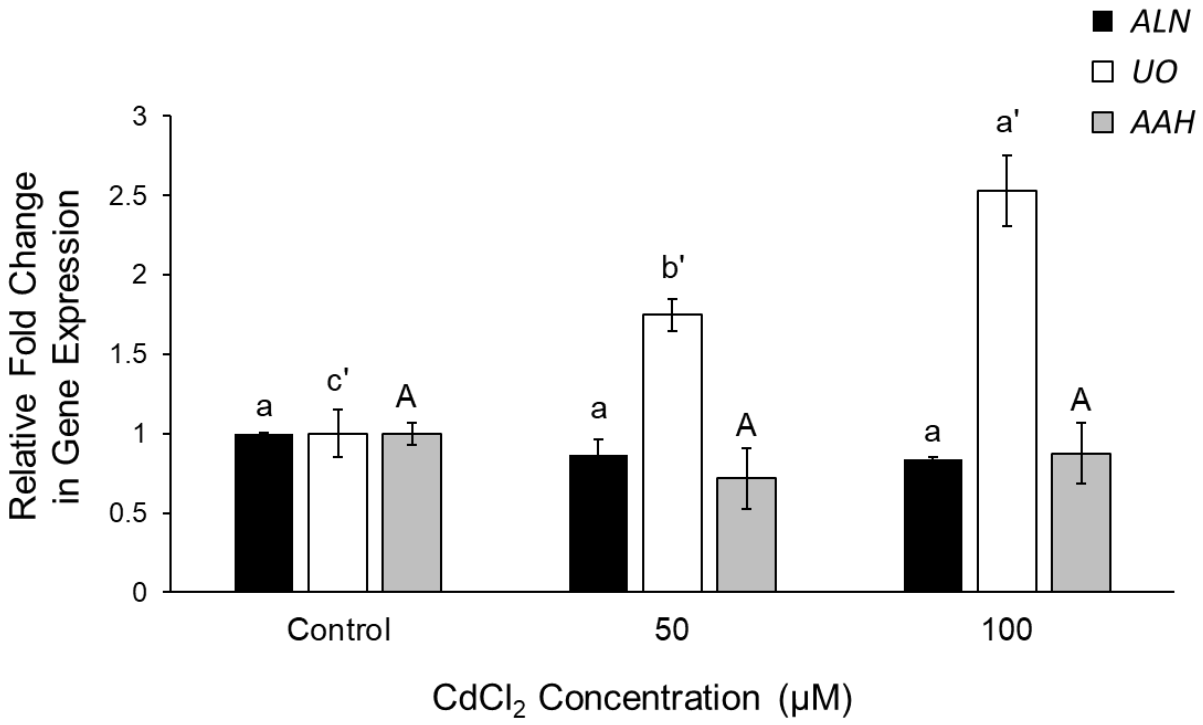

Figure 3.4. qRT-PCR analysis of ureides genes in Col-0 Arabidopsis following Cd treatment. Values are the mean of three independent replicates ± SE. Different letters (a,b,c for *ALN*; a',b',c' for *UO* and A,B,C for *AAH*) show significant differences in each group ($P \leq 0.05$). *AAH*, allantoate amidohydrolase; *ALN*, allantoinase; *UO*, uricase. *ALN*, *AAH* and *UO* were normalized to *ACT2* expression as an internal control.

3.2. Allantoinase-negative *aln-3* mutants in response to cadmium treatment

3.2.1. Two-week old *aln-3* seedlings show more cadmium tolerance than Col-0 Arabidopsis.

aln-3 mutants, which show constitutive accumulation of allantoin (Irani and Todd, 2016), were also exposed to the same Cd concentrations and compared with Col-0 samples (Fig. 3.5). *aln-3* seed germination was not influenced by Cd treatment, even at 200 µM (Fig. 3.6), while increasing Cd concentration decreased plant growth and root elongation, specifically at 100 and 200 µM $CdCl_2$ (Fig. 3.7). However, comparison between Col-0 and *aln-3* seedlings showed that the mutants were more resistant to Cd, reflected in greater seed germination and root elongation at higher Cd concentrations compared with Col-0 seedlings exposed to the same treatment. Consistent with Col-0 plants, *aln-3* mutants also accumulated allantoin in response to Cd treatment, showing the greatest concentration at 100 and 200 µM $CdCl_2$ (Fig. 3.8). Uric acid concentration did not change significantly in Cd-treated mutants (data not shown).

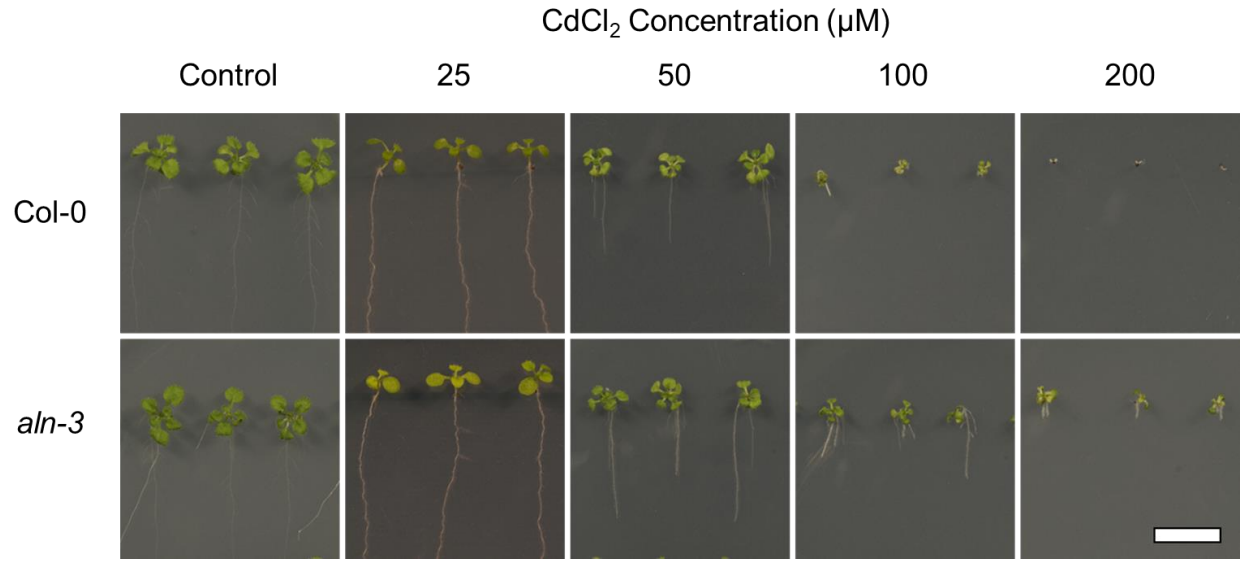

Figure 3.5. Seedling growth of Col-0 and *aln-3* Arabidopsis in response to Cd treatment. Picture is representative of three independent experiments. Scale bar = 1 cm.

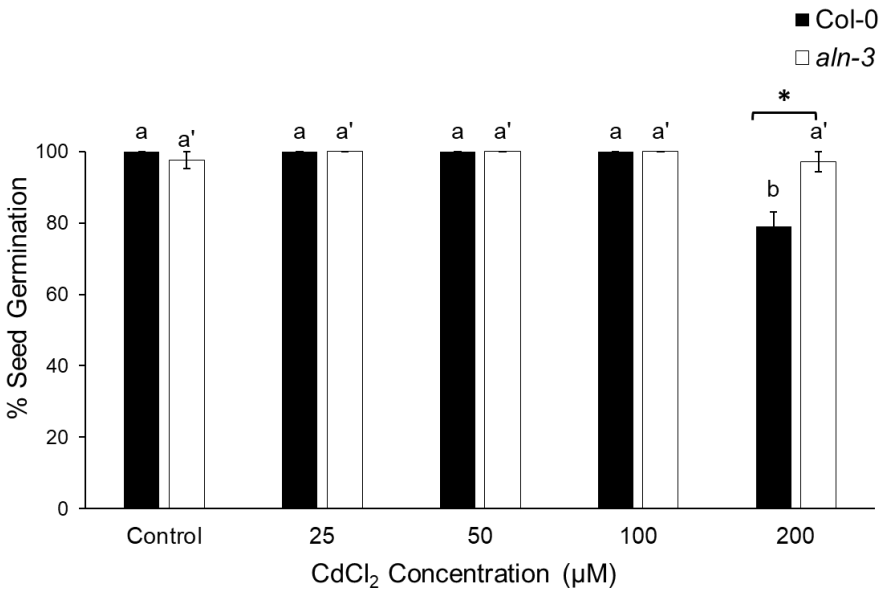

Figure 3.6. Effect of Cd on seed germination of Col-0 and *aln-3* Arabidopsis. Values shown are the mean of three independent replicates ± SE. Different letters (a,b,c for Col-0 and a',b',c' for *aln-3*) show significant differences in each group ($P \leq 0.05$). Asterisks indicate significant differences obtained by a t-test ($*P \leq 0.05$).

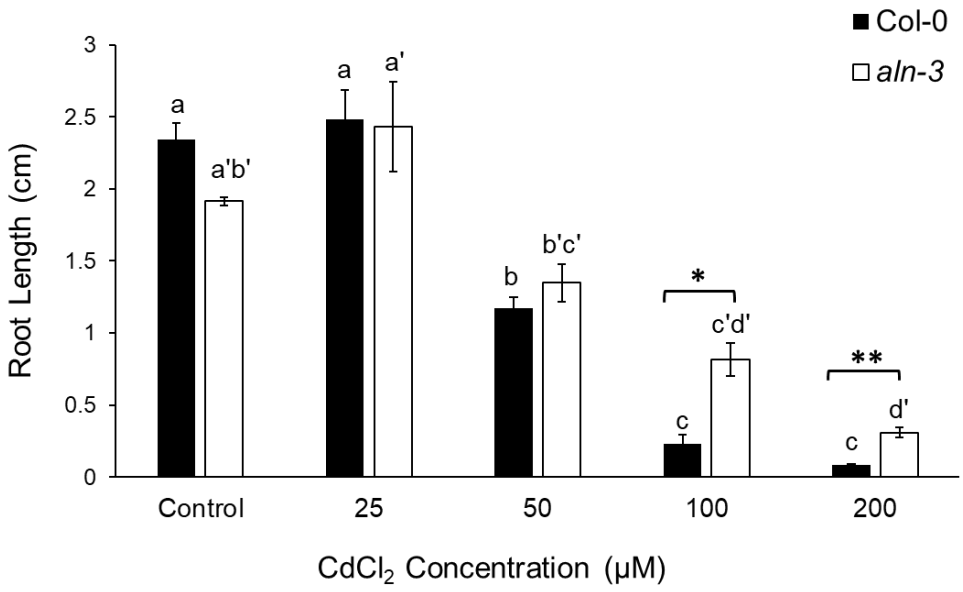

Figure 3.7. Root length of Col-0 and *aln-3* Arabidopsis in response to Cd treatment. Values shown are the mean of three independent replicates ± SE. Different letters (a,b,c for Col-0 and a',b',c' for *aln-3*) show significant differences in each group ($P \leq 0.05$). Asterisks indicate significant differences obtained by a t-test (*$P \leq 0.05$, ** $P \leq 0.01$).

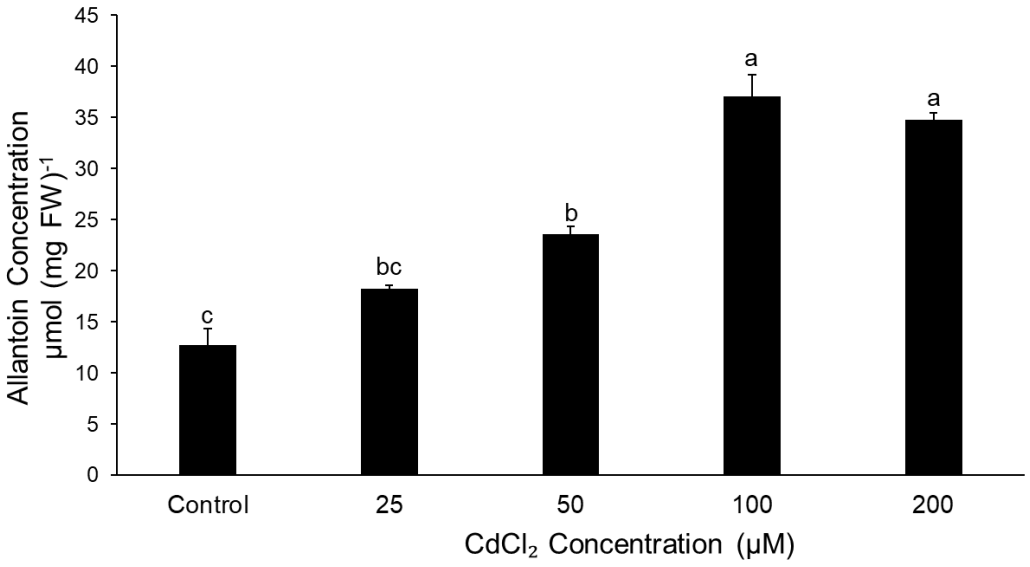

Figure 3.8. HPLC analysis of allantoin in *aln-3* seedlings in response to Cd treatment. Values shown are the mean of three independent replicates ± SE. Different letters show significant differences in each group ($P \leq 0.05$).

3.3. Comparison of soil-grown Col-0 and *aln-3* Arabidopsis shoots

To study Cd-induced allantoin accumulation in more detail and clarify the potential effect of allantoin on stress responses, Col-0 and *aln-3* plants were grown in soil, and leaves and roots were harvested separately for biochemical and molecular analyses.

3.3.1. *aln-3* shoots grow more than Col-0 shoot following cadmium treatment.

Cd treatment decreased plant growth and leaf expansion, especially at higher concentrations (1000 and 1500 µM $CdCl_2$) (Fig. 3.9). However, *aln-3* mutants exhibited better plant growth compared with Col-0 samples exposed to the same treatment, reflected in greater fresh and dry weight of leaf tissue (Figs. 3.10 and 3.11). HPLC analysis showed that both Col-0 and *aln-3* leaves accumulated the highest amounts of allantoin in response to 1000 µM $CdCl_2$ treatment, with nearly 10-fold difference between their allantoin content (Fig. 3.12).

3.3.2. Cadmium affects gene expression and enzyme activity.

Consistent with previous experiment on seedlings, leaves of Cd-treated Col-0 plants demonstrated a noticeable decrease in *ALN* expression at 1000 and 1500 µM $CdCl_2$ (Fig. 3.13). Additionally, Cd treatment triggered an increase in the transcript levels of *UO* in Col-0 and *aln-3* samples in the 500 and 1000 µM $CdCl_2$ treated samples (Fig. 3.13). Using qRT-PCR, transcript level of *ALN* and *UO* were quantified. In Col-0 samples, transcript levels of *ALN* decreased to approximately 70% and 15% of those at control conditions in response to 500 and 1000 µM $CdCl_2$, while *UO* increased nearly 6- and 8-fold, respectively (Fig. 3.14A). In *aln-3* mutants, no meaningful *ALN* mRNA abundance was detected, whereas *UO* transcript level exhibited 4- and 8-fold increase at 500 and 1000 µM $CdCl_2$ treatment (Fig. 3.14B). In addition to the transcription level of *ALN*, activity of this enzyme was also measured in Col-0 Arabidopsis in response to Cd treatment. As shown in Fig. 3.15, all three applied Cd concentrations dramatically decreased specific activity of the enzyme ALN in respect with no Cd (control) treated samples. ALN enzyme activity showed 3.5-fold decline at 500 µM, while it reached the lowest activity at 1000 and 1500 µM $CdCl_2$ treatment.

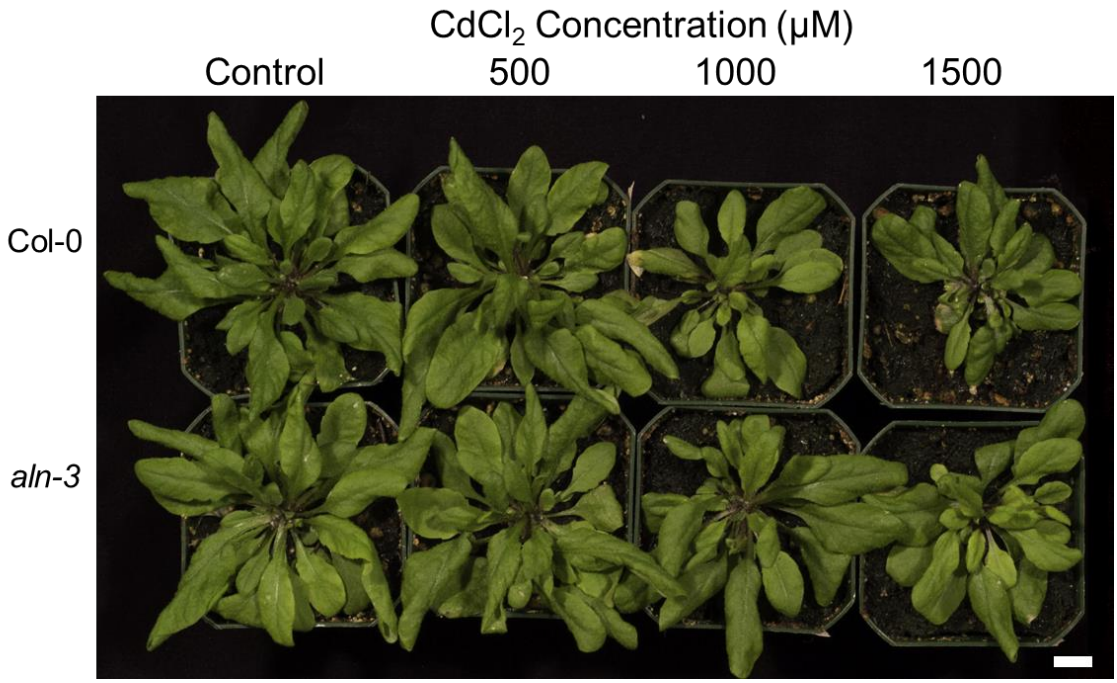

Figure 3.9. Effect of Cd treatment on Col-0 and *aln-3* Arabidopsis growth. Picture is representative of three independent experiments. Scale bar = 1 cm.

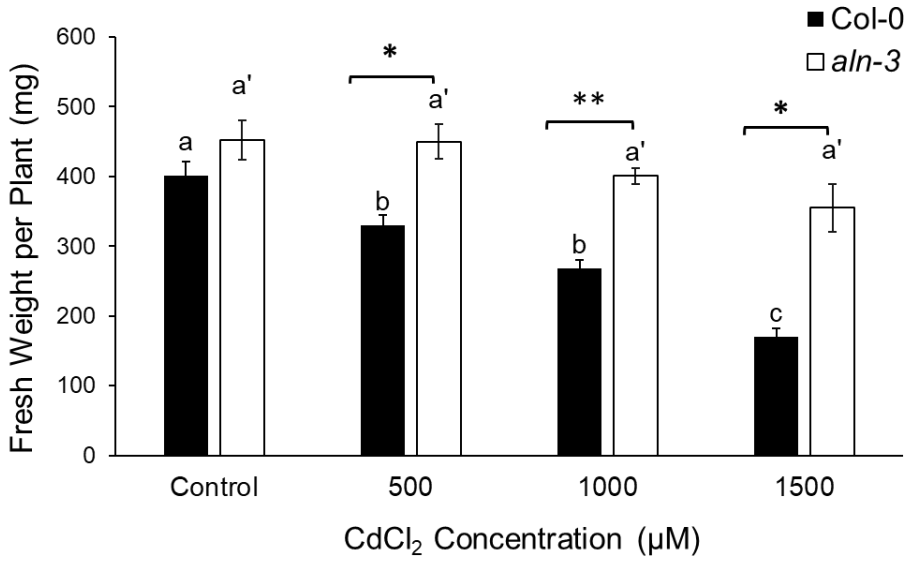

Figure 3.10. Fresh weight of Col-0 and *aln-3* Arabidopsis in response to Cd treatment. Values shown are the mean of three independent replicates ± SE. Different letters (a,b,c for Col-0 and a',b',c' for *aln-3*) show significant differences in each group ($P \leq 0.05$). Asterisks indicate significant differences obtained by a t-test (*$P \leq 0.05$, **$P \leq 0.01$).

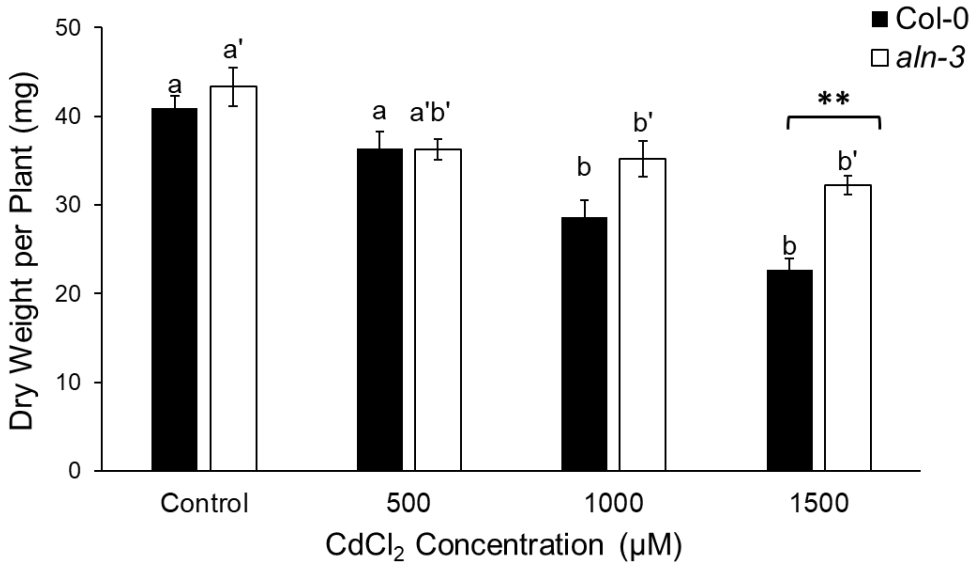

Figure 3.11. Dry weight of Col-0 and *aln-3* Arabidopsis in response to Cd treatment. Values shown are the mean of three independent replicates ± SE. Different letters (a,b,c for Col-0 and a',b',c' for *aln-3*) show significant differences in each group ($P \leq 0.05$). Asterisks indicate significant differences obtained by a t-test (**$P \leq 0.01$).

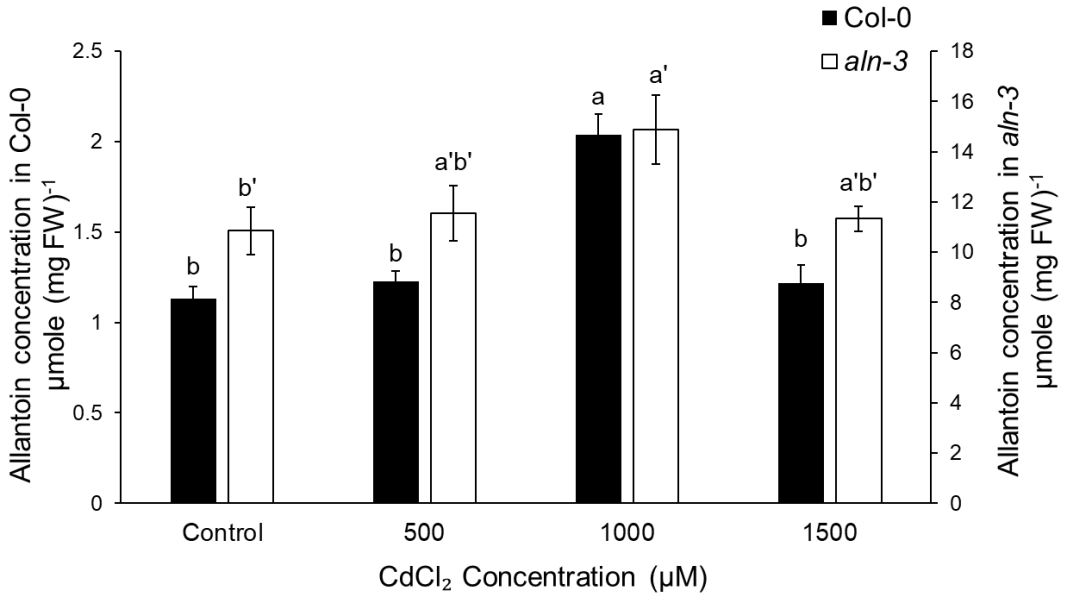

Figure 3.12. Effect of Cd treatment on allantoin content of Col-0 and *aln-3* leaves. Values shown are the mean of six independent replicates ± SE. Values for Col-0 (black bars) are shown on the left axis; values for *aln-3* (white bars) are shown on the right axis. Different letters (a,b,c for Col-0 and a',b',c' for *aln-3*) indicate significant differences in each group ($P \leq 0.05$).

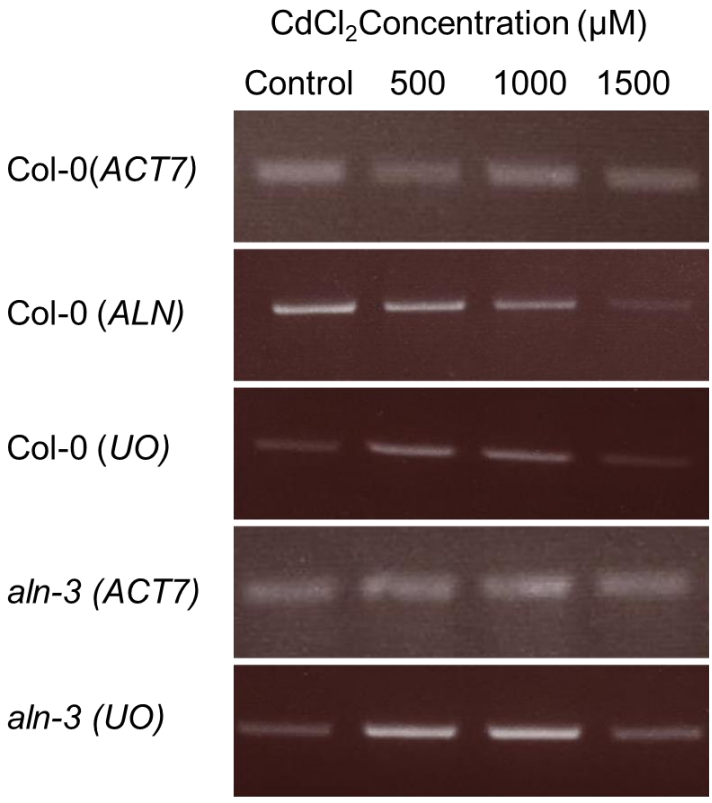

Figure 3.13. RT-PCR analysis of ureides genes in Col-0 and *aln-3* Arabidopsis leaves in response to Cd. Picture is representative of three independent experiments. *AAH*, allantoate amidohydrolase; *ACT7*, actin7 (reference gene); *ALN*, allantoinase; *UO*, uricase.

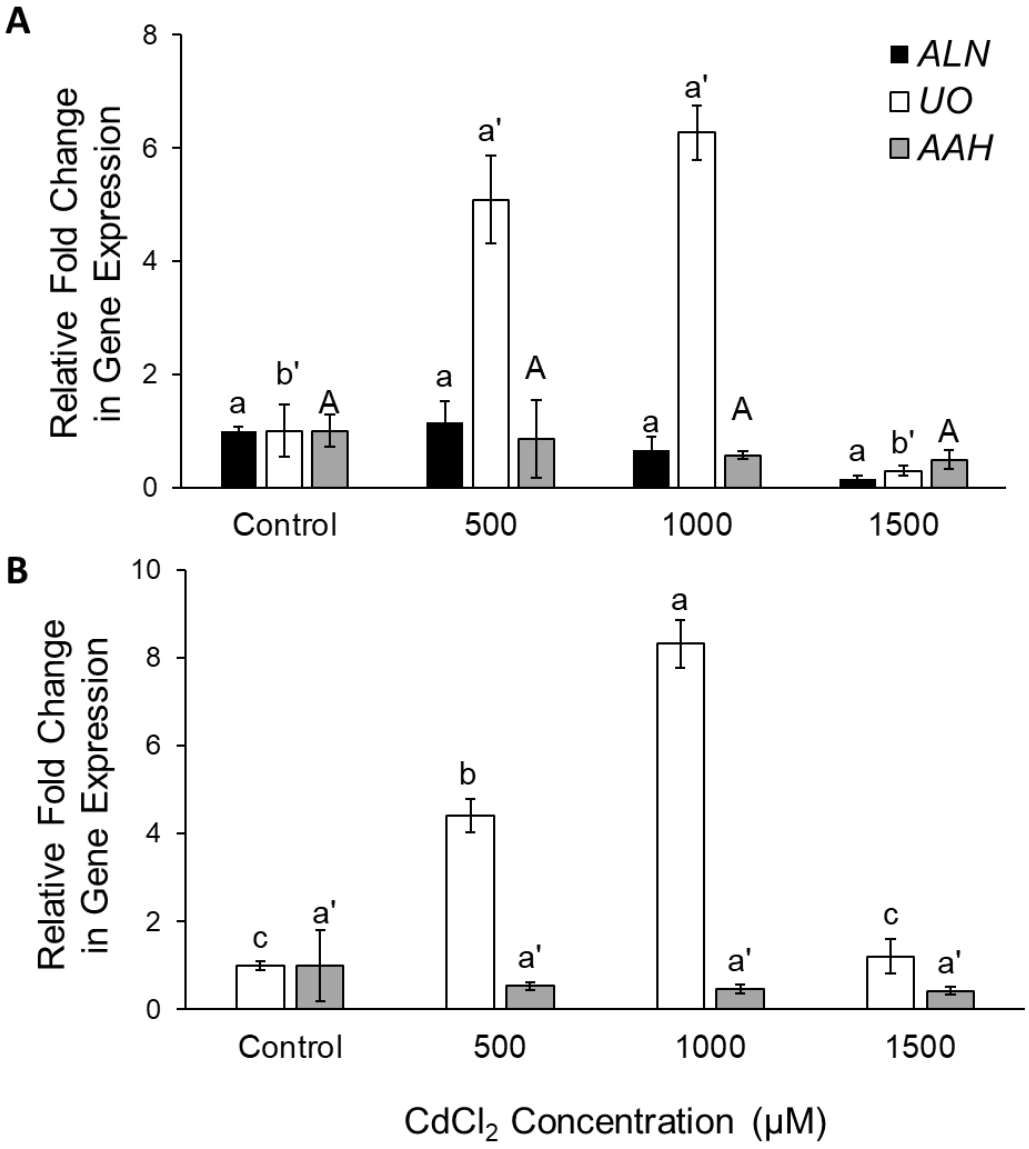

Figure 3.14. qRT-PCR analysis of ureides genes in (A) Col-0 and (B) *aln-3* Arabidopsis exposed to Cd treatment. Values shown are the mean of three independent replicates ± SE. Different letters show significant differences in each group ($P \leq 0.05$). In the top graph a,b,c for *ALN* and a',b',c' for *UO* and A,B,C for *AAH* show the difference in transcript levels. In the bottom graph a,b,c for *UO* and a',b',c' for *AAH* show the difference in transcript levels. *AAH*, allantoate amidohydrolase; *ALN*, allantoinase; *UO*, uricase. *ALN*, *AAH* and *UO* were normalized to *ACT2* expression as an internal control.

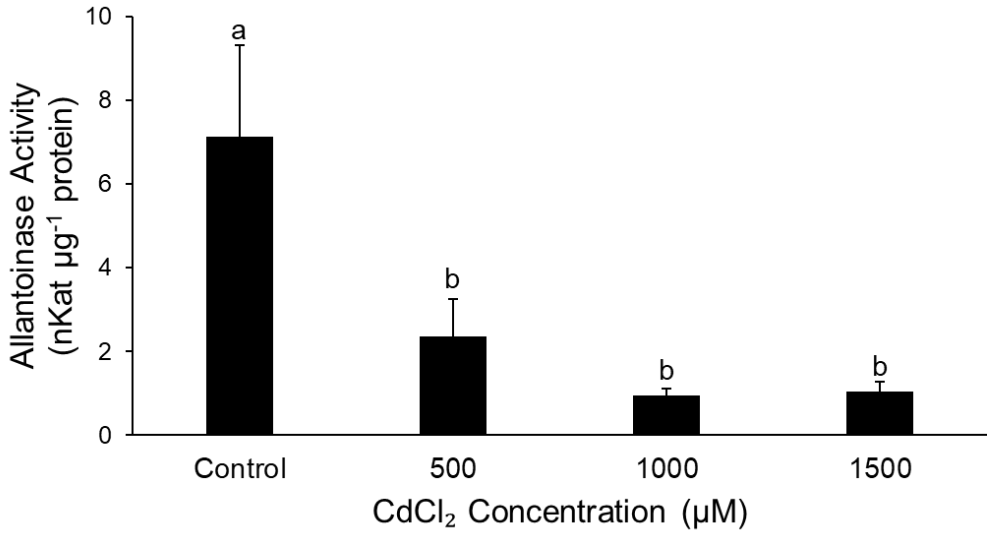

Figure 3.15. Effect od Cd treatment on allantoinase enzyme activity in Col-0 Arabidopsis. Values shown are the mean of six independent replicates ± SE. Different letters show significant differences in each group ($P \leq 0.05$).

3.3.3. *aln-3* leaves store higher amount of cadmium.

Using atomic absorption spectroscopy (AAS), Cd concentration within leaf tissue was quantified to determine the effect of genotype on Cd uptake and impact of leaf Cd concentration on ROS accumulation and stress response. Cd concentration of Col-0 leaves significantly increased at 500 µM $CdCl_2$ and decreased in response to 1000 and 1500 µM $CdCl_2$ treatment (Fig. 3.16). In *aln-3* mutants, concentration of Cd inside leaf tissue showed an increasing trend up to 1000 µM $CdCl_2$, but went down to a lower level following 1500 µM $CdCl_2$ treatment. It is worth noting that although *aln-3* leaves contained higher amount of Cd than Col-0 samples throughout the experiment, they exhibited significantly lower Cd concentration in the leaf tissue at control condition than Col-0 leaves.

3.3.4. Concentration of hydrogen peroxide and superoxide radicals in *aln-3* shoots is lower than that of Col-0 plants.

To gain a better understanding of how genotype and increased allantoin concentration influence Cd-derived ROS accumulation, hydrogen peroxide and superoxide radicals were measured in control and treated samples, comparing Col-0 and *aln-3*. As shown in Fig. 3.17A, H_2O_2 accumulation was higher in both genotypes, at higher concentrations (1000 and 1500 µM $CdCl_2$). However, these numbers are slightly lower in *aln-3* mutants. Consistently, O_2^- accumulation was observed in response to Cd treatment in Col-0 and *aln-3* mutants with significantly lower concentration of superoxide radicals in *aln-3* leaves at 1000 µM $CdCl_2$ treatment (Fig. 3.17B).

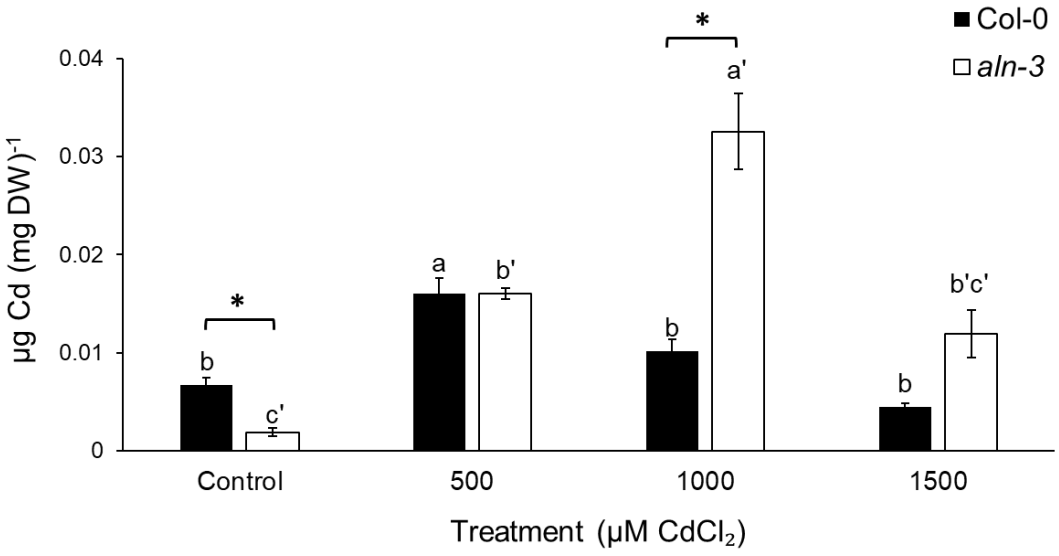

Figure 3.16. Effect of Cd treatment on Cd content of Col-0 and *aln-3* leaves. Values shown are the mean of three independent replicates ± SE. Different letters (a,b,c for Col-0 and a',b',c' for *aln-3*) show significant differences in each group ($P \leq 0.05$). Asterisks indicate significant differences obtained by a t-test (*$P \leq 0.05$).

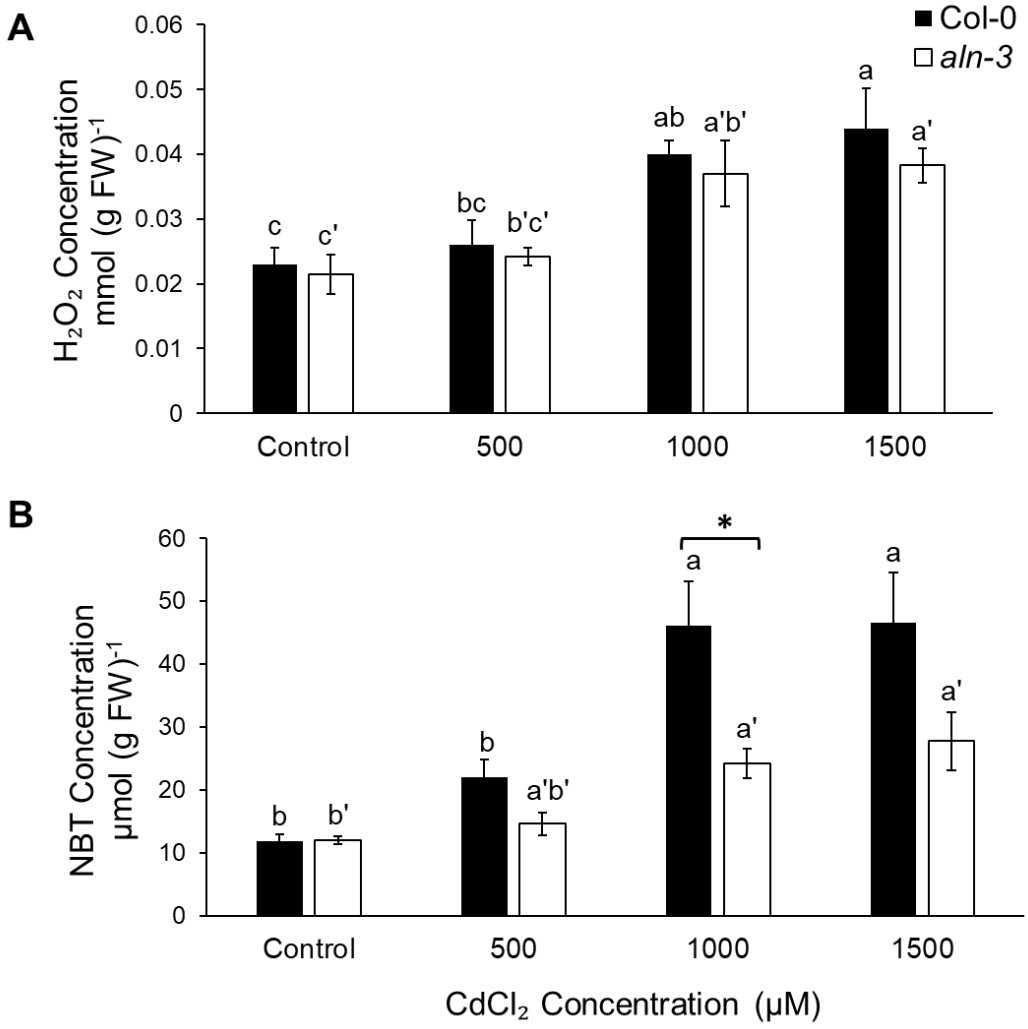

Figure 3.17. Quantification of (A) hydrogen peroxide and (B) superoxide radical accumulation in Cd-treated Col-0 and *aln-3* Arabidopsis leaves. Values shown are the mean of four independent replicates ± SE. Different letters (a,b,c for Col-0 and a',b',c' for *aln-3*) show significant differences in each group ($P \leq 0.05$). Asterisks indicate significant differences obtained by a t-test (*$P \leq 0.05$).

3.3.5. Antioxidant enzymes are more active in *aln-3* shoots.

Previous experiments showed that Cd-derived ROS accumulation is restricted in *aln-3* mutants compared with Col-0 Arabidopsis at the same condition. This finding proposed the hypothesis that these mutants benefit from a more active antioxidant system, resulting in increased ROS detoxification and consequently decreased ROS accumulation in response to Cd treatment. To test this hypothesis and to clarify the effect of genotype on antioxidant systems, the activities of superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) were measured in control and treated samples. 500 and 1000 µM $CdCl_2$ induced SOD activity in Col-0 leaves while 1500 µM $CdCl_2$ caused a slight decrease in SOD activity (Fig. 3.18). Comparatively, in *aln-3* mutants SOD activity did not change at 500 µM $CdCl_2$, reaching the highest value at 1000 and 1500 µM $CdCl_2$. Comparison between Col-0 and mutants revealed that SOD was generally more activated in *aln-3* mutants, even at control treatment.

APX activity of both genotypes showed an increasing trend in response to Cd treatment with the highest activity at 1000 µM $CdCl_2$, followed by a significant decline at 1500 µM $CdCl_2$ (Fig. 3.19). Consistent with SOD results, APX activity of *aln-3* mutants was significantly higher than that of Col-0 samples throughout the experiment.

Cd treatment also stimulated CAT activity in Col-0 and *aln-3* leaves specifically at higher Cd concentrations, 1000 and 1500 µM $CdCl_2$ (Fig. 3.20). However, a significant difference between Col-0 and *aln-3* samples was not detected for CAT activity measurement.

3.3.6. Proline content of *aln-3* shoots is slightly higher than that of Col-0 plants in response to cadmium treatment.

Proline accumulation in plants is also an important indicator of stress, also serves as a defence mechanism under this condition. In response to Cd treatment, both Col-0 and *aln-3* increased leaf proline levels following treatment with 1000 and 1500 µM $CdCl_2$ (Fig. 3.21). This Cd-induced proline accumulation was slightly higher in Cd-treated *aln-3* mutants, however the difference between Col-0 and mutants was not significantly different in this experiment.

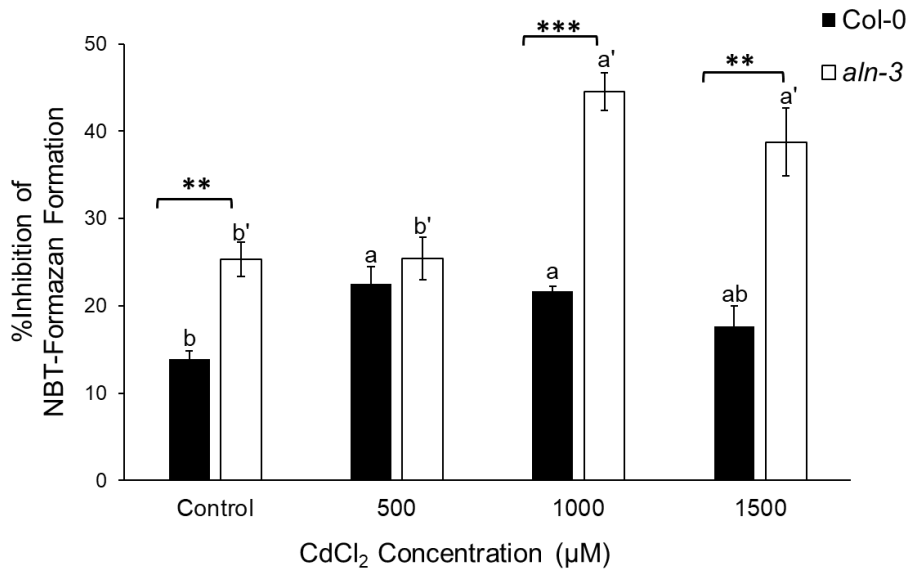

Figure 3.18. Effect of Cd on the activity of superoxide dismutase (SOD) in Col-0 and *aln-3* Arabidopsis leaves. Values shown are the mean of five independent replicates ± SE. Different letters (a,b,c for Col-0 and a',b',c' for *aln-3*) show significant differences in each group ($P \leq 0.05$). Asterisks indicate significant differences obtained by a t-test (**$P \leq 0.01$, ***$P \leq 0.001$).

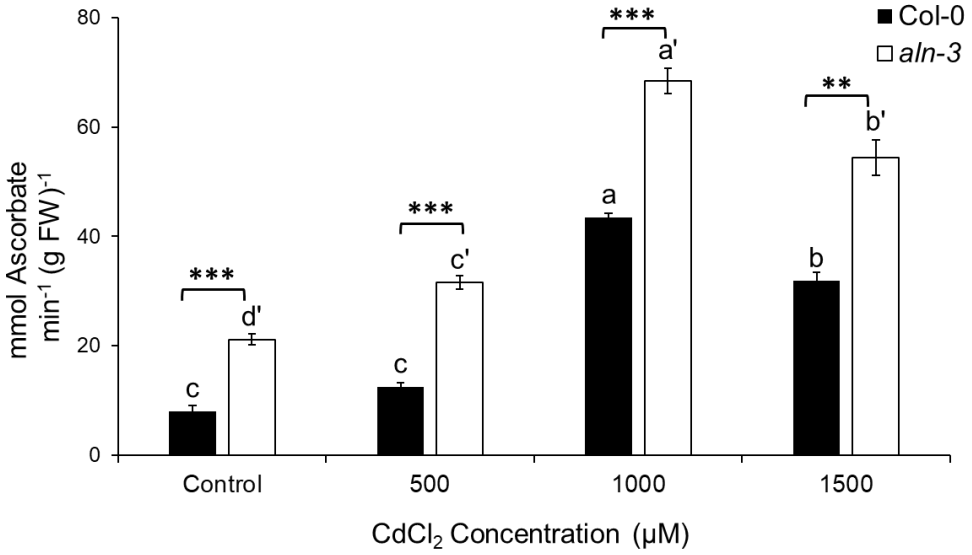

Figure 3.19. Effect of Cd on the activity of ascorbate peroxidase (APX) in Col-0 and *aln-3* Arabidopsis leaves. Values shown are the mean of four independent replicates ± SE. Different letters (a,b,c for Col-0 and a',b',c' for *aln-3*) show significant differences in each group ($P \leq 0.05$). Asterisks indicate significant differences obtained by a t-test (**$P \leq 0.01$, ***$P \leq 0.001$).

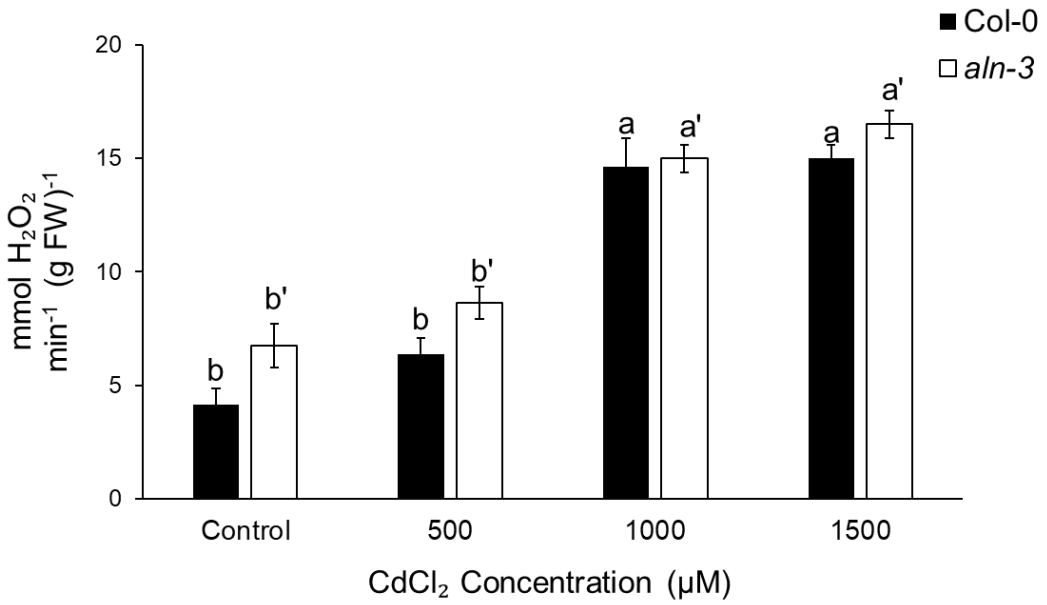

Figure 3.20. Effect of Cd on the activity of catalase (CAT) in Col-0 and *aln-3* Arabidopsis leaves. Values shown are the mean of four independent replicates ± SE. Different letters (a,b,c for Col-0 and a',b',c' for *aln-3*) show significant differences in each group ($P \leq 0.05$).

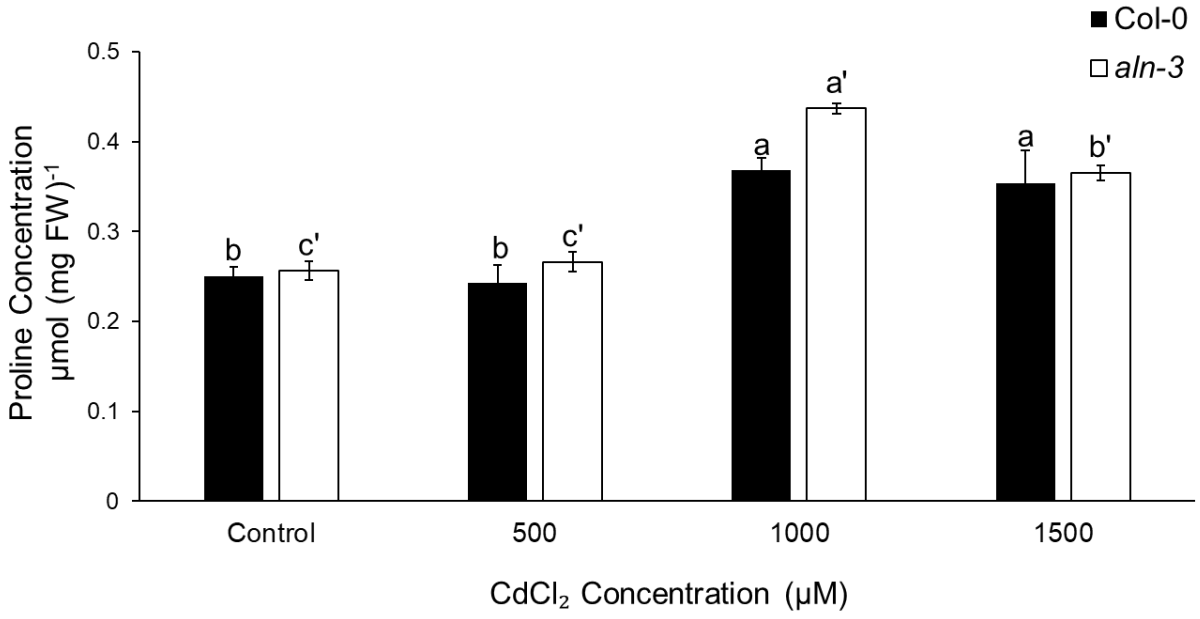

Figure 3.21. Proline accumulation in Col-0 and *aln-3* leaves in response to Cd. Values shown are the mean of three independent replicates ± SE. Different letters (a,b,c for Col-0 and a',b',c' for *aln-3*) show significant differences in each group ($P \leq 0.05$).

3.4. Comparison of soil-grown Col-0 and *aln-3* Arabidopsis roots

The above experiments on the effect of Cd on Col-0 and *aln-3* mutant leaves indicated that *aln-3* Arabidopsis is more resistant to Cd when compared with Col-0 samples exposed to the same condition. ROS and antioxidant assays also demonstrated that activated antioxidant enzymes in *aln-3* mutants are a potential response to Cd-induced ROS generation and may confer Cd tolerance to these plants. However, the root is the first defense line against many soil pollutants (including Cd), altering the physiological/molecular mechanism to combat stress. To evaluate root function at Cd treatment and to clarify the effect of genotype on root growth and stress response, the same experimental conditions were applied and roots of Col-0 and *aln-3* Arabidopsis were harvested for molecular and biochemical measurements.

3.4.1. *aln-3* plants show higher root growth than Col-0 samples.

Cd diminished root growth and weight in both Col-0 and *aln-3* samples (Fig. 3.22). Measuring fresh and dry weight of control and Cd-treated roots in both genotypes indicated that in spite of Cd-derived decrease in root weight, *aln-3* mutants were able to grow more than Col-0 roots, reflected in their higher fresh and dry weight, the difference between these two groups being statistically significant for dry weight (Fig. 3.23A,B).

3.4.2. Cadmium decreases allantoin content of Col-0 roots while triggers allantoin accumulation in *aln-3* roots.

Quantification of allantoin in control and treated plants demonstrated that 500 µM $CdCl_2$ did not influence allantoin content of Col-0 roots while 1000 and 1500 µM $CdCl_2$ significantly decreased allantoin concentration in these samples (Fig. 3.24). In *aln-3* roots no considerable change was observed at 500 µM $CdCl_2$, whereas 1000 µM $CdCl_2$ treatment enhanced allantoin content in the mutant roots, decreasing to a lower level at 1500 µM $CdCl_2$.

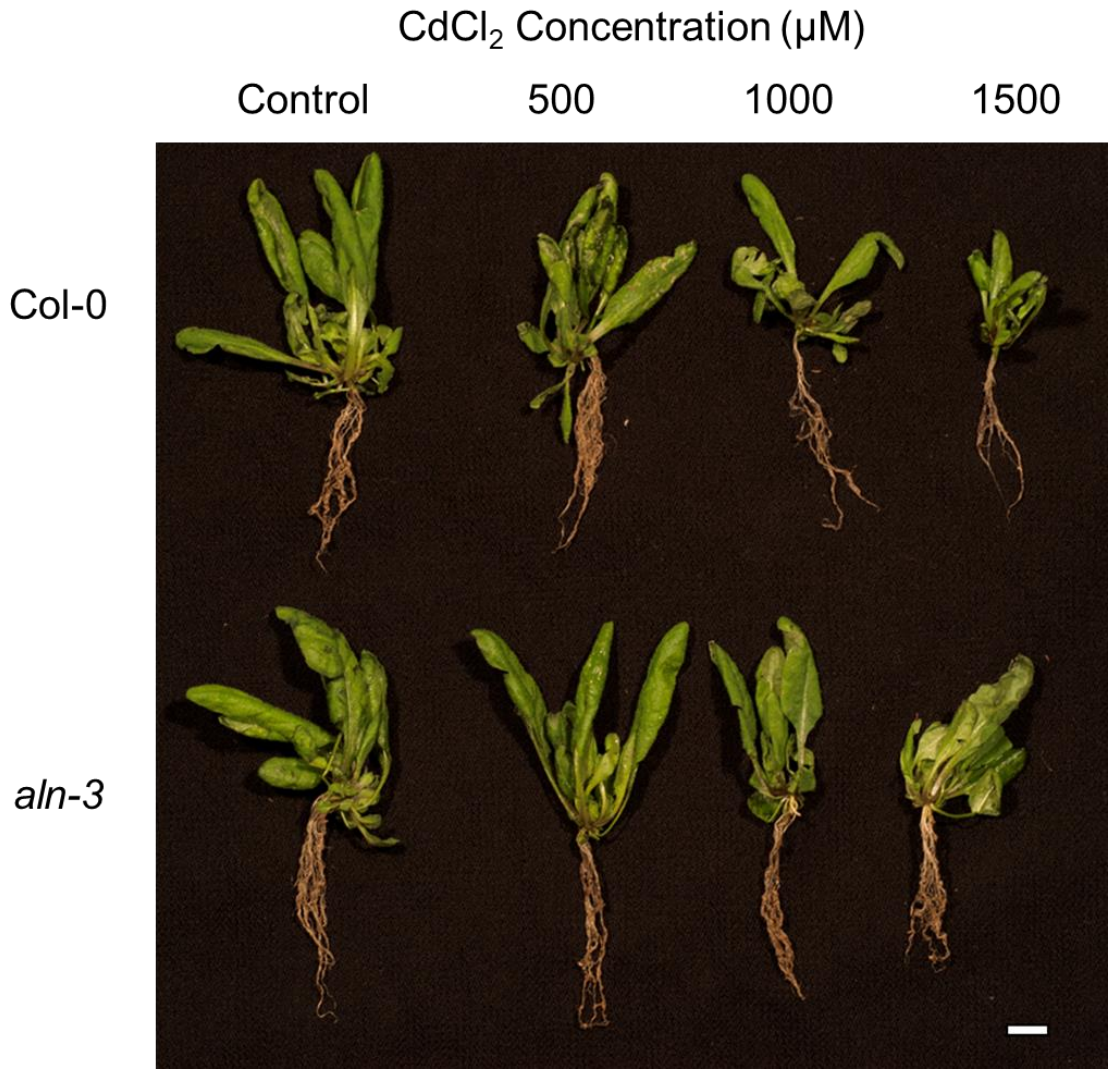

Figure 3.22. Col-0 and *aln-3* Arabidopsis root growth in response to Cd treatment. Picture is representative of three independent experiments. Scale bar = 1 cm.

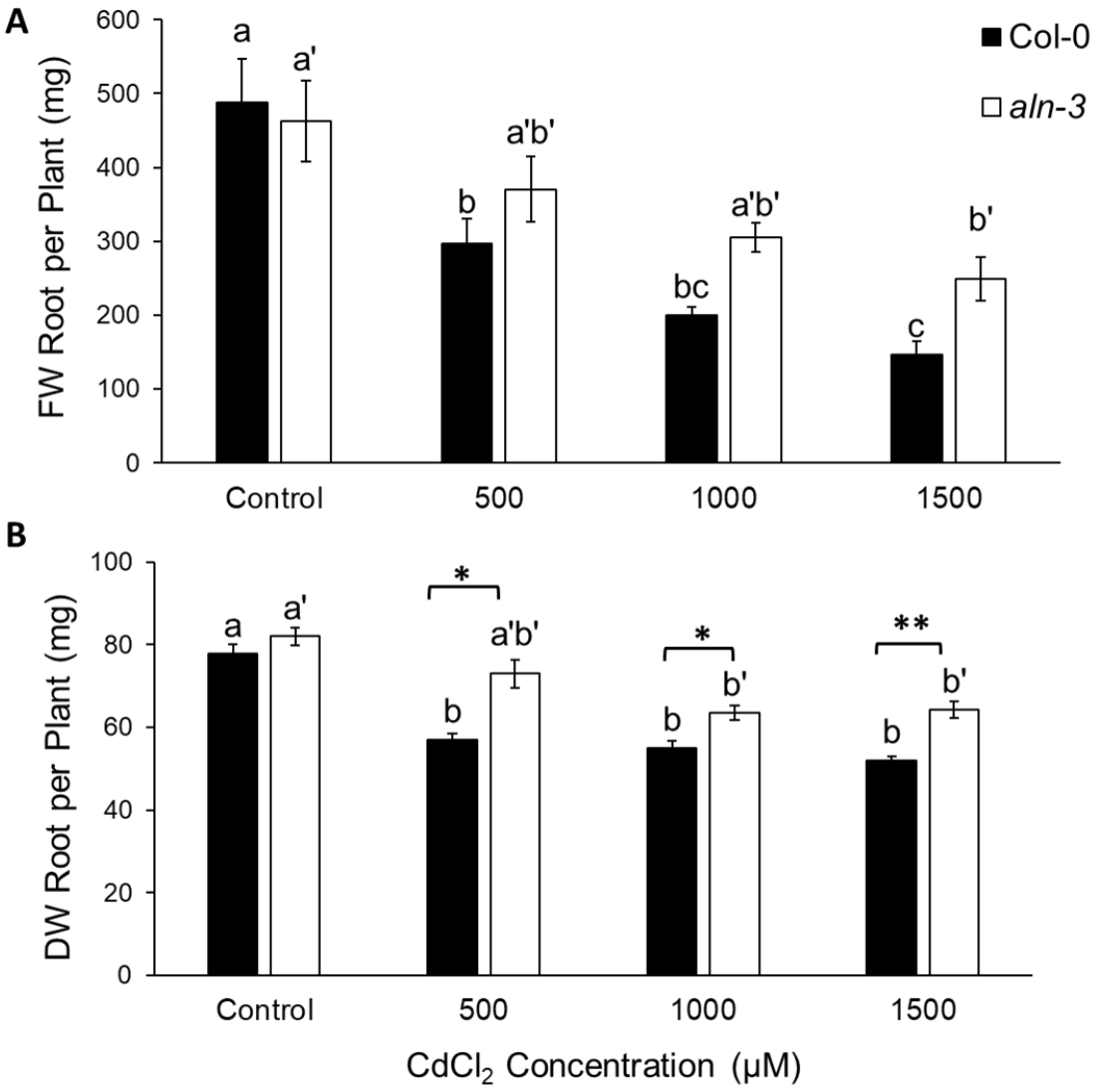

Figure 3.23. Effect of Cd on (A) fresh and (B) dry weight of Col-0 and *aln-3* roots. Values shown are the mean of four independent replicates ± SE. Different letters (a,b,c for Col-0 and a',b',c' for *aln-3*) show significant differences in each group ($P \leq 0.05$). Asterisks indicate significant differences obtained by a t-test (*$P \leq 0.05$, **$P \leq 0.01$).

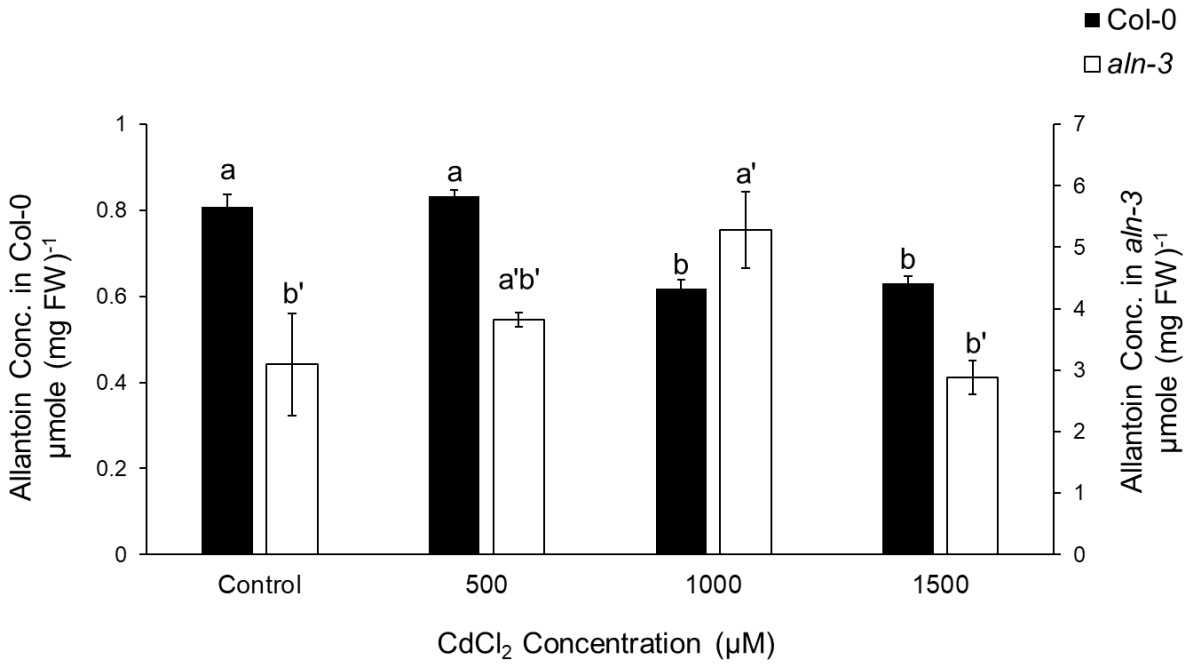

Figure 3.24. Effect of Cd treatment on allantoin content of Col-0 and *aln-3* roots. Represented values are the mean of six independent replicates ± SE. Left axis demonstrates data for Col-0 Arabidopsis and right axis shows *aln-3* data. Different letters (a,b,c for Col-0 and a',b',c' for *aln-3*) indicate significant differences in each group ($P \leq 0.05$).

3.4.3. Transcription of allantoinase and uricase in Col-0 and *aln-3* roots is influenced by cadmium.

Analyses the expression of ureide metabolic genes (*XDH1*, *UO*, *ALNS*, *ALN* and *AAH*) showed that expression of *ALN* in Col-0 roots and *UO* in Col-0 and *aln-3* mutants were influenced by Cd treatment (Fig. 3.25). In Col-0 roots *ALN* expression showed a noticeable decrease in response to Cd treatment (500-1500 µM $CdCl_2$). *UO* in Col-0 Arabidopsis was also downregulated at 1000 and 1500 µM $CdCl_2$. In *aln-3* roots expression of *UO* exhibited a considerable increase at higher Cd concentrations (1000 and 1500 µM $CdCl_2$).

Evaluating *ALN* and *UO* expression using qRT-PCR revealed that in Col-0 roots, three applied Cd concentrations decreased transcript level of *ALN* to 50% of that at control condition (Fig. 3.26A). *UO* expression of Col-0 samples did not change significantly at 500 µM $CdCl_2$, but went down dramatically in response to 1000 and 1500 µM $CdCl_2$ treatments. In *aln-3* mutants, mRNA abundance of *UO* remained unchanged at 500 µM $CdCl_2$, rising significantly at 1000 µM $CdCl_2$ and falling to a lower level at 1500 µM $CdCl_2$ (Fig. 3.26B).

3.4.4. *aln-3* roots show higher cadmium content than Col-0 roots.

Using AAS, Cd concentrations of root tissue were measured in Col-0 and mutant samples. Cd content of both Col-0 and *aln-3* roots increased in response to Cd treatment up to 1000 µM $CdCl_2$, followed by a considerable decrease at 1500 µM $CdCl_2$ (Fig. 3.27). However, Cd concentrations of *aln-3* mutant roots were greater than Col-0 samples throughout the experiment and the difference between these two genotypes is significantly different at control condition as well as 1000 and 1500 µM $CdCl_2$. It is also worth noting that contrary to the same experiment in leaf tissue (Fig. 3.16) *aln-3* roots at control treatment contained significantly higher amount of Cd when compared with Col-0 samples exposed the same condition.

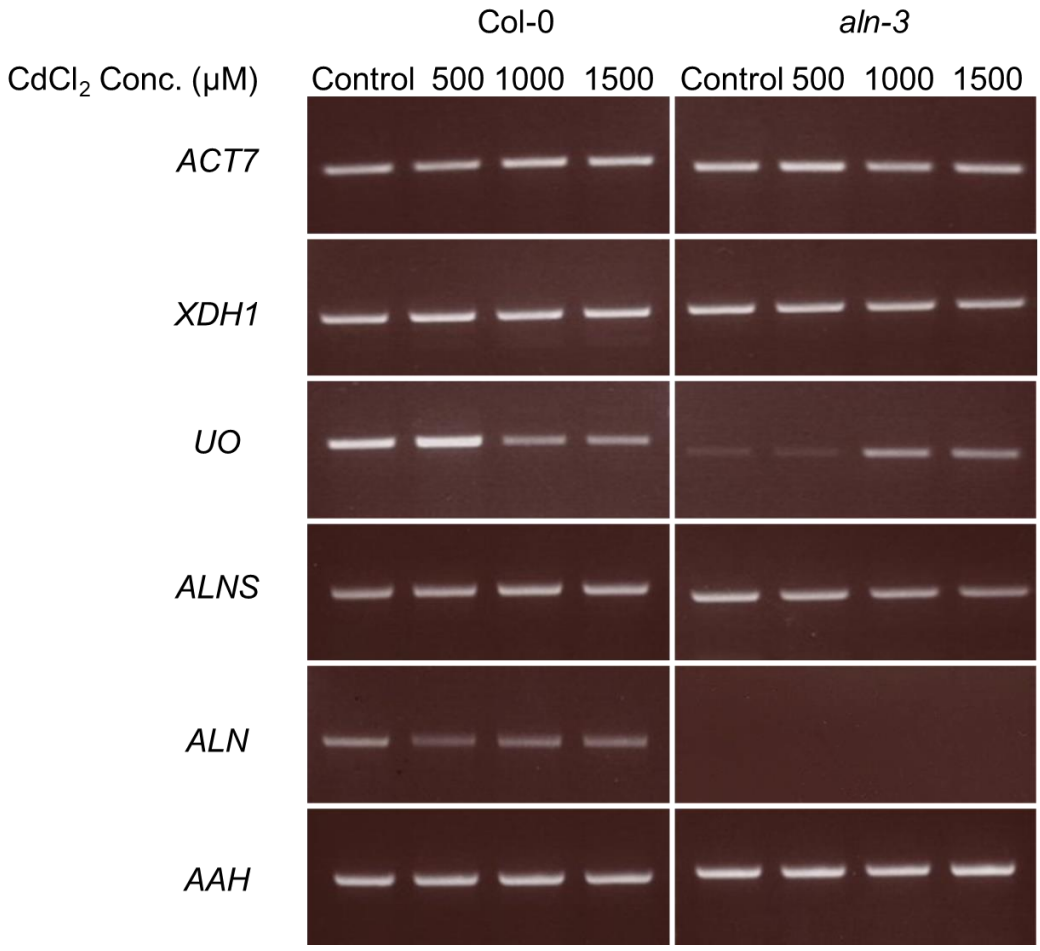

Figure 3.25. RT-PCR analysis of ureides genes in Col-0 and *aln-3* Arabidopsis roots in response to Cd. Picture is representative of three independent experiments. *AAH*, allantoate amidohydrolase; *ACT7*, actin7 (reference gene); *ALN*, allantoinase; *ALNS*, allantoin synthase; *UO*, uricase; *XDH1*, xanthine dehydrogenase 1.

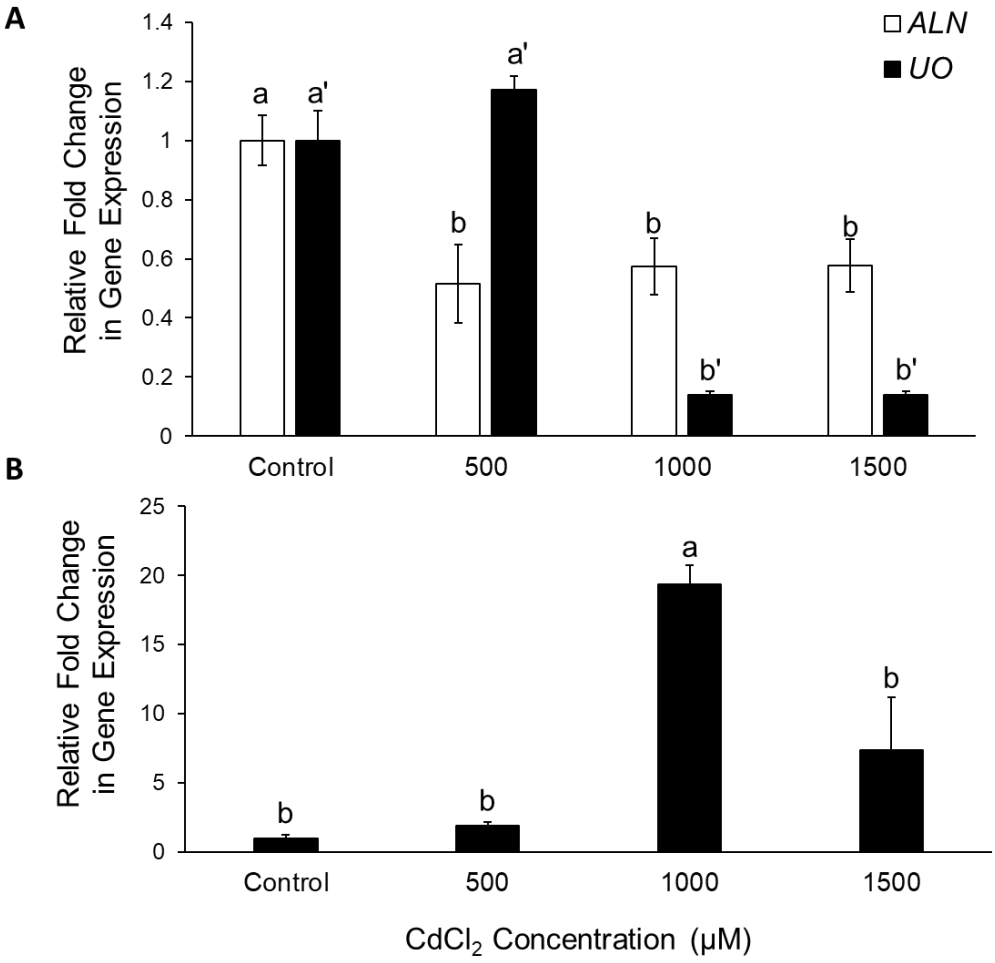

Figure 3.26. qRT-PCR analysis of ureides genes in (A) Col-0 and (B) *aln-3* Arabidopsis following Cd treatment. Values shown are the mean of three independent replicates ± SE. Different letters show significant differences in each group ($P \leq 0.05$). In the top graph a,b,c for *ALN* and a',b',c' for *UO* show the difference in transcript levels. In the bottom graph a,b,c for *UO* shows the difference in transcript levels. *ALN*, allantoinase; *UO*, uricase. *ALN* and *UO* were normalized to *ACT2* expression as an internal control.

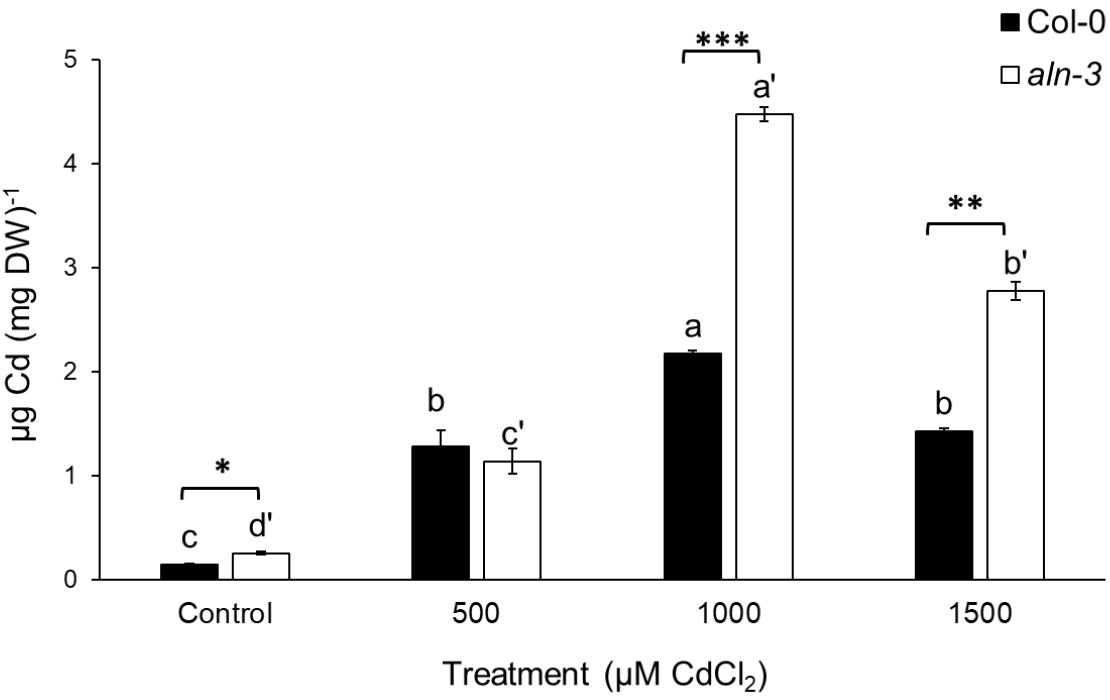

Figure 3.27. Effect of Cd treatment on Cd content of Col-0 and *aln-3* roots. Values shown are the mean of four independent replicates ± SE. Different letters (a,b,c for Col-0 and a',b',c' for *aln-3*) show significant differences in each group ($P \leq 0.05$). Asterisks indicate significant differences obtained by a t-test (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$).

3.4.5. *aln-3* roots accumulate lower reactive oxygen species in response to Cd treatment.

Quantification of hydrogen peroxide and superoxide in Col-0 and *aln-3* mutant roots following Cd treatment demonstrated that Cd (specifically 1000 and 1500 µM $CdCl_2$) stimulated H_2O_2 and O_2^- accumulation in Col-0 and *aln-3* roots (Fig. 3.28A,B). However, comparison between Col-0 and mutant roots showed that concentration of H_2O_2 and O_2^- in *aln-3* roots was lower than that of Col-0 samples with a significant difference between these two genotypes for superoxide radical measurement.

3.4.6. *aln-3* roots exhibit an increased antioxidant activity in response to cadmium.

Antioxidant assays in Col-0 roots indicated that superoxide dismutase was significantly activated at 500 µM $CdCl_2$, followed by a slightly decrease at 1000 and 1500 µM $CdCl_2$ (Fig. 3.29). Comparatively, SOD activity of *aln-3* roots increased in response to Cd treatment, reaching the highest value at 1000 and 1500 µM $CdCl_2$. SOD assay revealed that there is a significant difference between Col-0 and *aln-3* mutant roots, especially at higher Cd treatments (1000 and 1500 µM $CdCl_2$).

Consistent with Col-0 SOD, quantification of CAT activity showed that 500 µM $CdCl_2$ activated this antioxidant enzyme in Col-0 roots, while it decreased at 1000 and 1500 µM $CdCl_2$ (Fig. 3.30). In *aln-3* roots, CAT activity did not change at 500 µM $CdCl_2$, whereas 1000 and 1500 µM $CdCl_2$ significantly stimulated enzyme activity. There was a significant difference between CAT activity of Col-0 and *aln-3* roots exposed to Cd treatment.

APX activity in both genotypes showed an increasing trend in response to increasing Cd concentration, with the highest activity at 1500 µM $CdCl_2$ for both Col-0 and *aln-3* roots (Fig 3.31).

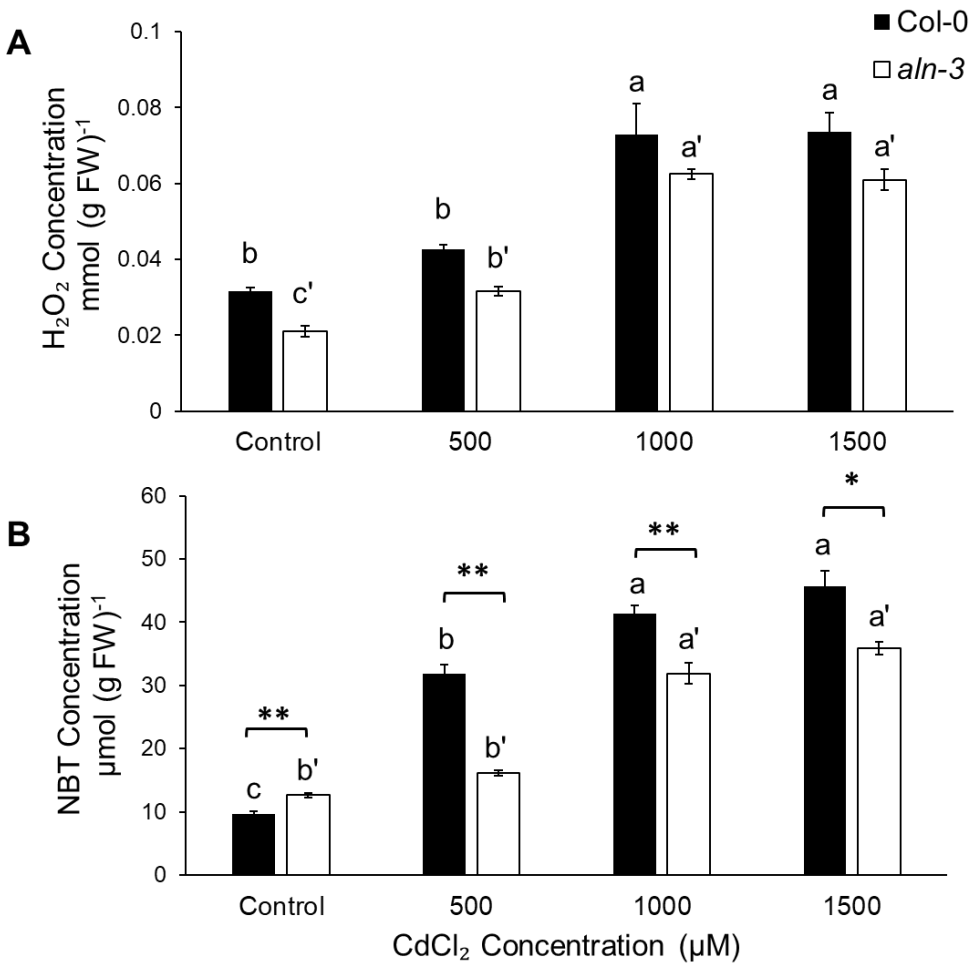

Figure 3.28. Quantification of (A) hydrogen peroxide and (B) superoxide radical accumulation in Cd-treated Col-0 and *aln-3* Arabidopsis roots. Values shown are the mean of four independent replicates ± SE. Different letters (a,b,c for Col-0 and a',b',c' for *aln-3*) show significant differences in each group ($P \leq 0.05$). Asterisks indicate significant differences obtained by a t-test (*$P \leq 0.05$, **$P \leq 0.01$).

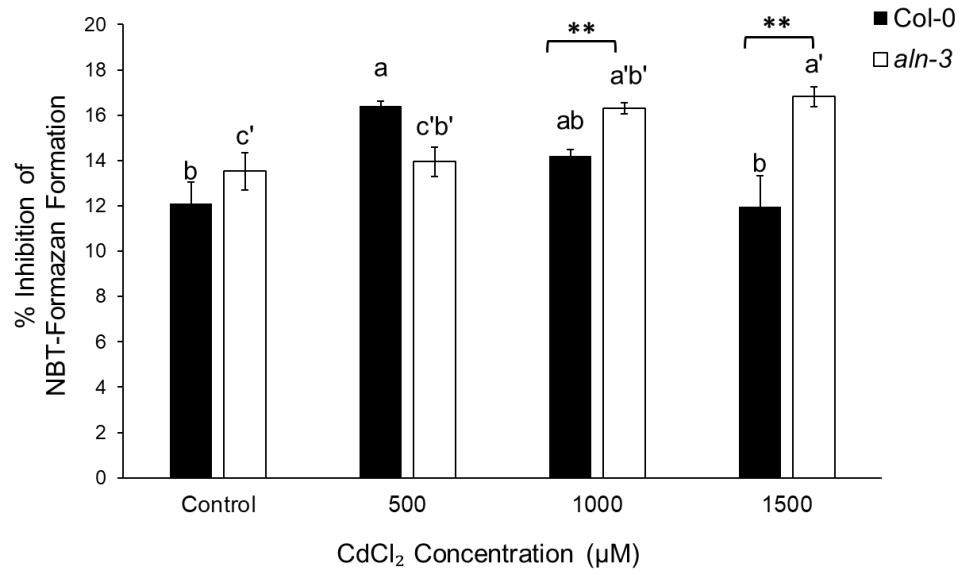

Figure 3.29. Effect of Cd on the activity of superoxide dismutase (SOD) in Col-0 and *aln-3* Arabidopsis roots. Values shown are the mean of four independent replicates ± SE. Different letters (a,b,c for Col-0 and a',b',c' for *aln-3*) show significant differences in each group ($P \leq 0.05$). Asterisks indicate significant differences obtained by a t-test (**$P \leq 0.01$).

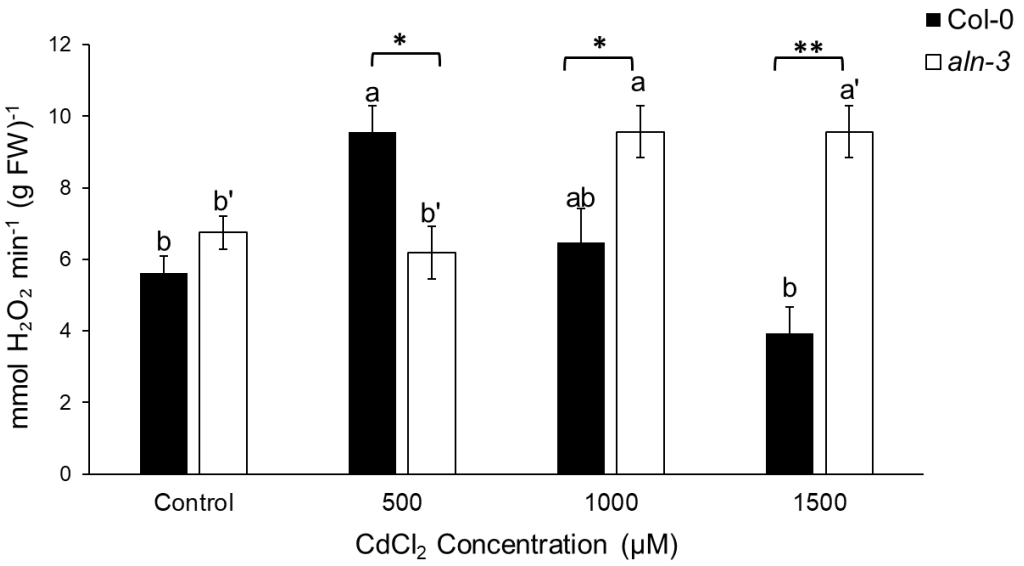

Figure 3.30. Effect of Cd on the activity of catalase (CAT) in Col-0 and *aln-3* Arabidopsis roots. Values shown are the mean of four independent replicates ± SE. Different letters (a,b,c for Col-0 and a',b',c' for *aln-3*) show significant differences in each group ($P \leq 0.05$). Asterisks indicate significant differences obtained by a t-test (*$P \leq 0.05$, **$P \leq 0.01$).

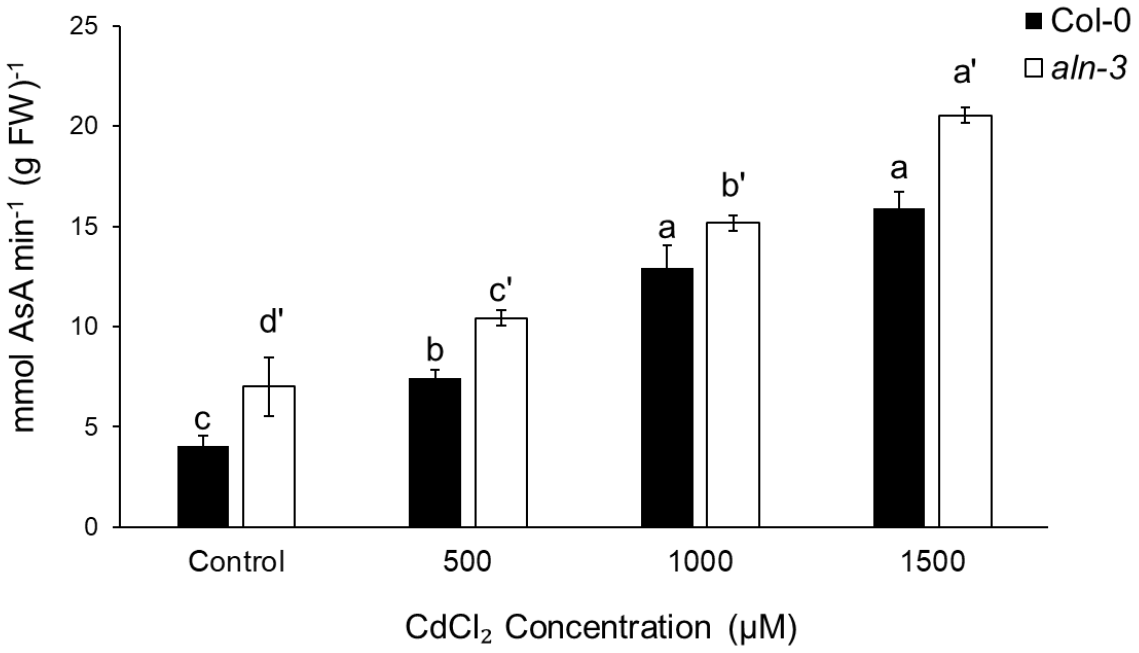

Figure 3.31. Effect of Cd on the activity of ascorbate peroxidase (APX) in Col-0 and *aln-3* Arabidopsis roots. Values shown are the mean of four independent replicates ± SE. Different letters (a,b,c for Col-0 and a',b',c' for *aln-3*) show significant differences in each group ($P \leq 0.05$).

3.5. Effect of exogenous allantoin on Col-0 Arabidopsis

Comparison between Cd-exposed Col-0 and *aln-3* mutants suggested that elevated amounts of allantoin in *aln-3* mutants improves plant growth and affords stress tolerance in response to Cd treatment. Results of these experiments prompts the question whether exogenous application of allantoin has the same protective effect on Col-0 plants. To answer this question Col-0 Arabidopsis seeds were allowed to germinate and grow on MS plates containing 10 mM allantoin (MS+Aln) and 50, 100 and 200 µM $CdCl_2$. After two weeks, root length obtained from these samples were compared with those grown in the absence of allantoin (MS).

3.5.1. Allantoin confers cadmium tolerance to Col-0 seedlings.

As shown in Figs. 3.32 and 3.33, although increasing Cd concentrations decreased plant growth and root elongation of both MS- and MS+Aln-grown seedlings, allantoin in the growth media increased root length of Cd exposed-Col-0 Arabidopsis and the difference between these two groups (MS- and MS+Aln-grown samples) was statistically significant following Cd treatment.

3.5.2. Allantoin is effective at the early stages of plant growth.

In the above experiments allantoin was present in the plant tissue (such as *aln-3* mutants) or in the growth media (such as MS+Aln plates) from the beginning and throughout the experiment. However, it is also important to know at which plant developmental stage allantoin applies its protective effect. To answer this question Col-0 Arabidopsis seeds were germinated on MS plates and transferred to MS+Aln, MS+Cd (50 and 100 µM $CdCl_2$) and MS+Aln+Cd (50 and 100 µM $CdCl_2$) on different days (days one, two, three, four and five after seed germination). At the end of the experiment (after two weeks) seedling root lengths were measured and compared with those grown on MS plates.

Consistent with previous observations, Cd decreased root length in all five experimental conditions and plants grown in the presence of allantoin showed relatively greater root length compared with those not exposed to allantoin (Figs. 3.34 – 3.36). However, the difference between MS and MS+Aln plates was statistically significant for the samples transferred at early stages of plant development, on days one and two after germination, but not on days three, four, and five.

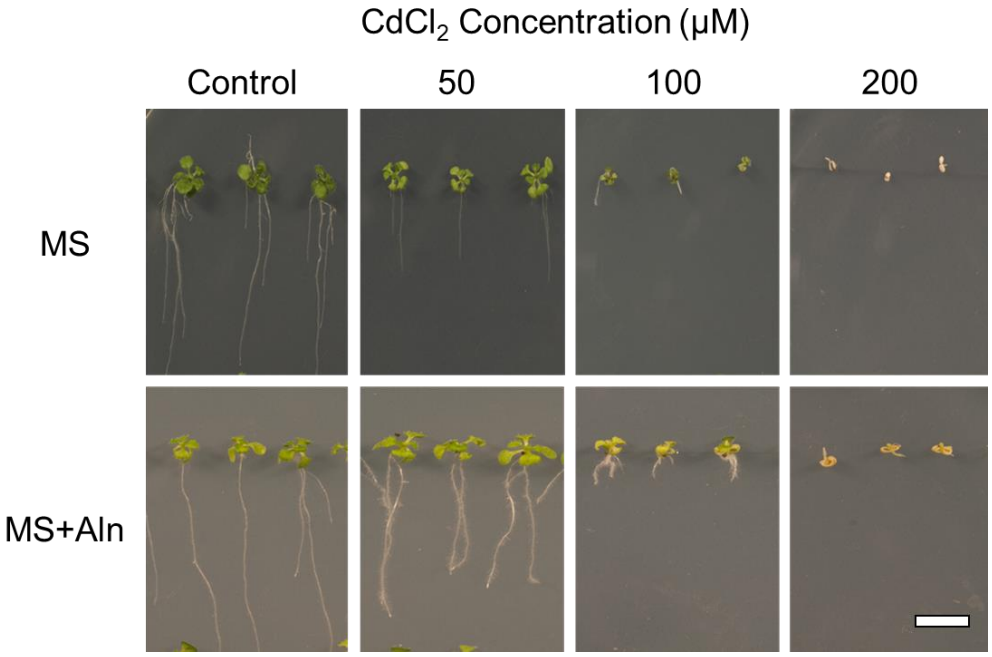

Figure 3.32. Effect of exogenous allantoin on seedling growth of Col-0 Arabidopsis in response to Cd treatment. Picture is representative of three independent experiments. Scale bar = 1cm.

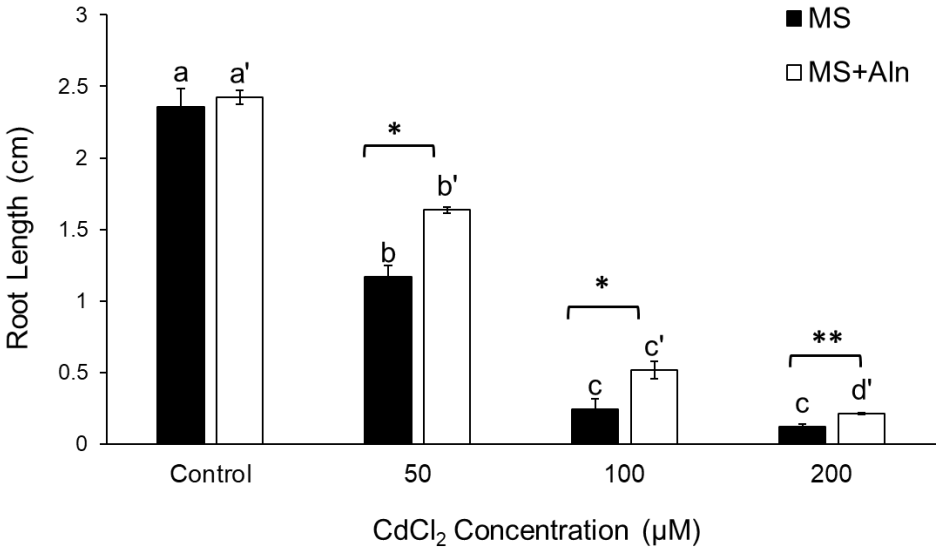

Figure 3.33. Effect of exogenous allantoin on root length of Col-0 Arabidopsis in response to Cd treatment. Values shown are the mean of three independent replicates ± SE. Different letters (a,b,c for Col-0 and a',b',c' for *aln-3*) show significant differences in each group ($P \leq 0.05$). Asterisks indicate significant differences obtained by a t-test (*$P \leq 0.05$, **$P \leq 0.01$).

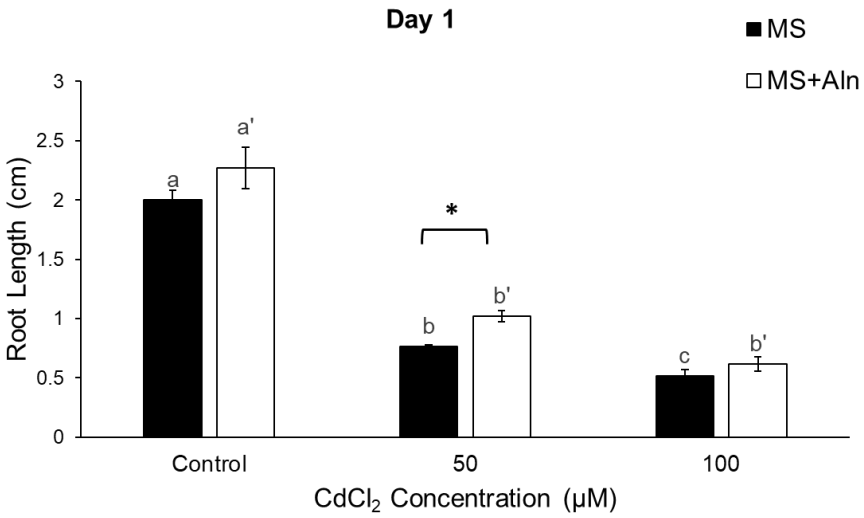

Figure 3.34. Effect of exogenous allantoin on the root length of Cd-exposed Col-0 Arabidopsis on day one after seed germination. Values shown are the mean of three independent replicates ± SE. Different letters (a,b,c for MS and a',b',c' for MS+Aln) show significant differences in each group ($P \leq 0.05$). Asterisks indicate significant differences obtained by a t-test (*$P \leq 0.05$).

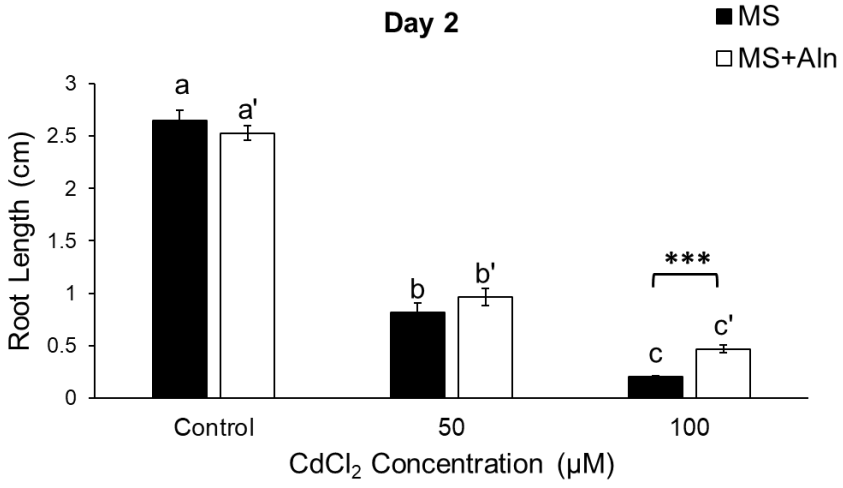

Figure 3.35. Effect of exogenous allantoin on the root length of Cd-exposed Col-0 Arabidopsis on day two after seed germination. Values shown are the mean of three independent replicates ± SE. Different letters (a,b,c for MS and a',b',c' for MS+Aln) show significant differences in each group ($P \leq 0.05$). Asterisks indicate significant differences obtained by a t-test (***$P \leq 0.001$).

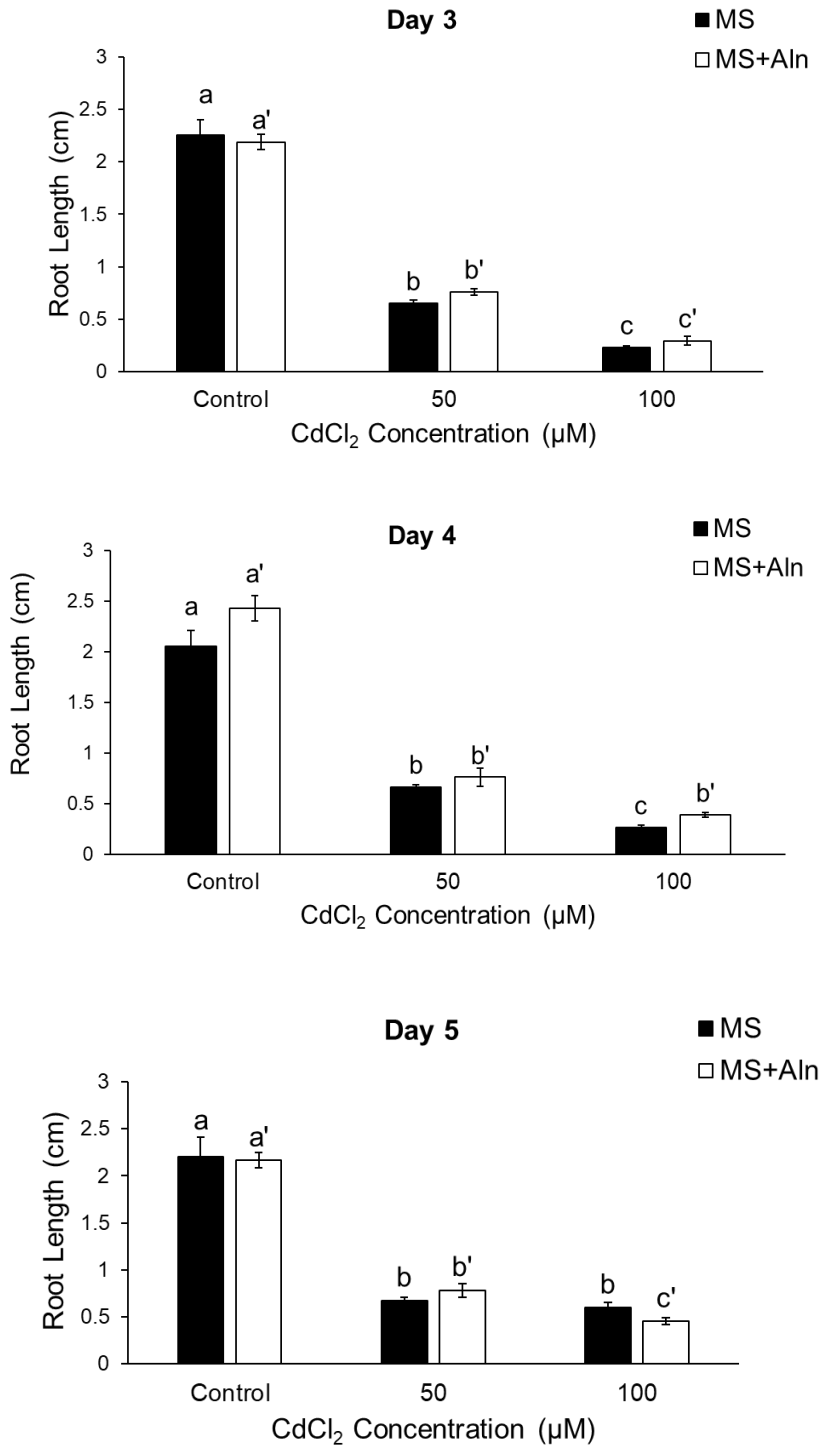

Figure 3.36. Effect of exogenous allantoin on the root length of Cd-exposed Col-0 Arabidopsis on days three, four and five after seed germination. Values shown are the mean of three independent replicates ± SE. Different letters (a,b,c for MS and a',b',c' for MS+Aln) show significant differences in each group ($P \leq 0.05$).

3.5.3. Allantoin treatment of Col-0 Arabidopsis seeds increases cadmium tolerance in seedlings.

Findings from the previous experiment revealed that allantoin is required at the early stages of plant growth in order to confer Cd-resistance to Col-0 seedlings. To clarify the effect of allantoin on Col-0 Arabidopsis seeds, seeds were soaked in 10 mM allantoin at 4° C for 48 hours (Aln-treated seeds), then transferred to MS, MS+Aln, MS+100 µM $CdCl_2$ and MS+Aln+100 µM $CdCl_2$ plates and allowed to germinate and grow for two weeks. Seed germination was determined on days 4, 8 and 12 after transferring to plates, and root length was measured at the end of the experiment. These results were compared with seeds soaked in dH_2O (H_2O-treated seeds) as a control condition.

Cd-induced decrease of plant growth in Aln- and H_2O-treated samples, reflected in their decreased root length in response to Cd treatment (Figs. 3.37 and 3.38). Additionally, allantoin in the growth media improved root length of both groups exposed to Cd treatment. However, Aln-treated samples exhibited better growth and longer root length at Cd treatment, and a significant difference was observed between Aln-treated and H_2O-treated Arabidopsis in response to MS+Cd and MS+Aln+Cd treatments.

Germination percent of Aln- and H_2O-treated seeds on day 4 were variable in response to different treatments, while reaching the same level on days 8 and 12 (Fig. 3.39). Comparison between seed germination of Aln- and H_2O-treated samples on day 4 indicated that allantoin treatment of seeds increased their germination in response to Cd treatment and a significant difference was observed between these two groups (Fig. 3.40).

3.5.4. Allantoin induces antioxidants in Col-0 seeds.

To determine how allantoin improves seed germination and seedling growth following Cd treatment, antioxidant assay was performed for Col-0 Arabidopsis seeds after 48 h of Aln treatment and results were compared with H_2O-treated seeds as a control. As shown in Figs. 3.41 and 3.42, allantoin treatment stimulated the activity of antioxidant enzymes (SOD, CAT and APX) with a significant difference between SOD activity of Aln- and H_2O-treated seeds (Fig. 3.41).

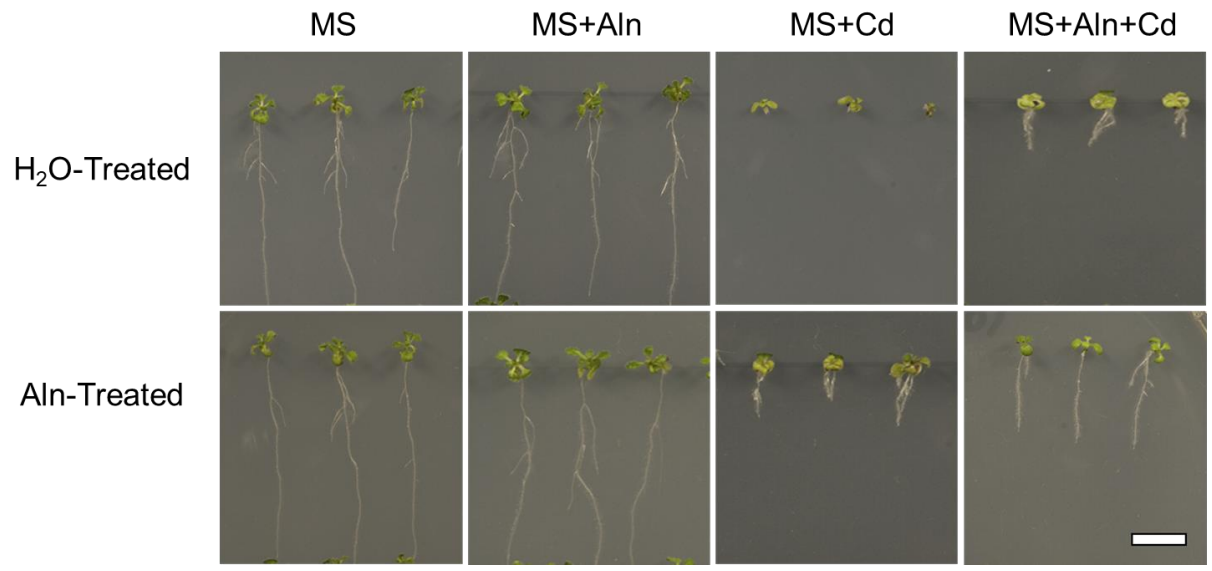

Figure 3.37. Effect of allantoin treatment of Col-0 seeds on the seedling growth in response to Cd. Picture is representative of three independent experiments. Scale bar = 1cm.

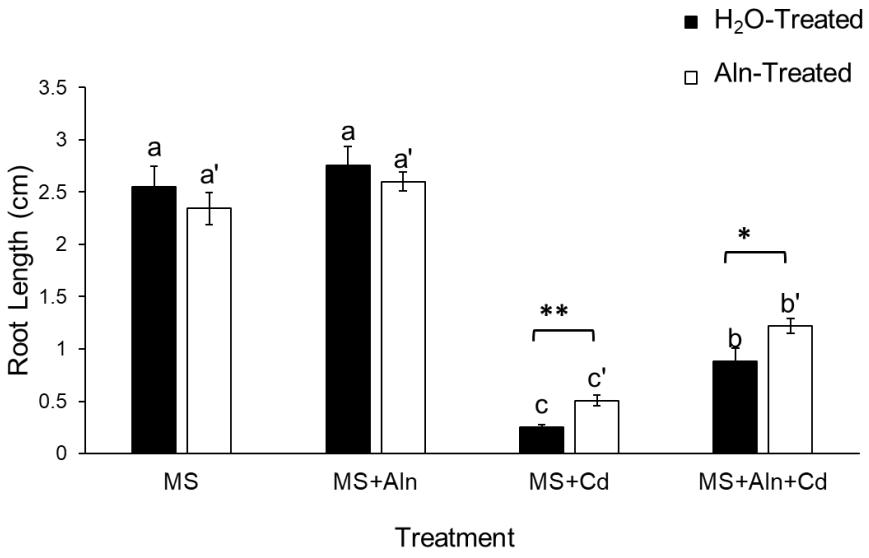

Figure 3.38. Effect of allantoin treatment of Col-0 seeds on the root length in response to Cd. Values shown are the mean of three independent replicates ± SE. Different letters (a,b,c for H_2O-treated and a',b',c' for Aln-treated samples) show significant differences in each group ($P \leq 0.05$). Asterisks indicate significant differences obtained by a t-test (*$P \leq 0.05$, **$P \leq 0.01$).

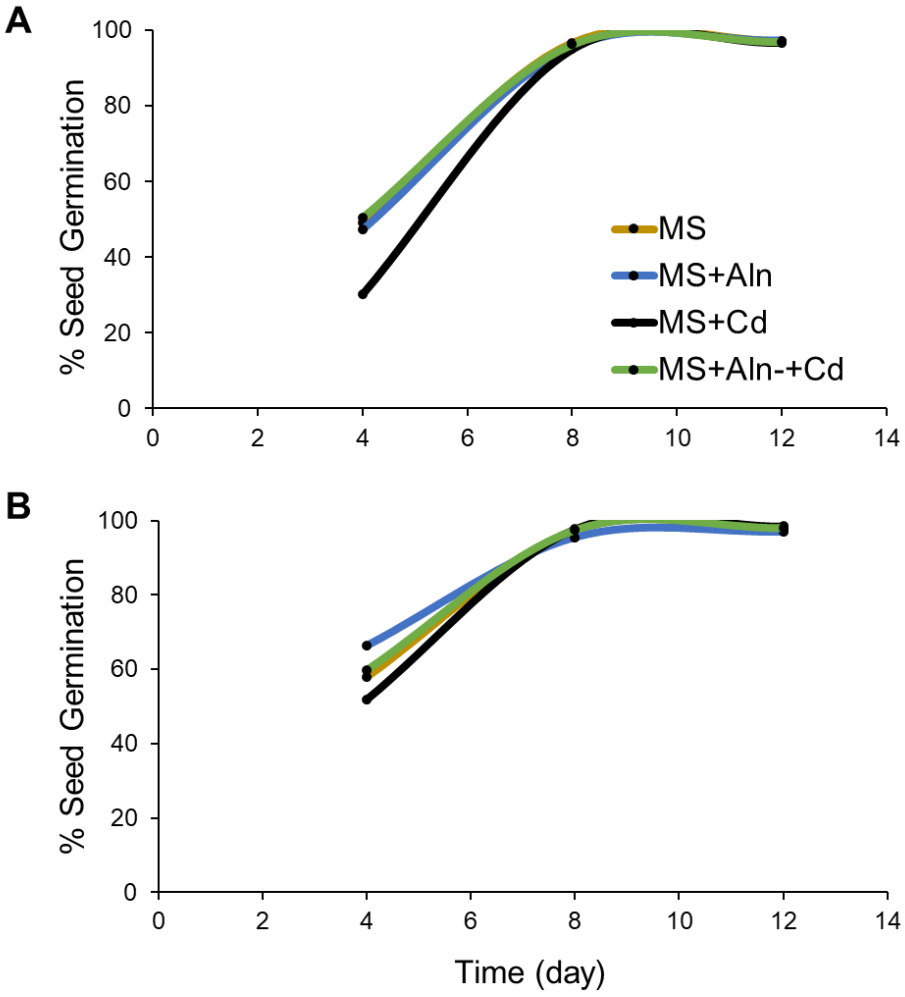

Figure 3.39. Effect of allantoin treatment on the seed germination of (A) H_2O-treated and (B) Aln-treated Col-0 seeds in response to Cd. Values shown are the mean of three independent replicates.

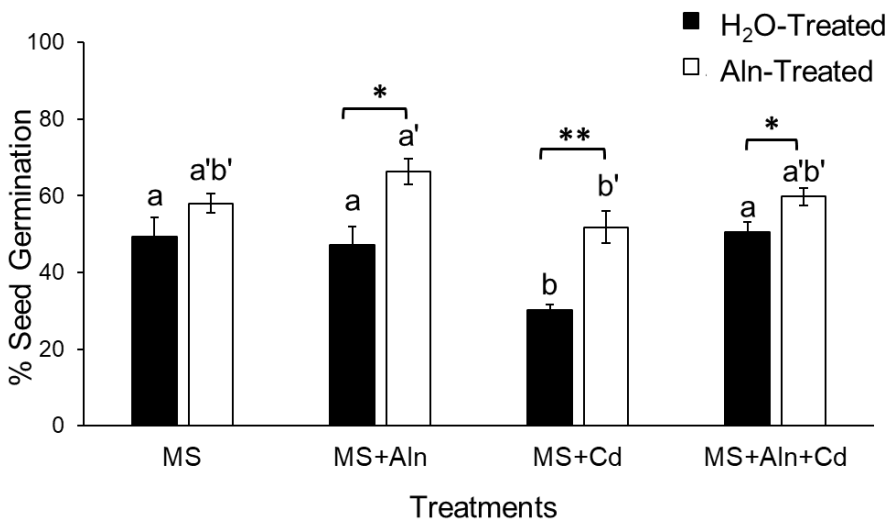

Figure 3.40. Effect of allantoin treatment on Col-0 seed germination on day four in response to Cd. Values shown are the mean of three independent replicates ± SE. Different letters (a,b,c for H_2O-treated and a',b',c' for Aln-treated samples) show significant differences in each group ($P \leq 0.05$). Asterisks indicate significant differences obtained by a t-test (*$P \leq 0.05$, **$P \leq 0.01$).

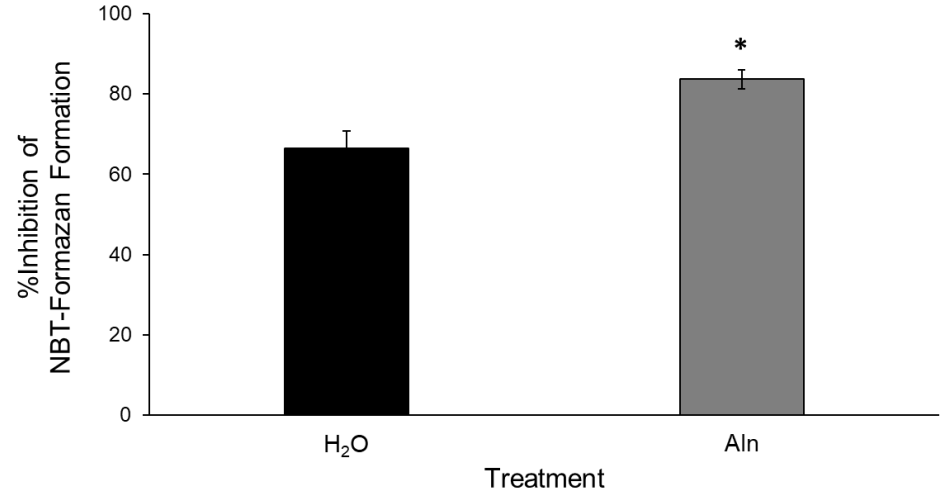

Figure 3.41. Effect of allantoin treatment on SOD activity of Col-0 Arabidopsis seeds. Values shown are the mean of four independent replicates ± SE. Asterisks indicate significant differences obtained by a t-test (*$P \leq 0.05$).

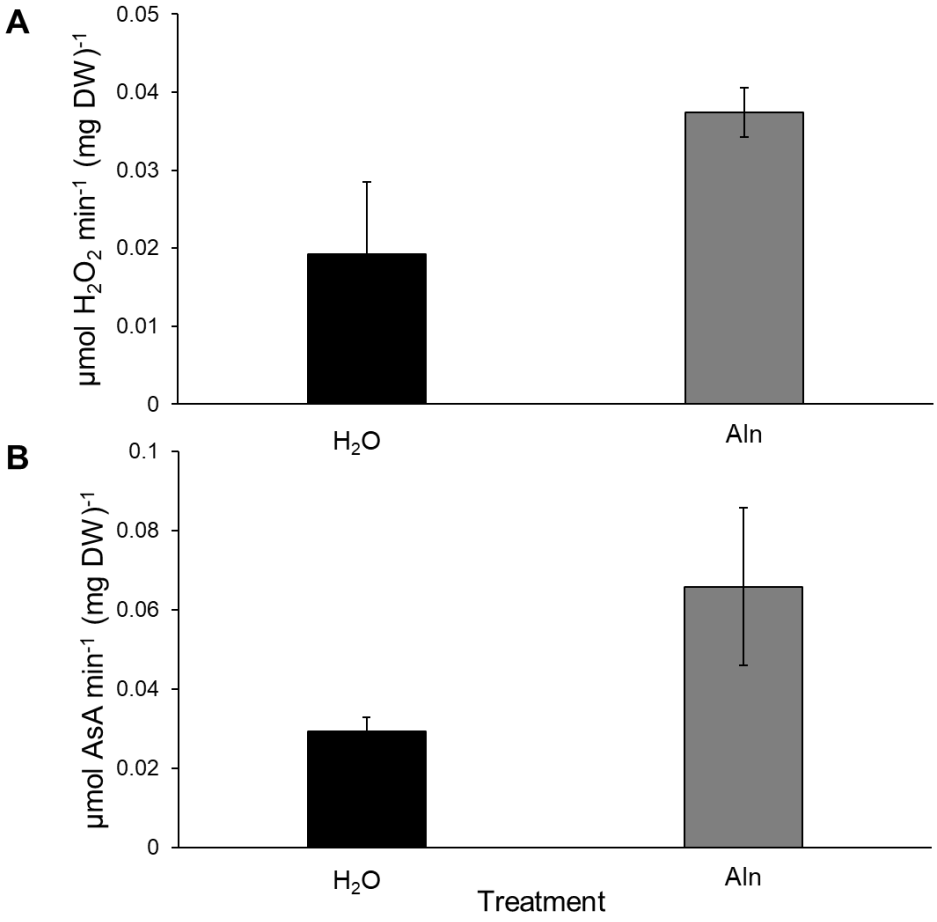

Figure 3.42. Effect of allantoin treatment on (A) CAT activity and (B) APX activity of Col-0 Arabidopsis seeds. Values shown are the mean of four independent replicates ± SE.

3.6. Characterization of *ALNox* lines

Employing *aln-3* mutants showed that this genotype is more tolerant in response to Cd treatment when compared with Col-0 Arabidopsis. This is probably related to increased amount of allantoin in these plants. These findings lead to the hypothesis that plants containing lower amount of allantoin, in comparison with Col-0 plants, are likely more susceptible in response to Cd. Therefore, plant lines overexpressing *ALN* from the constitutive CaMV 35S promoter were generated to test this hypothesis, and named, *ALNox*. Insertion of *ALN* coding sequence (*ALN-CDS*) into pCAMBIA 1303 plasmid, containing CaMV 35S promoter, and experimental procedure for the generation of *ALNox* lines are described in "Material and Methods", section 2.2.

3.6.1. Overexpression of *ALN* causes a noticeable change in *ALN* transcription and enzyme activity as well as allantoin content.

Expression level of *ALN* in overexpressed lines are significantly higher than those in Col-0 samples at control condition, confirming that the *35S::ALN* cassette caused increased expression of *ALN* in these plants (Fig. 3.43). Moreover, ALN enzyme activity assay indicated that this enzyme is considerably more activate in the mutants at control treatment in respect with Col-0 seedlings grown under the same condition (Fig. 3.44). HPLC analysis also demonstrated the lack of detectable amount of allantoin in these mutants when compared with Col-0 seedlings (Fig. 3.45).

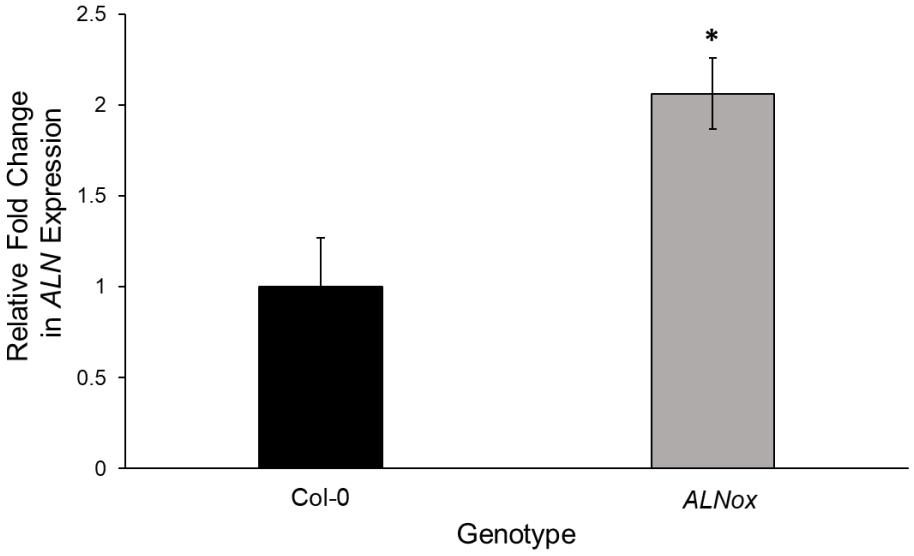

Figure 3.43. Evaluation of *ALN* gene expression in Col-0 and *ALNox* lines at control condition. Values shown are the mean of three independent replicates ± SE. Asterisks indicate significant differences obtained by a t-test (*$P \leq 0.05$). *ALN* (allantoinase) was normalized to *ACT2* expression as an internal control.

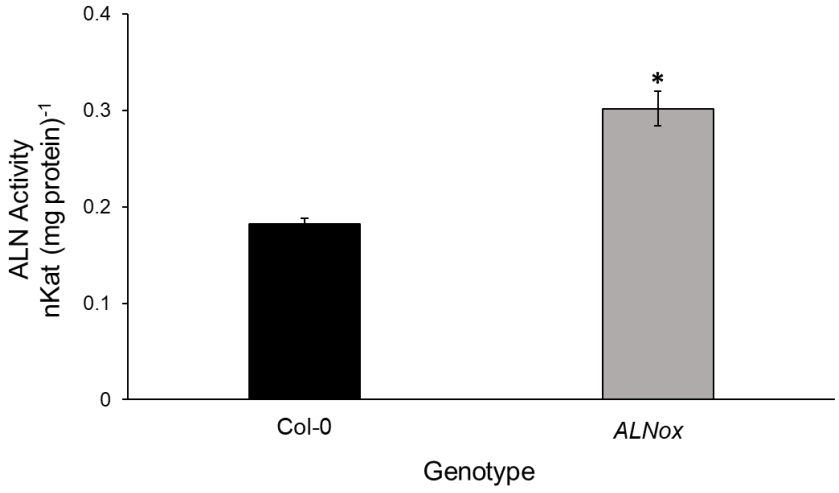

Figure 3.44. Evaluation of ALN enzyme activity in Col-0 and *ALNox* lines at control condition. Values shown are the mean of three independent replicates ± SE. Asterisks indicate significant differences obtained by a t-test (*$P \leq 0.05$). ALN, Allantoinase.

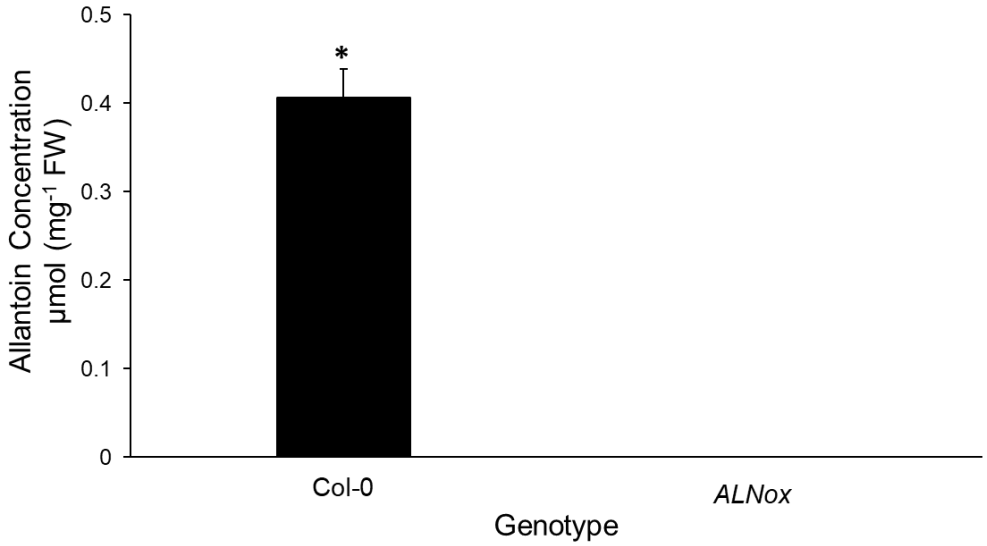

Figure 3.45. Measurement of allantoin content in Col-0 and *ALNox* lines at control condition. Values shown are the mean of three independent replicates ± SE. Asterisks indicate significant differences obtained by a t-test (*$P \leq 0.05$).

3.6.2. *ALNox* lines are more susceptible to cadmium than Col-0 plants.

Seed germination and root length was tested under control conditions and with 50 and 100 µM $CdCl_2$ treatment. In addition to the general reduction in plant growth due to Cd toxicity, overexpressers were more sensitive to Cd treatment and Cd decreased plant growth in the mutants was more than that observed in Col-0 seedlings (Fig. 3.46). Compromised function of *ALNox* lines was reflected in their lower seed germination upon Cd exposure (Fig. 3.47A), while, as mentioned above, these Cd concentrations did not have a significant effect on the germination of Col-0 seeds (Fig. 3.6). Additonally, root length measurement indicated that there is a significant difference between root elongation of Col-0 and *ALNox* lines, even at control treatment (Fig. 3.47B).

Based on these results 100 µM $CdCl_2$ treatment was selected to perform HPLC analysis. Since this Cd concentration was shown to be toxic enough to differentiate between Col-0 and mutants and also sympotoms of Cd toxicity such as decreased seed germination and decreased root length was observed at this Cd treatment. 100 µM $CdCl_2$ treatment did not induce allantoin accumulation in *ALNox* lines, whereas allantoin conentration of Col-0 seedling showed a significant increase at this Cd treatment (Fig. 3.48A). Furthermore, Allantoinase acitivty in both genotypes exhibited a slight decrease in response to Cd exposure, with camparatively higher values for the mutants (Fig. 3.48B). This observation is consistent with declined ALN activity in soil-grown Col-0 leaves in response to 500-1500 µM $CdCl_2$ treatmen (Fig. 3.15), though in the present assay neither decreases were statistically significant.

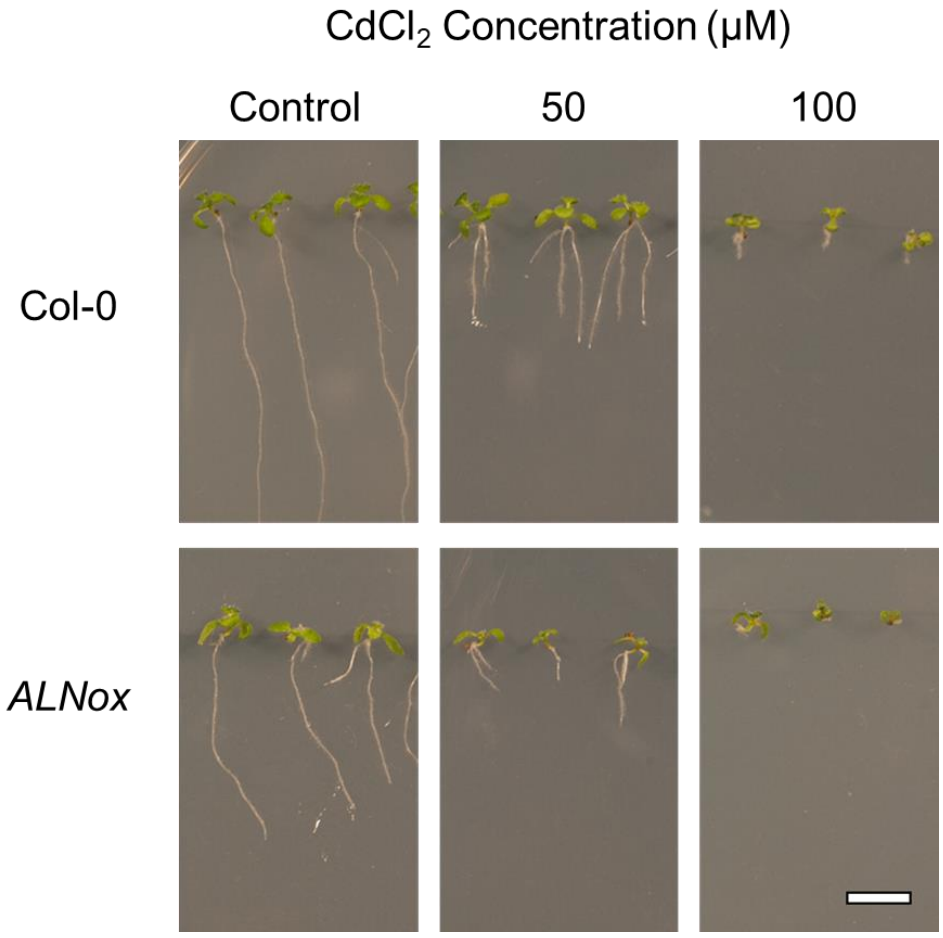

Figure 3.46. Effect of *ALN* overexpression on plant growth of Col-0 and *ALNox* lines at 50 and 100 µM $CdCl_2$ treatments. Picture is representative of three independent experiments. Scale bar = 1cm.

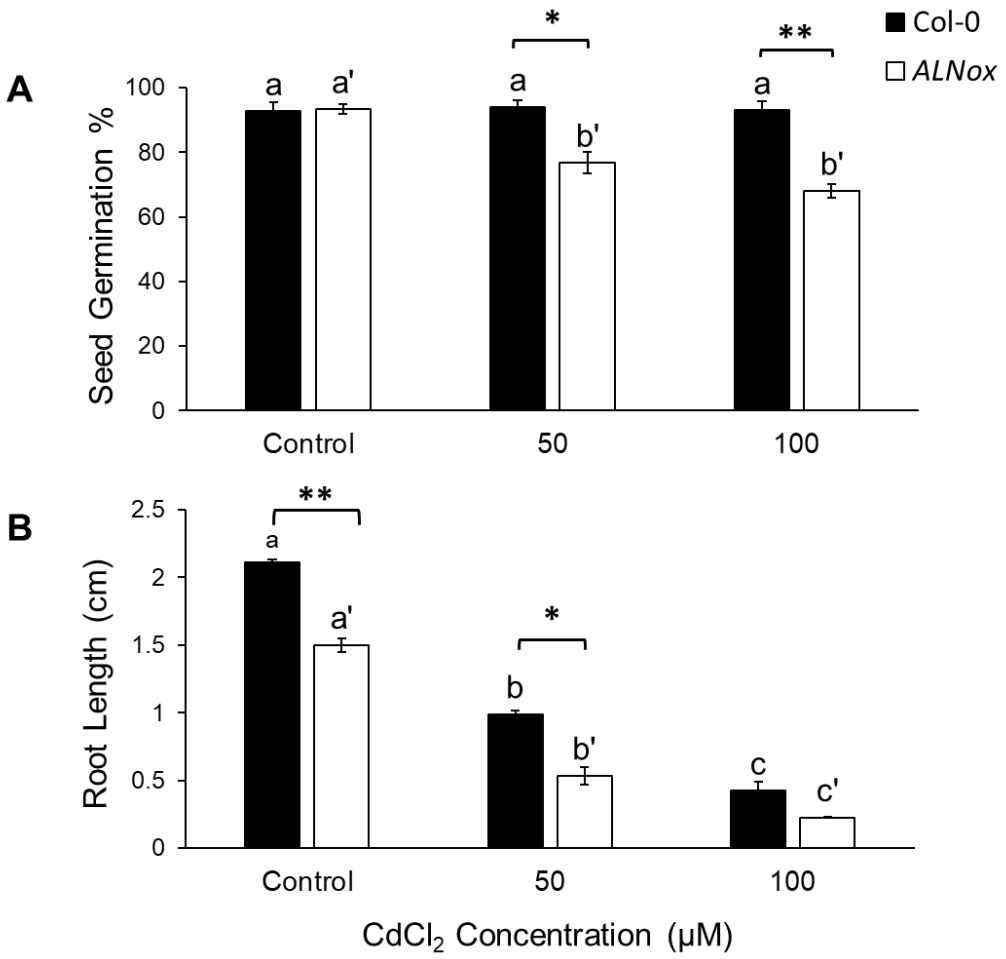

Figure 3.47. Effect of *ALN* overexpression on (A) seed germination and (B) root length of Col-0 and *ALNox* lines at 50 and 100 µM $CdCl_2$ treatments. Values shown are the mean of three independent replicates ± SE. Different letters (a,b,c for Col-0 and a',b',c' for *ALNox* samples) show significant differences in each group ($P \leq 0.05$). Asterisks indicate significant differences obtained by a t-test (*$P \leq 0.05$, **$P \leq 0.01$).

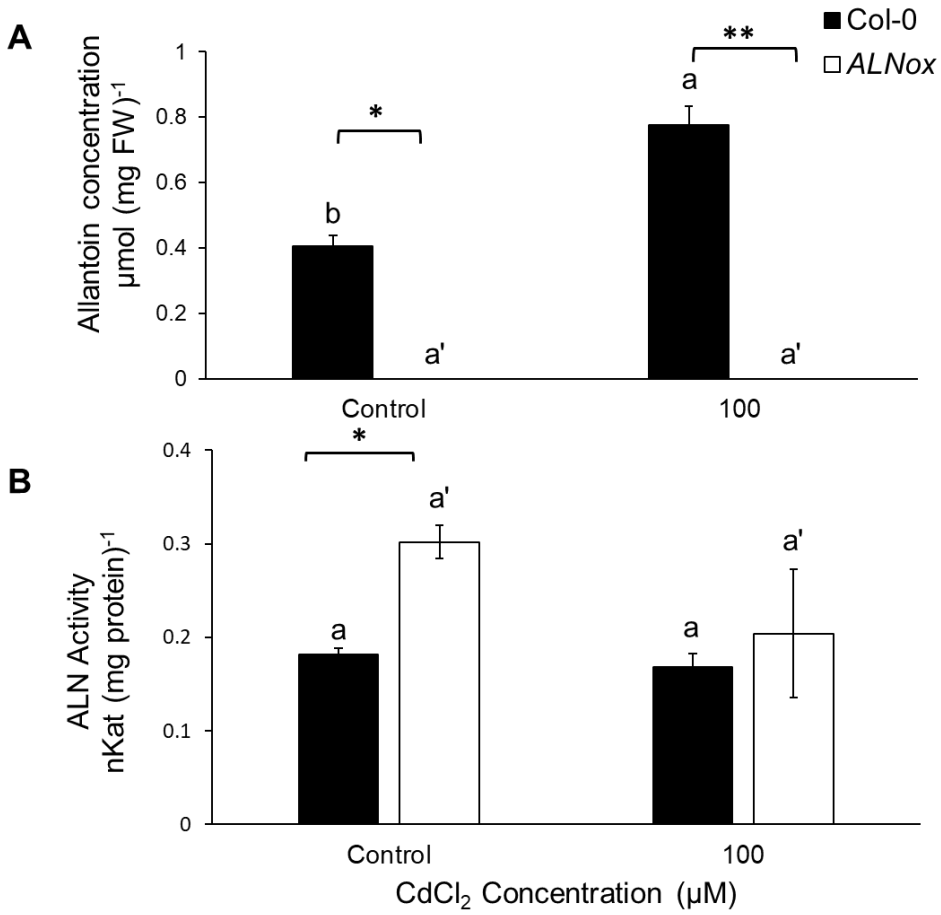

Figure 3.48. Effect of *ALN* overexpression on (A) allantoin content and (B) allantoinase enzyme activity in Col-0 and *ALNox* lines in response to Cd treatment. Values shown are the mean of three independent replicates ± SE. Different letters (a,b,c for Col-0 and a',b',c' for *ALNox* samples) show significant differences in each group ($P \leq 0.05$). Asterisks indicate significant differences obtained by a t-test (*$P \leq 0.05$, **$P \leq 0.01$).

3.6.3. Exogenous allantoin does not rescue *ALNox* lines from Cd toxicity.

Showing that *ALNox* lines are more susceptible to Cd than Col-0 seedling raised this question whether exogenous allantoin improve plant growth in these transformed lines or not. To this end, *ALNox* lines were allowed to grow on media plates containing 10 mM allantoin (MS+Aln) and different Cd concentrations (50 and 100 µM $CdCl_2$) and their root length were compared with those grown in the absence of allantoin (MS). Root length measurement indicated that Cd treatment significantly decreased root length of *ALNox* lines under both treatments (MS and MS+Aln). Comparison between MS- and MS+Aln-grown seedlings revealed that exogenous application of allantoin did not influence root length and there was not a significant difference between MS and MS+Aln treatments in this experiment (Fig. 3.49). It is worth noting that Col-0 samples showed an improved plant growth and root length when grown on MS+Aln plate as previously shown in Fig. 3.33.

Quantification of allantoin in Col-0 and mutant seedlings grown on MS+Aln and MS+Aln+Cd plates (100 µM $CdCl_2$) indicated that application of allantoin in growth media increased allantoin content of both genotypes (Fig. 3.50A). Agreeing with allantoin content of treated seedlings, activity of the enzyme allantoinase showed a dramatic increase for both genotypes, however this raise in enzyme activity was significantly higher in *ALNox* (Fig. 3.50B).

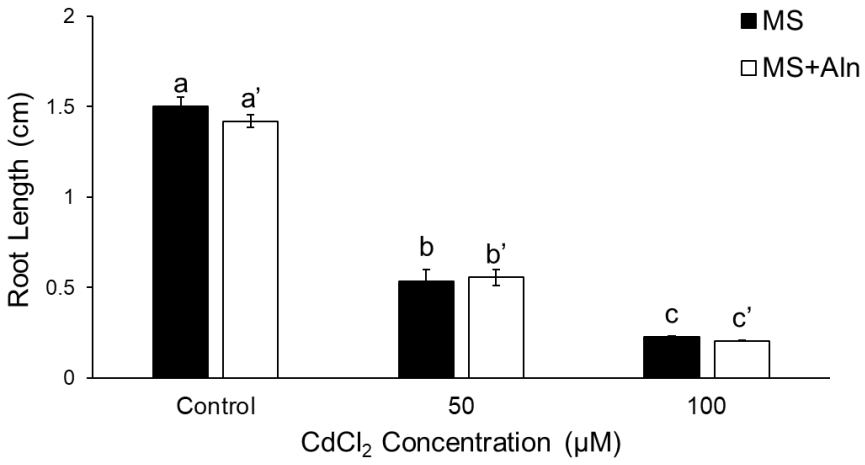

Figure 3.49. Effect of exogenous allantoin on root length in *ALNox* lines in response to Cd treatment. Values shown are the mean of three independent replicates ± SE. Different letters (a,b,c for MS and a',b',c' for MS+Aln) show significant differences in each group ($P \leq 0.05$).

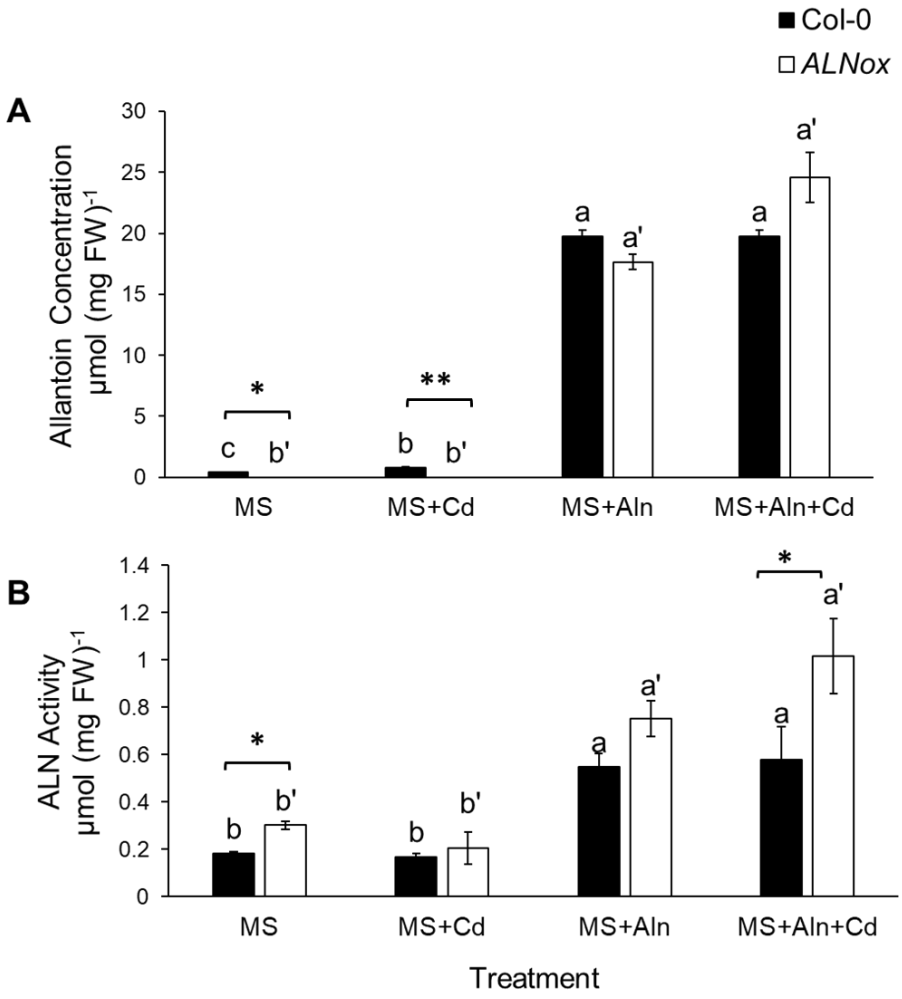

Figure 3.50. Effect of exogenous allantoin on (A) allantoin content and (B) allantoinase activity in Col-0 and *ALNox* lines at Cd treatment. Values shown are the mean of three independent replicates ± SE. Different letters (a,b,c for Col-0 and a',b',c' for *ALNox* samples) show significant differences in each group ($P \leq 0.05$). Asterisks indicate significant differences obtained by a t-test (*$P \leq 0.05$, **$P \leq 0.01$).

3.7. Evaluation of *abi* mutants in response to cadmium and allantoin treatment

Plant growth and seed germination assessment of *aln-3*, *ALNox* and Col-0 Arabidopsis exposed to Cd treatment along with exogenous application of allantoin revealed that allantoin at high concentrations is associated with Cd-tolerance of Arabidopsis seedlings. Additionally, involvement of allantoin in enhanced ABA production and accumulation in allantoinase-negative mutants reported by Watanabe *et al*. (2014) raised this question whether there is a link between allantoin and ABA signalling pathway in Arabidopsis. To determine whether allantoin functions through an ABA-dependent or -independent mechanism, ABA-insensitive (*abi*) mutants were recruited. Exposing these mutants to allantoin and Cd and monitoring seed germination, seedling growth and antioxidant gene expression would be informative in this regard. Assuming that regulatory function of allantoin is ABA-dependent, allantoin should not be able to play a protective role and enhance plant growth in *abi* mutants exposed to stress. In other words, if the function of allantoin is ABA-dependent *abi* mutants are will not be able to employ allantoin to abolish Cd toxicity.

3.7.1. Exogenous allantoin increases seed germination, while does not improve root length in Cd-treated *abi* mutants.

Three *abi* mutants along with their wild-type backgrounds were grown on MS plates and the effect of 10 mM allantoin and 100 µM Cd on their seed germination and root length was evaluated after two weeks and compared with their corresponding wild-type. 100 µM $CdCl_2$ (MS+Cd) caused a slight decrease in the seed germination of Col-0, Ler-0 and three *abi* mutants when compared with control samples (MS) (Fig. 3.51). However, this Cd-induced decline in seed germination was only statistically significant for *abi1*. Exogenous allantoin (MS+Aln) did not have a significant effect on seed germination in any of the genotypes, while combination effect of allantoin and Cd (MS+Aln+Cd) increased seed germination of *abi* mutants in comparison with MS+Cd-grown samples. This observation was consistent with the germination of Col-0 and Ler-0 seeds at MS+Aln+Cd treatment. Root length measurements indicated that in the absence of Cd (MS and MS+Aln treatments) there was not a considerable change in the root length of *abi* mutants which is consistent with results obtained from wild-type controls (Ler-0 and Col-0) (Fig 3.52). Exposure to Cd (MS+Cd treatment) lower root length in all tested genotypes. The combined effect of allantoin and Cd (MS+Aln+Cd treatment) increased the root length in Col-0 and Ler-0 samples

when it is compared with their MS+Cd root length. However, the difference between MS+Cd- and MS+Aln+Cd-grown seedlings were not statistically significant in *abi* mutants.

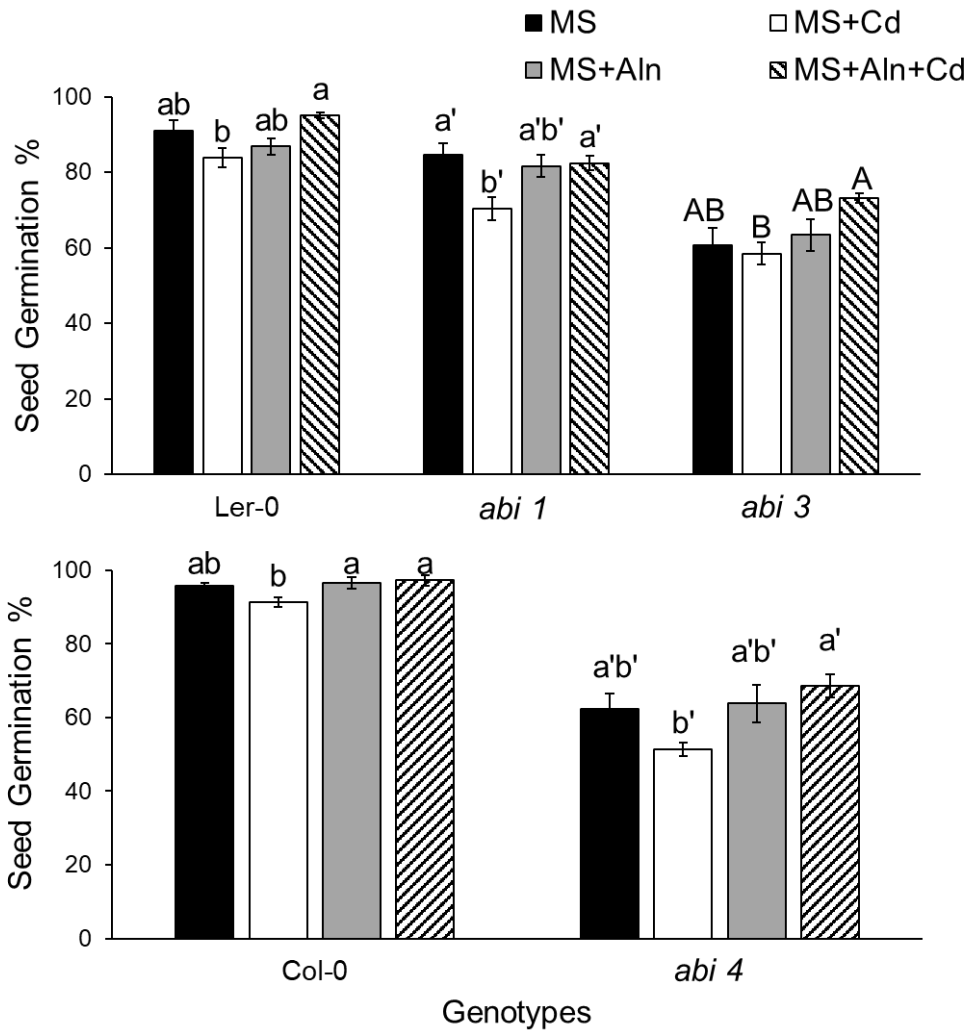

Figure 3.51. Effect of Cd and allantoin on the seed germination of *abi* mutants (*abi1*, *abi3* and *abi4*) and wild-type Arabidopsis (Ler-0 and Col-0). Values shown are the mean of five independent replicates ± SE. Different letters show significant differences in each group ($P \leq 0.05$). In the top graph a,b,c for Ler-0; a',b',c' for *abi1* and A,B,C for *abi3* shows the differences. In the bottom graph a,b,c for Col-0; a',b',c' for *abi4* shows the differences.

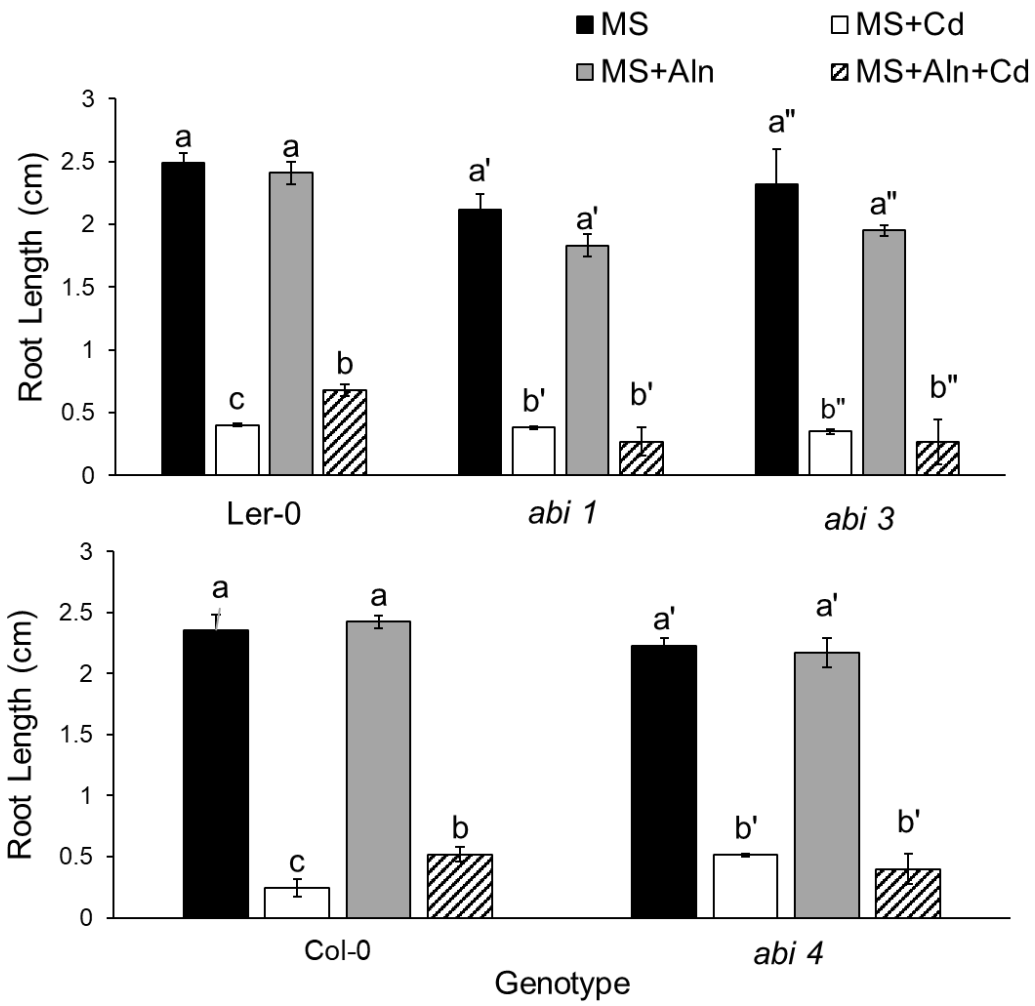

Figure 3.52. Effect of Cd and allantoin on the root length of *abi* mutants (*abi1*, *abi3* and *abi4*) and wild-type Arabidopsis (Ler-0 and Col-0). Values shown are the mean of three independent replicates ± SE. Different letters show significant differences in each group ($P \leq 0.05$). In the top graph a,b,c for Ler-0; a',b',c' for *abi1* and A,B,C for *abi3* shows the differences. In the bottom graph a,b,c for Col-0; a',b',c' for *abi4* shows the differences.

3.7.2. Transcription of antioxidant genes show differential responses to exogenous allantoin and Cd treatment in *abi* mutants

To reveal the differences between *abi* mutants and wild-type samples in response to allantoin and Cd treatment and also to evaluate the effect of these treatments on antioxidant systems of these genotypes, transcript level of six antioxidant enzymes were quantified in each genotype to see the effect of treatment and also compared with that of Col-0 or Ler-0 seedlings.

mRNA abundance of *APX1* increased significantly in Ler-0 and Col-0 in response to allantoin treatment (MS+Aln) (Fig. 3.53). Cd treatment did not influence *APX1* transcription and no significant difference was observed between MS+Cd and MS+Aln+Cd treatments in Col-0 and Ler-0 samples. As well, in *abi1* and *abi4* allantoin did not change *APX1* expression, while this gene is downregulated in *abi3* at the same condition. Cd exposure did not affect the transcript level of *APX1* in *abi1* and *abi3*, but decreased this value in *abi4*. Except *abi* exhibiting a dramatic increase in *APX1* transcription at MS+Aln+Cd treatment, neither *abi3* and *abi4* showed a considerable difference between *APX1* expressions obtained from MS+Cd and MS+Aln+Cd.

In agreement with *APX1* in wild-type seedlings (Col-0 and Ler-0), transcript level of *APX2* show a noticeable rise in response to allantoin (MS+Aln) and no significant change was observed between MS+Cd- and MS+Aln+Cd-treated Col-0 and Ler-0 plants (Fig. 3.54). Allantoin and Cd exposure (MS+Aln and MS+Cd) did not change *APX2* expression in *abi1* and *abi3* mutants. In *abi4*, *APX2* was not influenced by allantoin, while decreased following Cd treatment. *abi3* and *abi4* showed no change between MS+Cd and MS+Aln+Cd treatments, but *APX2* transcription of *abi1* exhibited a considerable raise at MS+Aln+Cd.

MSD1 expression increased in MS+Aln-treated Col-0 and Ler-0. MS+Cd and MS+Aln+Cd did not affect this gene in Ler-0 but caused a significant induction and reduction, respectively, in the transcription of *MSD1* in Col-0 (Fig. 3.55). *MSD1* transcription did not change in *abi4* in response to these treatments, while in *abi1* the only effective treatment was MS+Aln+Cd that induce *MSD1* expression. *MSD1* expression of *abi3* decreased significantly at MS+Aln treatment and a noticeable difference was observed between MS+Cd and MS+Aln+Cd with lower value at MS+Aln+Cd treatment.

In Ler-0, *CSD1* was not influenced by allantoin treatment, decreased at Cd treatment and no difference was noticed between MS+Cd and MS+Aln+Cd treatments (Fig. 3.56). In contrast, transcription of *CSD1* decreased at MS+Aln and MS+Cd and similar to Ler-0, no difference was observed between MS+Cd and MS+Aln+Cd. In *abi1*, both allantoin (MS+Aln) and Cd (MS+Cd) decreased *CSD1* expression, while MS+Aln+Cd increased its expression when compared with MS+Cd. *CSD1* transcription of *abi3* and *abi4* was not influenced by allantoin exposure, remained unchanged in MS+Cd-treated *abi4* while decreased in MS+Cd-treated *abi3*. In both *abi3* and *abi4* a significant difference was recognized between MS+Cd and MS+Aln+Cd treatment, showing increased value at MS+Aln+Cd.

Allantoin treatment increased mRNA abundance of *FSD1* in Ler-0, whereas did not have any impact in that of Col-0 (Fig. 3.57). *FSD1* expression remained unchanged in response to Cd treatment in Ler-0 and Col-0 while a significant rise was detected in both plant at MS+Aln+Cd when compared with their MS+Cd treatment. MS+Aln did not change *FSD1* expression in *abi1* and *abi4* and increased that of *abi3*. MS+Cd treatment decreased this value in abi1, while did not have any impact on *FSD1* transcript level of *abi3* and *abi4*. The difference between MS+Cd and MS+Aln+Cd was not noticeable in *abi3* and *abi4*, while MS+Aln+Cd induced *FSD1* in *abi1* when compared with its MS+Cd data.

Neither Ler-0 and Col-0 were affected by allantoin exposure for *CAT3* transcription (Fig. 3.58). This gene remained unaffected in MS+Cd-exposed Ler-0 while increased in Col-0 under the same condition. Although, no significant difference was observed between MS+Cd and MS+Aln+Cd in Col-0 plants, MS+Aln+Cd slightly increased *CAT3* expression in Ler-0. Transcription of *CAT3* decreased dramatically in *abi1*, did not change in *abi3* and enhanced significantly following allantoin exposure. Cd treatment downregulated this gene in *abi1* and *abi3*, while did not change that of *abi4*. MS+Aln+Cd induced *CAT3* expression in *abi1* and *abi3* in comparison with their MS+Cd measurements but did not have any impact in *abi4*. Table 3.1 summarizes different responses of antioxidant genes to applied treatments in *abi* mutants and wild-type samples.

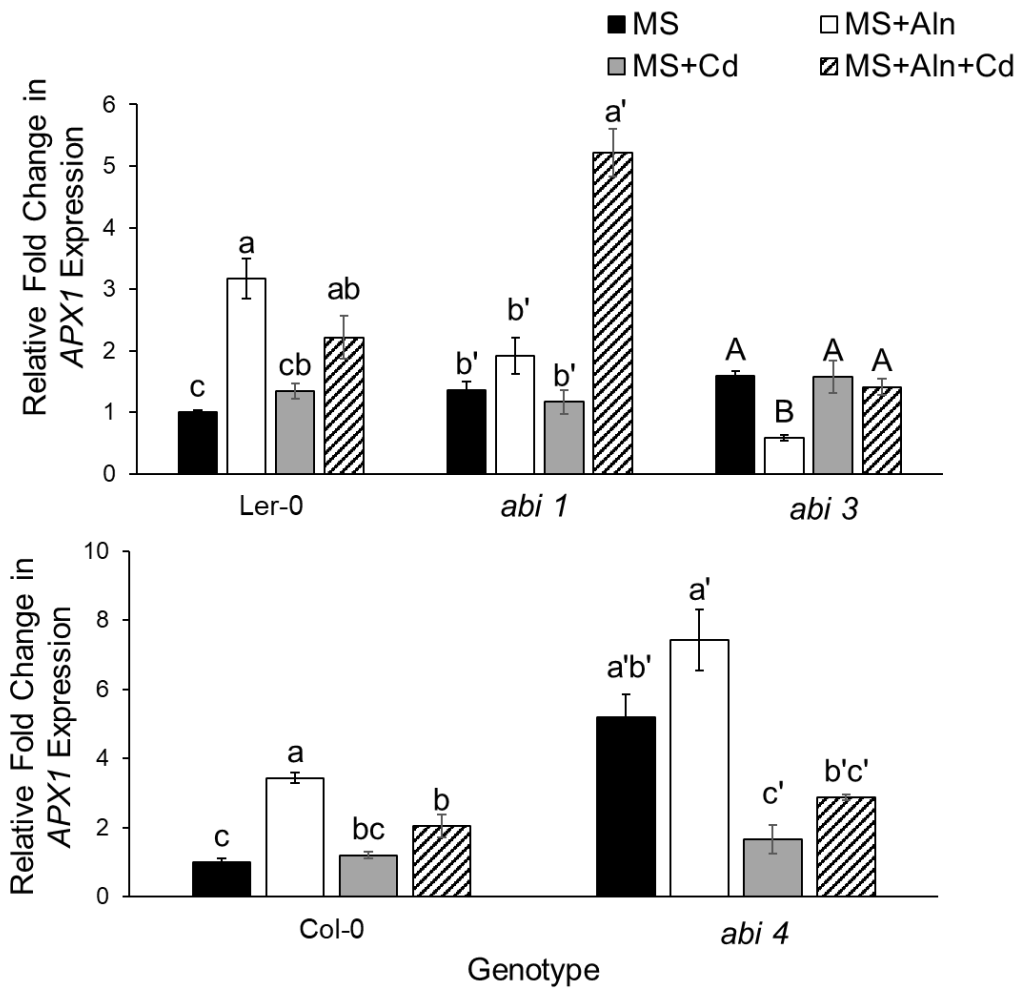

Figure 3.53. qRT-PCR analysis of antioxidant gene *APX1* in *abi* mutants and wild-type Arabidopsis seedlings in response to 10 mM allantoin (MS+Aln), 100 µM $CdCl_2$ (MS+Cd) and the combination of allantoin and cadmium (MS+Aln+Cd). Values shown are the mean of three independent replicates ± SE. Different letters show significant differences in each genotype ($P \leq 0.05$). In the top graph a,b,c for Ler-0; a',b',c' for *abi1* and A,B,C for *abi3* shows the differences. In the bottom graph a,b,c for Col-0; a',b',c' for *abi4* shows the differences. *APX1* expressions was normalized to *ACT2* expression as an internal control and also normalized to their MS-treated corresponding wild-type. *APX1*, ascorbate peroxidase1.

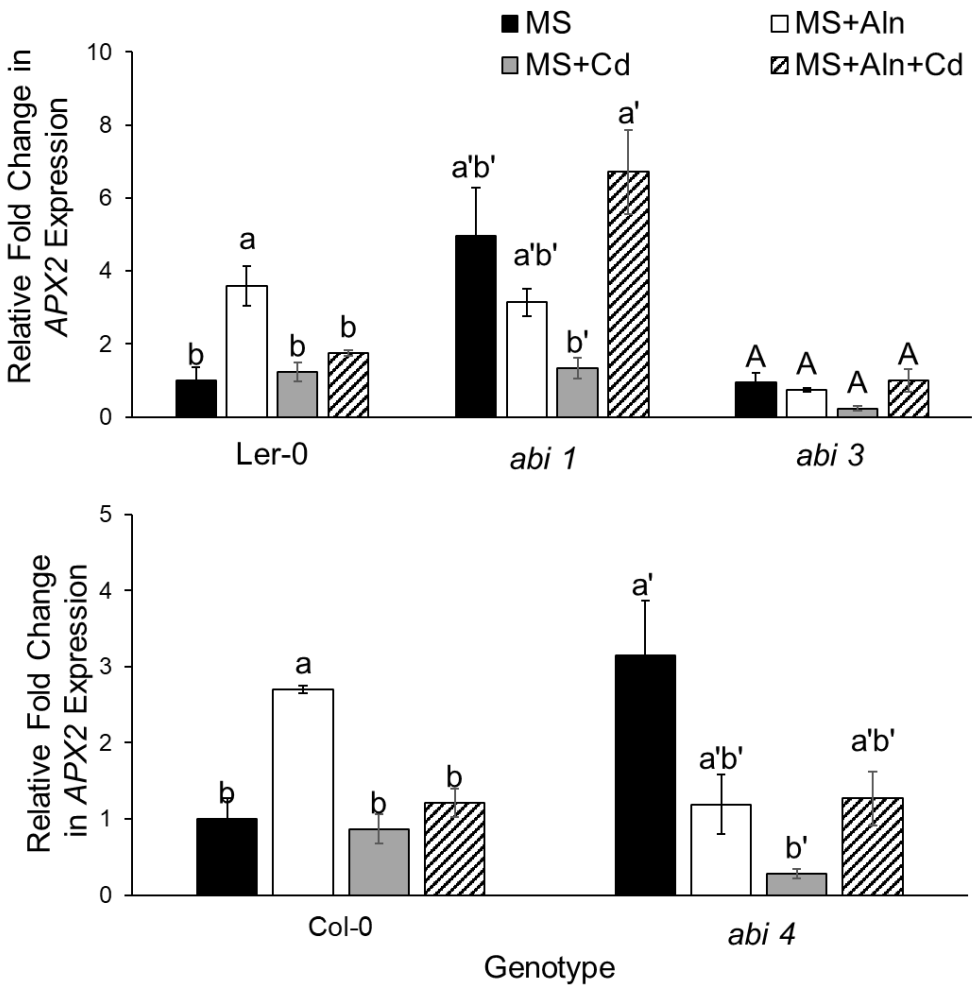

Figure 3.54. qRT-PCR analysis of antioxidant gene *APX2* in *abi* mutants and wild-type Arabidopsis seedlings in response to 10 mM allantoin (MS+Aln), 100 µM $CdCl_2$ (MS+Cd) and the combination of allantoin and cadmium (MS+Aln+Cd). Values shown are the mean of three independent replicates ± SE. Different letters show significant differences in each genotype ($P \leq 0.05$). In the top graph a,b,c for Ler-0; a',b',c' for *abi1* and A,B,C for *abi3* shows the differences. In the bottom graph a,b,c for Col-0; a',b',c' for *abi4* shows the differences. *APX2* expressions was normalized to *ACT2* expression as an internal control and also normalized to their MS-treated corresponding wild-type. *APX2*, ascorbate peroxidase2.

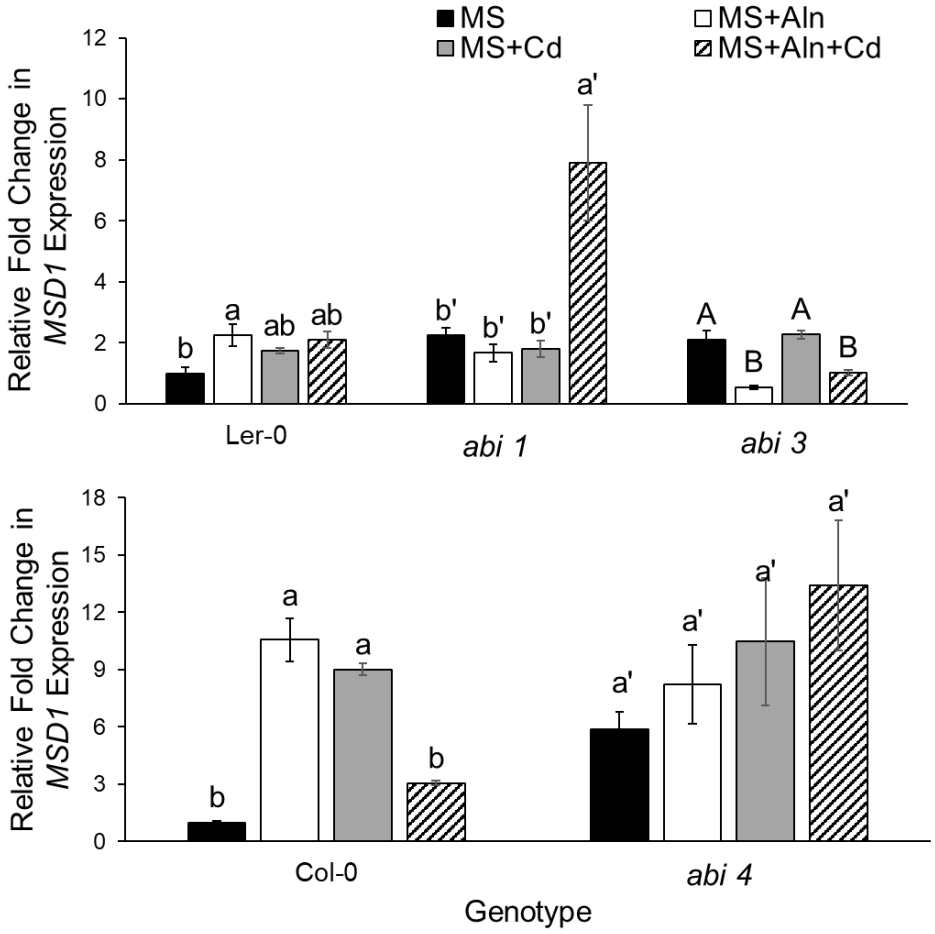

Figure 3.55. qRT-PCR analysis of antioxidant gene *MSD1* in *abi* mutants and wild-type Arabidopsis seedlings in response to 10 mM allantoin (MS+Aln), 100 µM $CdCl_2$ (MS+Cd) and the combination of allantoin and cadmium (MS+Aln+Cd). Values shown are the mean of three independent replicates ± SE. Different letters show significant differences in each genotype ($P \leq 0.05$). In the top graph a,b,c for Ler-0; a',b',c' for *abi1* and A,B,C for *abi3* shows the differences. In the bottom graph a,b,c for Col-0; a',b',c' for *abi4* shows the differences. *MSD1* expressions was normalized to *ACT2* expression as an internal control and also normalized to their MS-treated corresponding wild-type. *MSD1,* Mn-Superoxide dismutase1.

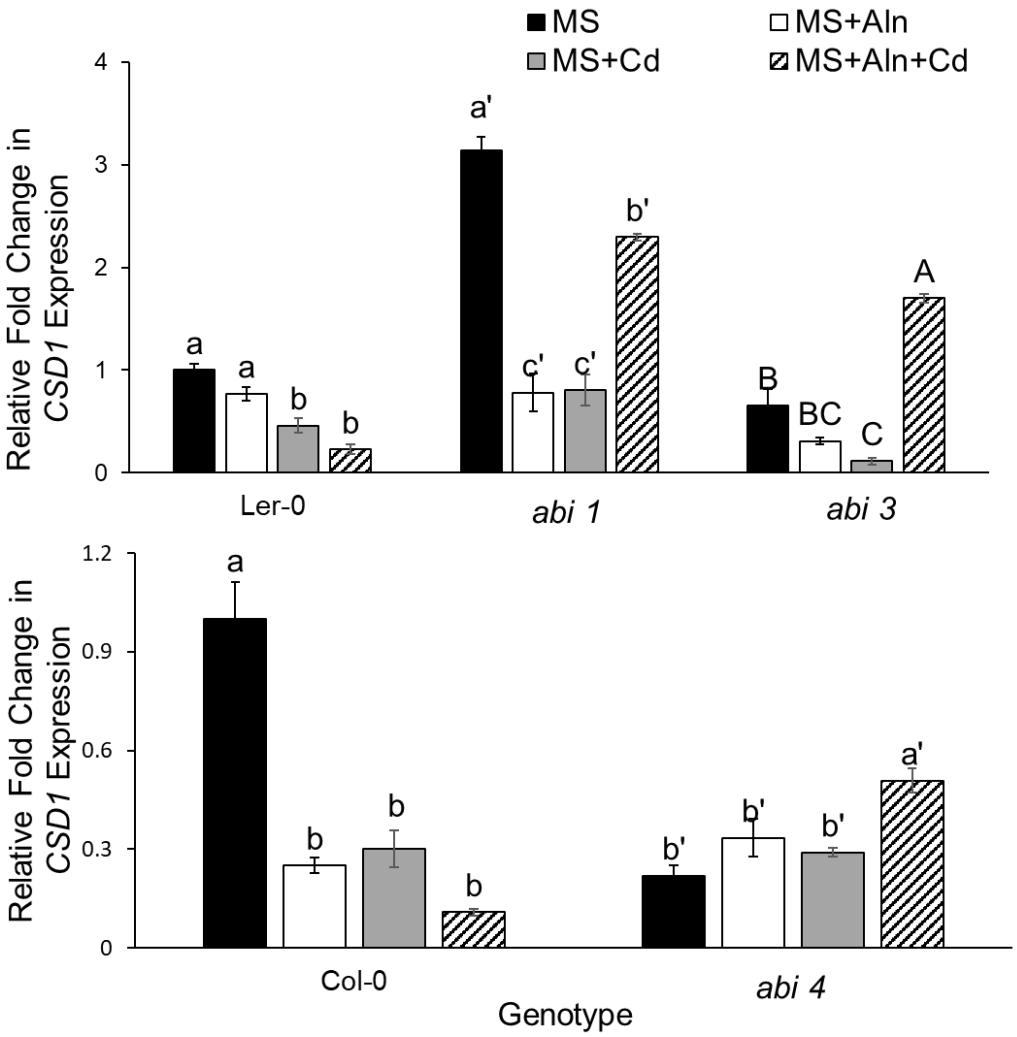

Figure 3.56. qRT-PCR analysis of antioxidant gene *CSD1* in *abi* mutants and wild-type Arabidopsis seedlings in response to 10 mM allantoin (MS+Aln), 100 µM $CdCl_2$ (MS+Cd) and the combination of allantoin and cadmium (MS+Aln+Cd). Values shown are the mean of three independent replicates ± SE. Different letters show significant differences in each genotype ($P \leq 0.05$). In the top graph a,b,c for Ler-0; a',b',c' for *abi1* and A,B,C for *abi3* shows the differences. In the bottom graph a,b,c for Col-0; a',b',c' for *abi4* shows the differences. *CSD1* expressions was normalized to *ACT2* expression as an internal control and also normalized to their MS-treated corresponding wild-type. *CSD1*, Cu-Superoxide dismutase1.

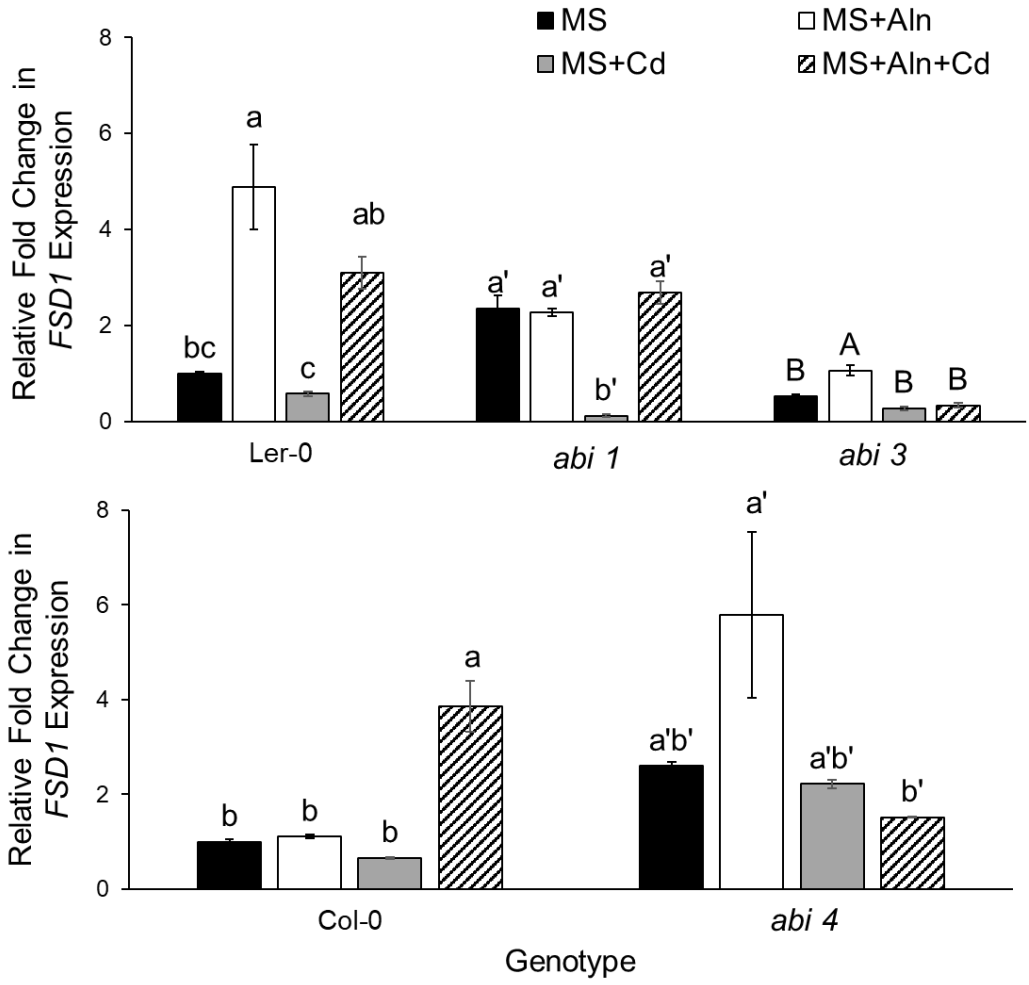

Figure 3.57. qRT-PCR analysis of antioxidant gene *FSD1* in *abi* mutants and wild-type Arabidopsis seedlings in response to 10 mM allantoin (MS+Aln), 100 µM $CdCl_2$ (MS+Cd) and the combination of allantoin and cadmium (MS+Aln+Cd). Values shown are the mean of three independent replicates ± SE. Different letters show significant differences in each genotype ($P \leq 0.05$). In the top graph a,b,c for Ler-0; a',b',c' for *abi1* and A,B,C for *abi3* shows the differences. In the bottom graph a,b,c for Col-0; a',b',c' for *abi4* shows the differences. *FSD1* expressions was normalized to *ACT2* expression as an internal control and also normalized to their MS-treated corresponding wild-type. *FSD1*, Fe-Superoxide dismutase1.

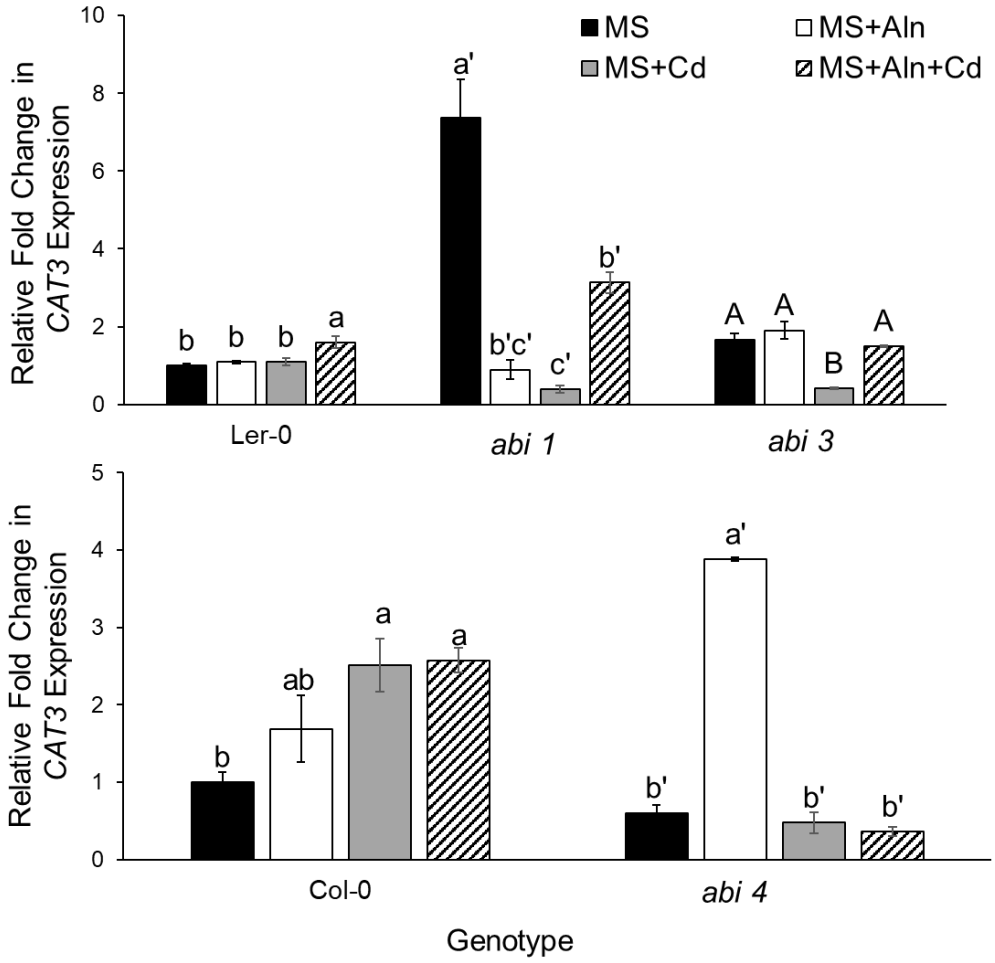

Figure 3.58. qRT-PCR analysis of antioxidant gene *CAT3* in *abi* mutants and wild-type Arabidopsis seedlings in response to 10 mM allantoin (MS+Aln), 100 µM $CdCl_2$ (MS+Cd) and the combination of allantoin and cadmium (MS+Aln+Cd). Values shown are the mean of three independent replicates ± SE. Different letters show significant differences in each genotype ($P \leq 0.05$). In the top graph a,b,c for Ler-0; a',b',c' for *abi1* and A,B,C for *abi3* shows the differences. In the bottom graph a,b,c for Col-0; a',b',c' for *abi4* shows the differences. *CAT3* expressions was normalized to *ACT2* expression as an internal control and also normalized to their MS-treated corresponding wild-type. *CAT3*, Catalase3.

Table 3.1. Antioxidant gene expression in *abi* mutants and wild-type Arabidopsis in response to MS, MS+10 mM allantoin (MS+Aln), MS+100 µM $CdCl_2$ (MS+Cd) and MS+10 mM allantoin+100 µM $CdCl_2$ (MS+Aln+Cd). ↑ shows increased transcription, ↓ indicates decreased transcription and ─ means no significant change was observed. *APX1*, ascorbate peroxidase1; *APX2*, ascorbate proxidase2; *CAT3*, Catalase3; *CSD1*, Cu-Superoxide dismutase1; *FSD1*, Fe-Superoxide dismutase1; *MSD1*, Mn-Superoxide dismutase1.

Antioxidant gene	Genotype	MS – MS+Aln	MS – MS+Cd	MS+Cd – MS+Aln+Cd
APX1	Ler-0	↑	─	─
	Col-0	↑	─	─
	abi1	─	─	↑
	abi3	↓	─	─
	abi4	─	↓	─
APX2	Ler-0	↑	─	─
	Col-0	↑	─	─
	abi1	─	─	↑
	abi3	─	─	─
	abi4	─	↓	─
MSD1	Ler-0	↑	─	─
	Col-0	↑	↑	↓
	abi1	─	─	↑
	abi3	↓	─	↓
	abi4	─	─	─
CSD1	Ler-0	─	↓	─
	Col-0	↓	↓	─
	abi1	↓	↓	↑
	abi3	─	↓	↑
	abi4	─	─	↑
FSD1	Ler-0	↑	─	↑
	Col-0	─	─	↑
	abi1	─	↓	↑
	abi3	↑	─	─
	abi4	─	─	─
CAT3	Ler-0	─	─	↑
	Col-0	─	↑	─
	abi1	↓	↓	↑
	abi3	─	↓	↑
	abi4	↑	─	─

3.8. Effect of lithium and nickel treatment on ureides in Col-0, *aln-3* and *aah* Arabidopsis

Effect of lithium (Li) and nickel (Ni) treatments was also evaluated on seed germination, seedling growth and ureide content of two mutants, *aln-3* and *aah*. The first Li experiment on Col-0 plants showed that Col-0 Arabidopsis accumulated allantoate in response to Li treatment. This finding suggested the idea that *aah* mutants, with constitutive allantoate accumulation, might be a potential stress-tolerant genotype in response to metal treatments.

3.8.1. *aln-3* and *aah* mutants are more resistant to lithium.

Col-0 samples, *aln-3* and *aah* mutants were exposed to 10 and 15 mM LiCl for two weeks and their seed germination and root length were measured under this condition. As shown in Fig. 3.59 LiCl at high concentration (15 mM) decreased seedling growth at all three genotypes, however their seed germination and root length measurement showed that *aln-3* and *aah* mutants are comparatively more resistant to Li than Col-0 seedlings (Figs. 3.60A,B). Seed germination of Col-0 Arabidopsis showed a considerable decrease at 15 mM LiCl, whereas *aah* mutant seeds were not influenced significantly by Li treatment. Li decreased the germination of *aln-3* mutants at 10 and 15 mM LiCl, however these values are considerably higher than that of Col-0 seeds with a significant difference at 15 mM LiCl. Root length of Col-0 Arabidopsis decreased noticeably at 10 mM LiCl, while this Li concentration did not change root growth of *aln-3* and *aah* samples. 15 mM LiCl caused a significant decrease in the root length of all three genotypes, however mutants exhibited a higher root length with a significant difference between each mutant and Col-0 samples.

3.8.2. Lithium induces allantoin and allantoate accumulation in Col-0 seedlings.

Quantification of ureides by HPLC demonstrated and increasing amount of allantoin in response to Li treatment in three Arabidopsis genotypes (Fig. 3.61). However, this Li-derived allantoin accumulation is only significant for Col-0 and *aah* mutants with highest allantoin content at 15 mM LiCl. In addition to allantoin, allantoate was also accumulated in Col-0 Arabidopsis with the highest value at 10 mM LiCl (Fig. 3.62). Allantoate content of *aah* mutants remained unchanged following Li treatment and *aln-3* mutants did not show a detectable amount of allantoate at this condition.

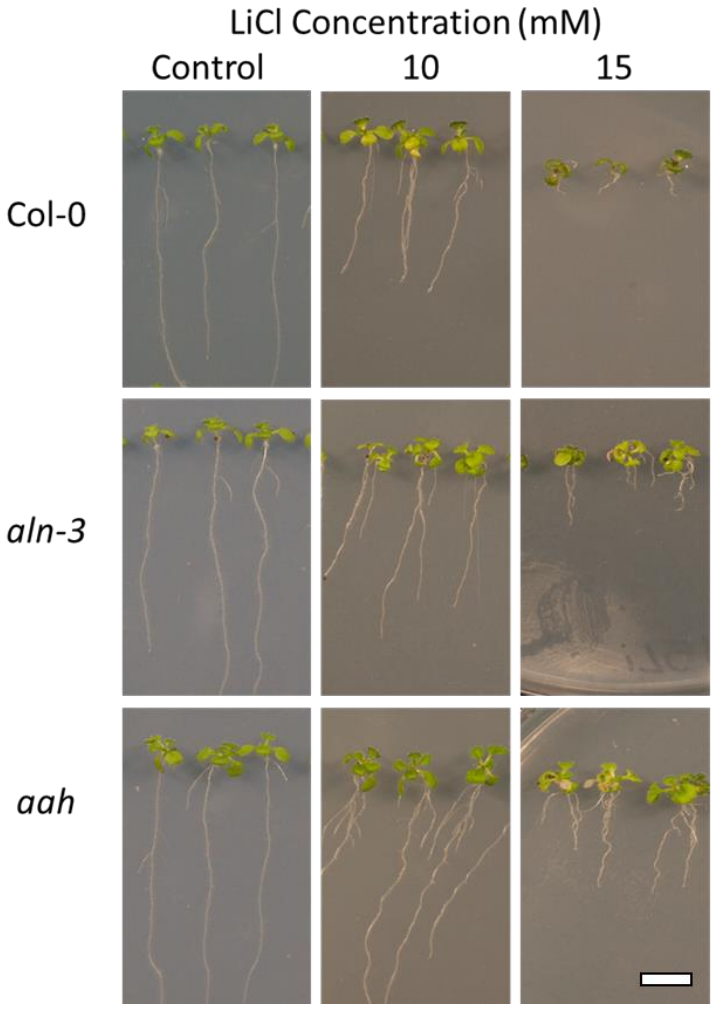

Figure 3.59. Effect of Li treatment on seedling growth of Col-0, *aln-3* and *aah* mutants. Picture is representative of three independent experiments. Scale bar = 1cm.

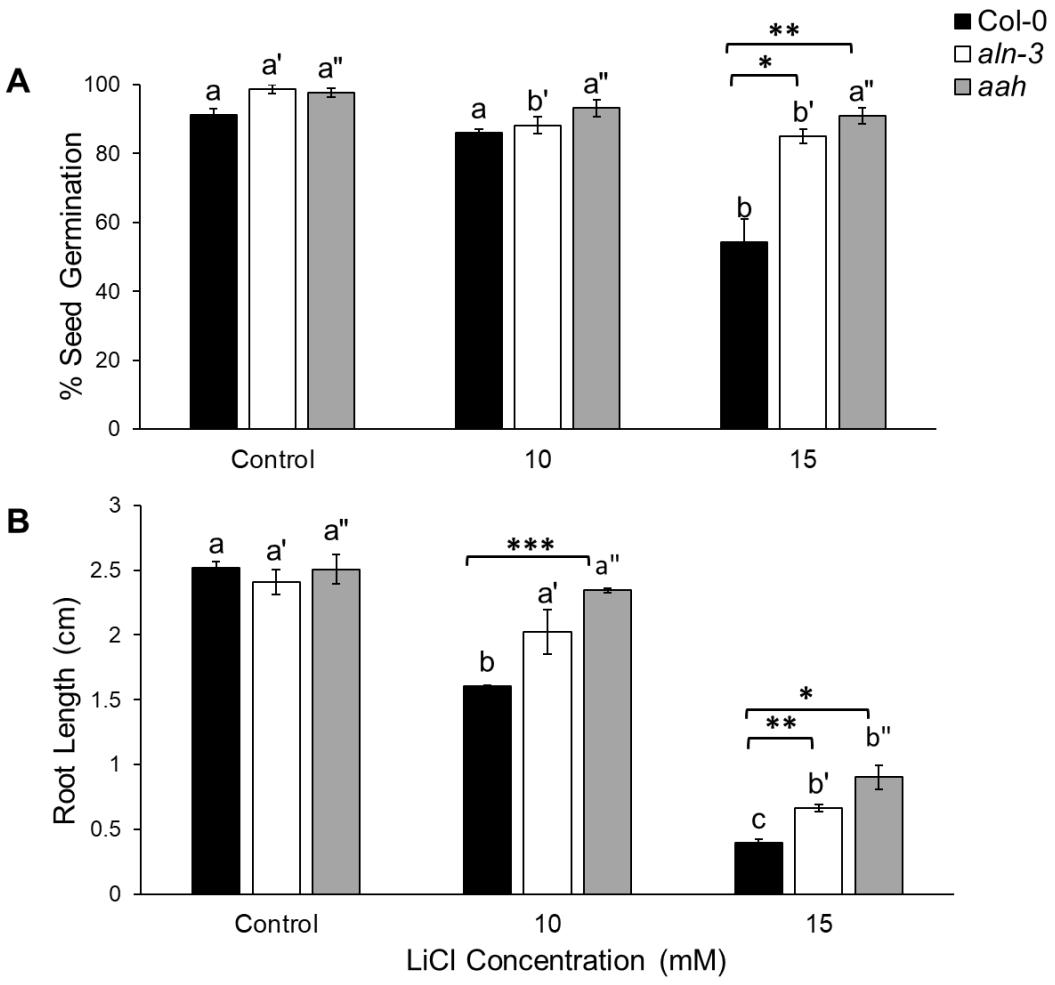

Figure 3.60. Effect of Li treatment on (A) seed germination and (B) root length of Col-0, *aln-3* and *aah* mutants. Values shown are the mean of three independent replicates ± SE. Different letters (a,b,c for Col-0; a',b',c' for *aln-3* and a",b",c" in *aah* samples) show significant differences in each group ($P \leq 0.05$). Asterisks indicate significant differences obtained by a t-test (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$).

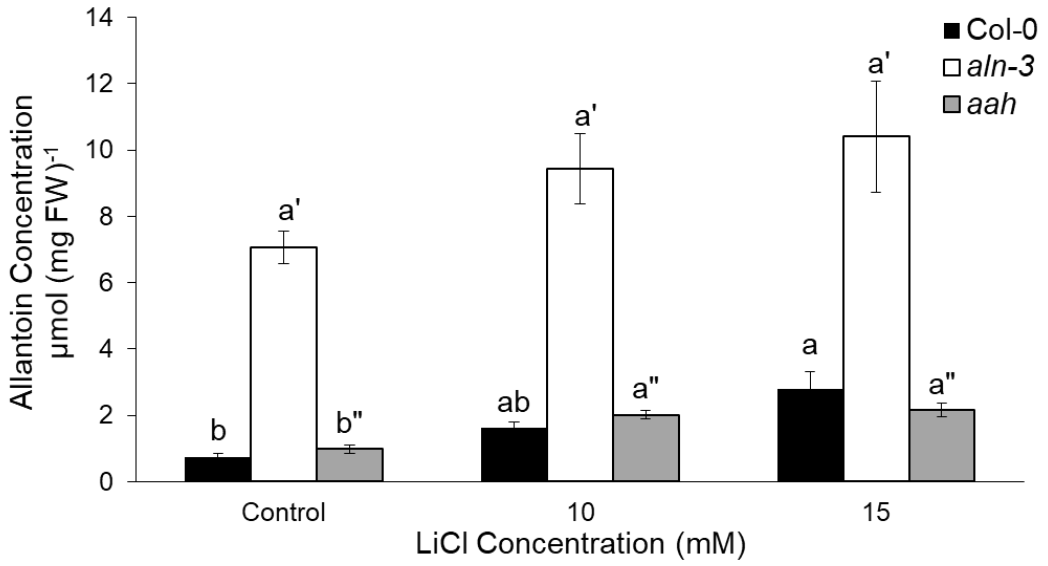

Figure 3.61. Effect of Li treatment on allantoin accumulation of Col-0, *aln-3* and *aah* Arabidopsis. Values shown are the mean of four independent replicates ± SE. Different letters (a,b,c for Col-0; a',b',c' for *aln-3* and a",b",c" for *aah* samples) show significant differences in each group ($P \leq 0.05$).

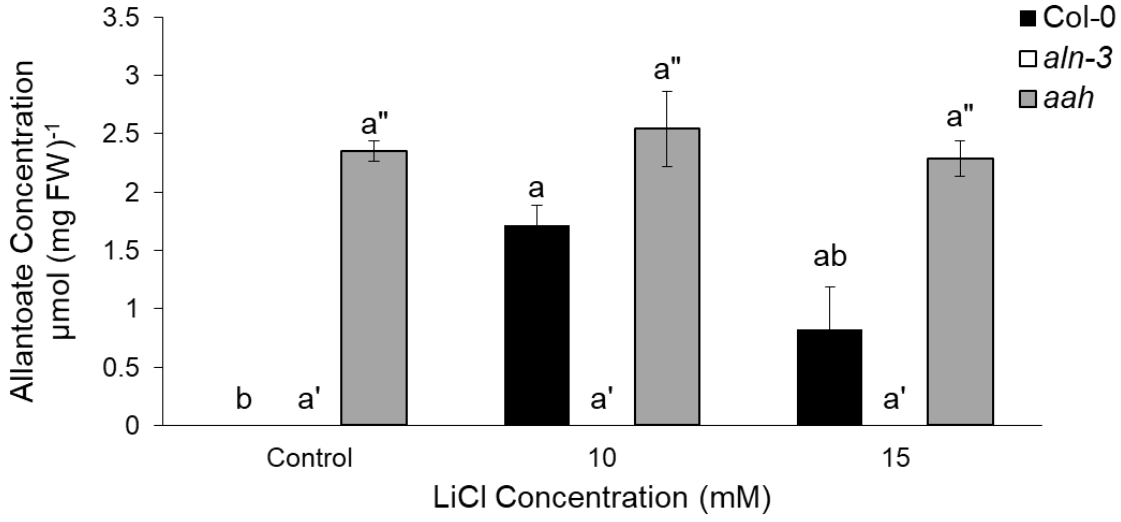

Figure 3.62. Effect of Li treatment on allantoate accumulation of Col-0, *aln-3* and *aah* Arabidopsis. Values shown are the mean of four independent replicates ± SE. Different letters

(a,b,c for Col-0; a',b',c' for *aln-3* and a",b",c" for *aah* samples) show significant differences in each group ($P \leq 0.05$).

3.8.3. *aah* and *aln-3* mutants show more nickel tolerance.

Growing Col-0, *aln-3* and *aah* mutants on 50 and 100 µM $NiCl_2$ plates showed that Ni treatment, specifically at higher concentration, decreased plant growth in three tested genotypes (Fig. 3.63). As shown in Fig. 3.64A 50 and 100 µM $NiCl_2$ significantly decreased seed germination in Col-0 Arabidopsis, however these two Ni concentrations did not have a significant impact on *aln-3* seed germination. Seed germination of *aah* mutants remained unchanged at 50 µM $NiCl_2$ treatment, while went down at 100 µM $NiCl_2$. Comparison among three tested mutants revealed that there is a significant difference between seed germination of Col-0 and two mutants, with higher values for *aln-3* and *aah* seeds especially at 100 µM $NiCl_2$. Consistently, root length measurement demonstrated that although Ni decreased root growth, *aln-3* and *aah* mutants exhibited a better growth and higher root length following Ni treatment compared with Col-0 samples exposed to the same treatment (Fig. 3.64B).

3.8.4. Nickel increases allantoin accumulation in Col-0 and *aah* Arabidopsis.

Ureides measurement in Ni-treated Col-0, *aln-3* and *aah* Arabidopsis showed that 100 µM $NiCl_2$ significantly induced allantoin accumulation in Col-0 and *aah* seedlings (Fig. 3.65). Allantoin content of *aln-3* mutants exhibited a slight decrease at 50 µM $NiCl_2$, followed by an increase in response to 100 µM $NiCl_2$ treatment. However, this Ni-derived fluctuation in the allantoin concentration of *aln-3* mutants was not statistically significant in respect with control treatment.

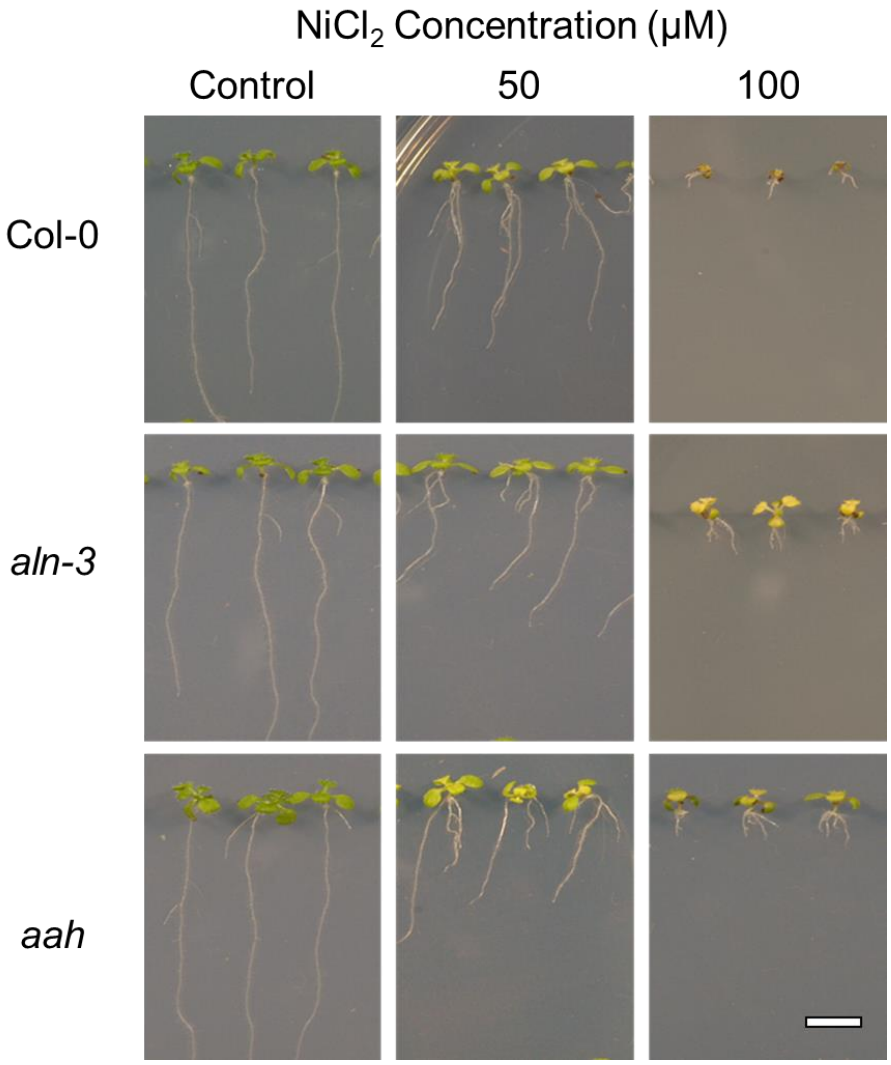

Figure 3.63. Effect of Ni treatment on seedling growth of Col-0, *aln-3* and *aah* mutants. Picture is representative of three independent experiments. Scale bar = 1cm.

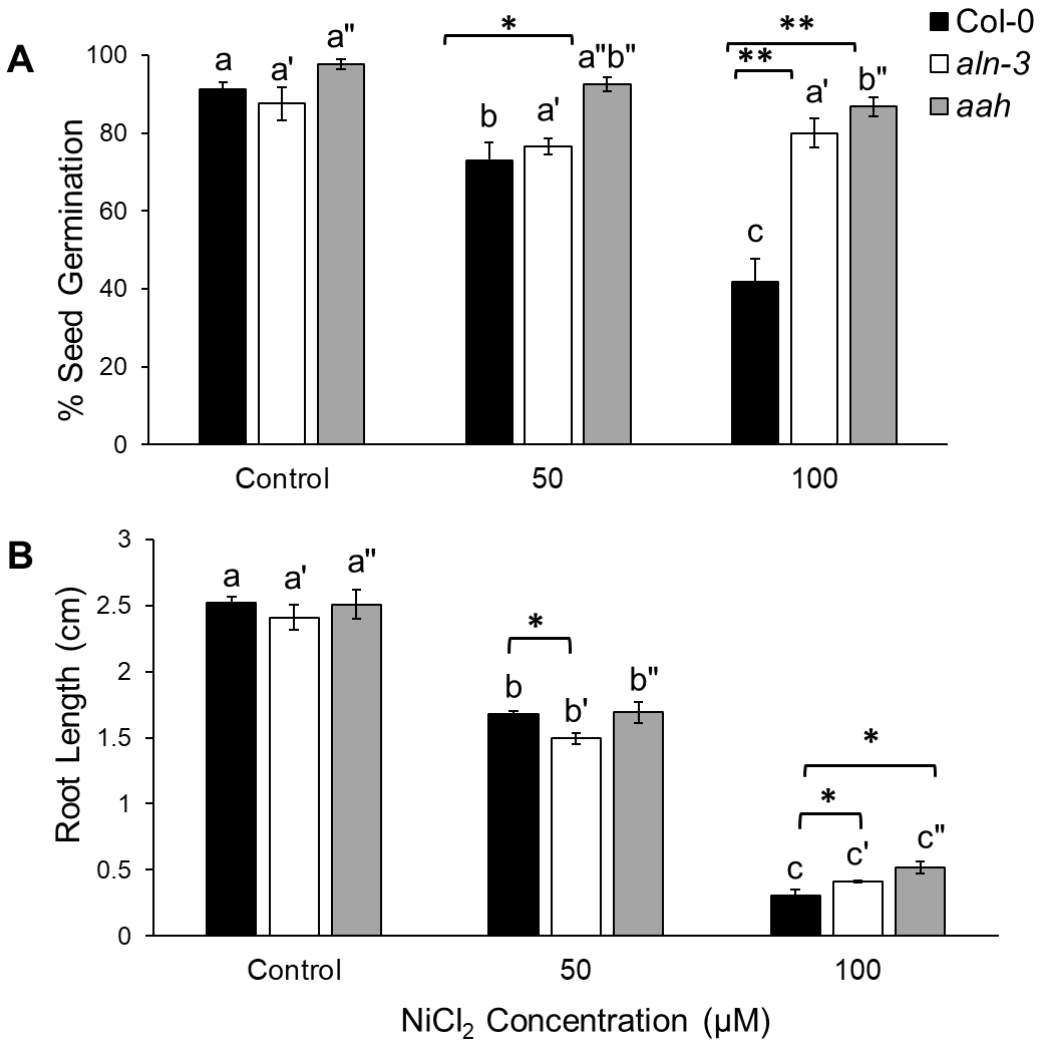

Figure 3.64. Effect of Ni treatment on (A) seed germination and (B) root length of Col-0, *aln-3* and *aah* mutants. Values shown are the mean of three independent replicates ± SE. Different letters (a,b,c for Col-0; a',b',c' for *aln-3* and a",b",c" for *aah* samples) show significant differences in each group ($P \leq 0.05$). Asterisks indicate significant differences obtained by a t-test (*$P \leq 0.05$, **$P \leq 0.01$).

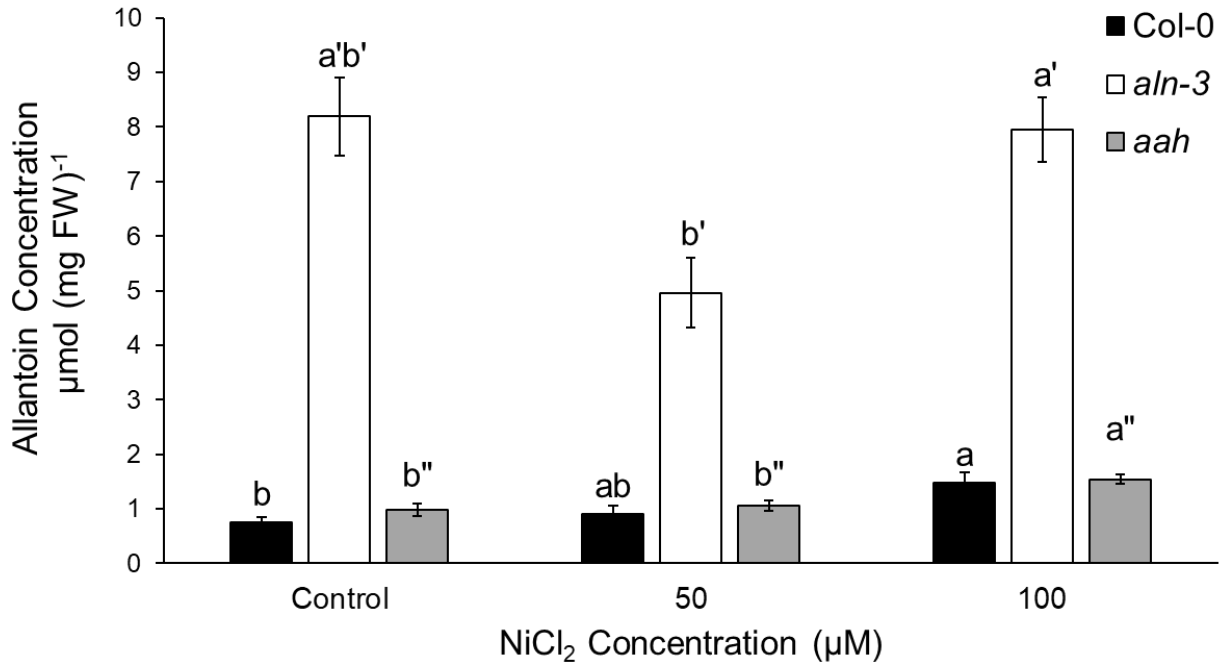

Figure 3.65. Effect of Ni treatment on allantoin accumulation of Col-0, *aln-3* and *aah* Arabidopsis. Values shown are the mean of four independent replicates ± SE. Different letters (a,b,c for Col-0; a',b',c' for *aln-3* and a",b",c" for *aah* samples) show significant differences in each group ($P \leq 0.05$).

4. Discussion

The primary goal of this project was to study ureide pathway and ureides accumulation in response to abiotic stresses with the purpose of clarifying how ureide metabolism and their intermediates are associated with stress response and tolerance in plants. In this work, cadmium (Cd) was used as an abiotic stressor that induces reactive oxygen species (ROS) generation and oxidative damage. Although no biological function has been found for Cd in plants, its high mobility in soil and low affinity for soil colloids makes Cd as one of the most toxic metals to plants (Alloway, 1995; Ross, 1978; Joshi *et al*., 2011). Considering Cd-derived ROS production in plants and involvement of ureide metabolism in abiotic stress responses and ROS scavenging suggested that ureides may be responsive to Cd. Therefore, current project relies on my first hypothesis that Cd influences ureide metabolism and causes ureides accumulation. Among ureides, allantoin, has been considered as an important compound accumulating in response to stress. Despite different reports indicating its involvement in stress conditions, the mechanism underlying its function is still unknown. Hence, these experiments were designed to address the following question:

a. Do ureides (specifically allantoin) accumulates in response to Cd treatment?
b. Is there a difference between growth of *aln* mutants and wild-type plants upon Cd exposure?
c. Does increased allantoin level in *aln* mutants influence their ROS content in response to Cd?
d. How does allantoin affect antioxidant enzymes in *aln* Arabidopsis?
e. What is the difference between root and shoot of wild-type and *aln* Arabidopsis at Cd treatment?
f. Does exogenous application of allantoin protects wild-type seedlings from Cd toxicity?
g. How do *ALN-overexpressed* (*ALNox*) lines with decreased amount of allantoin respond to Cd?

h. Is allantoin function coupled with the ABA signalling pathway?

i. What is the effect of other metals (such as Li and Ni) on ureides metabolism?

To answer these questions ureides overexpressed lines (*ALNox*) and deficient mutants (*aln-3*) as well as Col-0 Arabidopsis (as a wild-type sample) together with allantoin-treated Col-0 samples were exposed to Cd and their growth, ureide concentration, ureides gene expression, ROS content and antioxidant enzymes were tested. The present work contributes to our understanding of ureides engagement in stress responses and proposes a model explaining the mechanism for allantoin function in response to Cd.

4.1. Allantoinase mutation confers Cd-resistance to *aln-3* Arabidopsis seedlings.

Based on a European Union (EU) report, Cd at concentrations 1-3 mg kg^{-1} soil is safe and non-toxic to plants in agricultural soil. However, this limit is relatively lower than that of other metals such as Pb (50-300 mg kg^{-1} soil) and Cr (100-150 mg kg^{-1}), indicating its high toxicity at low concentrations (Commission of the European Communities, 1986; Renella *et al*., 2004). Cd treatment, specifically at higher concentrations, restricts seedling growth and seed germination. Diminished plant growth has been previously shown in response to metal (Pb, Cd, Hg, Cr and As) exposure (DalCorso, 2012). Cd-induced decrease in plant growth is mainly attributed to its chemical properties. Cd is highly soluble in water (135 g $CdCl_2$/100 ml water at 20° C), leading to its rapid movement in soil, fast uptake by roots, and transfer to shoots (DalCorso *et al*., 2010). Due to its similarity to essential cations, such as Mg^{2+}, Ca^{2+} and Fe^{2+}, Cd interferes with the absorption of these elements from the soil, causing nutrient deficiency (DalCorso *et al*., 2008). Cd also imposes imbalances on the function and structure of chloroplasts, resulting in chlorosis and restricted plant growth. Photosystems I and II (PS I and II) and light harvesting complexes (LHC) are susceptible to Cd at high concentrations. Additionally, Cd has a negative effect on chlorophyll content via substitution for Mg in chlorophyll generating non-functional molecules, inhibiting 5-aminolevulinic acid (ALA) biosynthesis which is a precursor to chlorophyll and inducing chlorophyll degradation through increasing lipid peroxidation (Somashekaraiah *et al*., 1992; Sanita di Toppi and Gabbrielli, 1999; Liu *et al*., 2014). A more detailed discussion regarding the effect of Cd treatment on photosynthesis can be found in Appendix B. In summary, the inhibitory effect of Cd on plant growth is contributed to its effect on chlorophyll and subsequently photosynthetic efficiency,

CO_2 fixation and C assimilation and interference in nutrient equilibrium in plants (Sanita di Toppi and Gabbrielli, 1999; Perfus-Barbeoch *et al*., 2002; DalCorso *et al*., 2008).

In response to Cd exposure, allantoin accumulates in Col-0 Arabidopsis which is driven by *UO* up-regulation, feeding the pathway with enough substrate, and down-regulation of *ALN*, minimizing allantoin breakdown. At the same time, the concentration of uric acid decreases, likely due to its enhanced conversion to allantoin. Ureides accumulation is also reported in *Thellungiella salsuginea* in response to high light irradiance (750 μmol photon m^{-2} s^{-1}) (Malik *et al*., 2016). Dark treatment increases allantoin and allantoate accumulation in wild-type Arabidopsis through upregulation of *XDH1* gene expression and enzyme activity. Moreover, mutation of *XDH1* in Arabidopsis, compromising allantoin and allantoate production causes early senescence, ROS accumulation and chlorophyll degradation (Brychkova *et al*., 2008). Irani and Todd (2016) showed that drought, salinity (NaCl) and osmotic stress stimulates allantoin accumulation via enhancing the transcript level of *UO* (Irani and Todd, 2016). From these studies, it can be concluded that ureides, specifically allantoin and allantoate, participate in plant stress response while playing an important role in attenuating negative effects of stress.

The role allantoin plays in response to abiotic stresses is likely associated with its protective function and/or regulatory effect in response to stress. Allantoin is suggested to have antioxidant properties quenching free radicals, decreasing ROS accumulation and alleviating cell death once administered to wild-type Arabidopsis leaf discs (Brychkova *et al*., 2008). Interestingly, allantoin quantification of different rice cultivars demonstrate that allantoin concentration in rice is related to stress tolerance in this plant. Rice cultivars with higher allantoin content are more resistant to chilling and dehydration stresses. However, exogenous application of allantoin does not reveal an antioxidant activity in rice, implying that protective role of allantoin is likely mediated through a regulatory mechanism (Wang *et al*., 2012). Allantoin is also proposed to function as a signalling molecule regulating plant stress-responsive machinery. Allantoin can stimulate abscisic acid (ABA) and jasmonic acid (JA) production in wild-type Arabidopsis. ABA and JA are the main components of two signalling pathways regulating the expression of stress-responsive genes. Involvement of allantoin in enhancing ABA/JA accumulation signifies an indirect effect of allantoin on stress response and tolerance in plants (Watanabe *et al*., 2014; Takagi *et al*., 2016). To elucidate Cd-derived

allantoin accumulation in Col-0 Arabidopsis, allantoinase-negative (*aln-3*) mutants, containing elevated concentration of allantoin (Irani and Todd, 2016), was employed to study the response of this mutant to Cd and unravel the potential function of allantoin under this condition.

My data indicate that despite decreased plant growth caused by Cd treatment, *aln-3* mutants manage to grow better, reflected in their higher root length and seed germination. Additionally, 50-200 µM $CdCl_2$ treatment induced allantoin accumulation in *aln-3* mutants, consistent with my observation in Col-0 samples. Improved growth and function of allantoinase-negative mutants was previously reported under different abiotic stresses. It has been indicated that *aln* mutants are more tolerant to drought, salinity (NaCl), high light and osmotic stress that is much likely associated with enhanced content of allantoin in these plants (Watanabe *et al*., 2014; Irani and Todd, 2016 and 2017). However, it is still not clear how allantoin participates in stress response and confers stress tolerance to plants. *aln* mutants are shown to be more resistant to abiotic stresses, demonstrated by their higher plant growth, more chlorophyll content and restricted ROS accumulation (Watanabe *et al*., 2014; Irani and Todd, 2016; Takagi *et al*., 2016). One proposed idea is that protective effect of allantoin on stressed plants is mediated through ROS scavenging at stress conditions. Studies on *aln* mutants as well as exogenous administration of allantoin show that elevated allantoin level (both in vivo and in vitro) is effectively related to declined ROS content in plants following stress exposure (Brychkova *et al*., 2008; Watanabe *et al*., 2010; Watanabe *et al*., 2014). Nonetheless, how allantoin minimizes ROS concentration and protects plants from negative effects of stress is still under debate. My results clearly demonstrate more Cd-resistance of two-week old *aln-3* mutants when compared with Col-0 Arabidopsis, while raised questions whether allantoin decreased ROS content in Cd-treated *aln-3* mutants, whether allantoin directly functions in ROS decomposition mechanism or it acts as a signalling molecule that activate ROS detoxification systems. Thus, to compare between the response of two-week old seedlings and mature plants to Cd treatment, and to illustrate the influence of allantoin on plant growth and ROS accumulation, these *aln-3* and Col-0 Arabidopsis were allowed to grow in soil and treated with different Cd concentration.

4.2. Cadmium treatment and ureides in soil-grown Arabidopsis shoots and roots

4.2.1. Enhanced allantoin concentration influences gene expression, metabolite accumulation and Cd content of *aln-3* mutants.

Growing Col-0 and *aln-3* mutants in soil and treating them with different Cd concentrations (500, 1000 and 1500 µM $CdCl_2$) revealed almost the same results as I observed in MS plates. Cd decreases plant growth, leaf expansion and root elongation at high concentrations, reflected in their lower biomass upon Cd treatment. Consistent with MS-grown samples, *aln-3* mutant Arabidopsis exhibit better growth and higher biomass when compared with Col-0 Arabidopsis grown under the same conditions. However, neither Col-0 nor *aln-3* leaves showed common visual symptoms of stress and Cd toxicity such as discoloration and yellowing. Growth conditions in this experiment seemed to have a considerable impact on the toxicity of Cd. A short-day photoperiod (8 h light/16 h dark) was employed to maintain the plants in the vegetative stage and avoid their early flowering (Putterill *et al*., 2004; Queval *et al*., 2007). Using this light regime, rather than more popular long-day photoperiod (16 h light/8 h dark), seems to be beneficial for plants in this experiment via limiting light-induced oxidative stress, mainly produced by the electron transport chain in chloroplasts, and may provide additional protection to the plant through detoxification of generated ROS during longer dark hours (Queval *et al*., 2007). Moreover, using potting soil as a growth base may minimize the severity of applied Cd. The nutrient composition of soil, along with its moisture, texture and pH influence Cd mobility and may change its availability for plant uptake (Chibuike and Obiora, 2014). I assume shorter light exposure, chemical properties of soil in addition to higher plant age when exposed to Cd treatment (Wieser *et al*., 2002) alleviates Cd toxicity, limits oxidative damage causing a generally improved plant growth in both Col-0 and *aln-3* mutants.

In agreement with MS-grown seedlings, Cd increased allantoin accumulation in Col-0 and *aln-3* leaves. Biochemical and molecular analyses indicated that this increase in allantoin concentration can be attributed to a significant increase in the transcript level of *UO* in both genotypes under Cd treatment. Additionally, in Col-0 leaves gene expression and enzyme activity of *ALN* remarkably decreased upon Cd exposure. Therefore, increased *UO* expression and decreased *ALN* expression and activity of leaves lead to enhanced allantoin production and restricted allantoin degradation, resulting in the accumulation of this ureide in response to Cd.

Cd-induced allantoin accumulation and *UO* upregulation is also observed in *aln-3* roots, while allantoin content of Cd-treated wild-type roots follow a different pattern. 1000 and 1500 µM $CdCl_2$ decreases allantoin concentration in Col-0 roots which is likely associated with a significant decrease of *UO* and *ALN* transcription at these Cd concentrations. Transcriptome analysis of *Salix matsudana* demonstrated that there is a different molecular response in the root and shoot at Cd treatment, probably related to different stress response mechanisms in these two tissues (Yang *et al*., 2015). Most of the genes upregulated in roots in response to Cd are related to the structural modifications, whereas Cd-induced genes in leaves orchestrate plant function (such as stimulating signalling pathways and metabolite regulation) (Yang *et al*., 2015). Another significant factor determining the metabolite content of roots and shoots is the regulation and function of transporters under stress conditions. Ureide permease 5 (UPS5), a ureide transporter with high affinity for allantoin, is highly expressed following NaCl treatment (Lescano *et al*. 2016). Considering that UPS5 is mainly present in Arabidopsis roots, this salt-induced upregulation results in enhanced root-to-shoot translocation of allantoin, decreased concentration of allantoin in root and lower allantoin root:shoot ratio at NaCl treatment (Lescano *et al*., 2016; Schmidt *et al*., 2004 and 2006). Allantoin transport may be the mechanism explaining decreased amount of allantoin in Cd-treated wild-type roots, along with a significant rise in allantoin content of Col-0 leaves, in my experiments. Moreover, comparison between Cd content of shoot and root in both genotypes shows that root tissue, in general, contains dramatically higher amounts of Cd than shoot (compare Figs. 3.16 and 3.27). Given that root is the first organ in direct contact with Cd and the primary defense line against Cd toxicity (Lux, 2014), it is reasonable to think that high concentrations of Cd absorbed by roots first impinges on their molecular mechanism, resulting in downregulation of ureide metabolic genes in Col-0 roots which consequently decreases allantoin content of wild-type roots at this condition.

Interestingly, *aln-3* mutants contain noticeably more amount of Cd in their roots and shoots when compared with Col-0 samples, but despite the elevated amount of Cd in their roots, these mutants exhibit the similar molecular and metabolic response in root and shoot. Cd-induced allantoin accumulation, which is likely driven by increased *UO* transcription in roots (and shoots), suggests this idea that basal high concentration of allantoin in *aln-3* mutants protects plant from the harmful effects of Cd toxicity, leading to improved plant growth, increased Cd

uptake from soil as well as stable ureide metabolism under this condition. It is also worth noting that the potential protective role allantoin plays in response to Cd seems to have an important contribution to the Cd content of root and shoot in both genotypes. In control-treated shoots, Col-0 leaves contain higher concentration of Cd than *aln-3* mutants, while the opposite result is observed in roots under the same condition. This pattern resembles the defense mechanism proposed in hyperaccumulators in which high amounts of metals are absorbed by root, stored in this tissue and root to shoot translocation is restricted. Hence, lower metal concentration is transported to shoot. This strategy helps these plants to withstand elevated amounts of toxic elements via protecting aboveground tissues, where photosynthetic machinery functions, from negative effects of stress (Nada *et al*., 2007; Emamverdian *et al*., 2015). Although my results don't point to the effect of allantoin on nutrient/element transportation, the observed difference between Col-0 and *aln-3* mutants at control conditions along with better function of *aln-3* samples in response to Cd suggests the possible influence of allantoin on Cd sequestration in root and its transportation.

4.2.2. Allantoin causes decreased O_2^- accumulation in Cd-treated *aln-3* Arabidopsis.

Measuring the concentration of reactive oxygen species (ROS), H_2O_2 and O_2^-, in Col-0 and *aln-3* roots and shoots showed that Cd induced ROS generation in both genotypes, specifically at higher concentrations. Although Cd, per se, is not able to react with oxygen through a redox reaction (Garnier *et al*., 2006), it induces ROS production via disrupting the function and structure of the photosynthetic apparatus, mitochondrial respiration and antioxidant mechanisms in wide variety of plants (Heyno *et al*., 2008; Groppa *et al*., 2012; Pérez-Chaca *et al*., 2014). ROS accumulation induced by Cd toxicity, in turn, stimulates signalling pathways and antioxidant systems. Prolonged ROS generation in plants causes decreased plant growth, oxidative damage, and cell death (Lin and Aarts, 2012; Matilla-Va´zquez and Matilla, 2012). However, comparison of ROS content in Col-0 and *aln-3* samples revealed that mutants accumulate relatively lower amounts of hydrogen peroxide and superoxide, with a statistically significant difference between O_2^- content of wild-type and *aln-3* root and shoot. These data suggest that *aln-3* mutants are more tolerant to Cd and can grow better under this condition because increased allantoin level in these plants decreases the amount of toxic ROS and consequently limits oxidative damage upon Cd exposure. Likewise, it was previously reported

that suppression of *XDH* in Arabidopsis decreases downstream ureide accumulation, generating drought susceptible plants due to increased ROS production and ROS-induced cell death (Watanabe *et al*., 2010). Additionally, it is demonstrated that allantoin treatment of Arabidopsis leaf discs minimizes ROS accumulation and alleviates oxidative damage in dark-treated plants via reducing chlorophyll degradation (Brychkova *et al*., 2008).

One pivotal mechanism that controls ROS accumulation and responds to it is mediated through the antioxidant network. Lower superoxide content of *aln-3* shoots and roots in response to Cd treatment proposed this hypothesis that allantoin has a stimulatory effect on antioxidant systems, leading to the measurement of superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT) enzyme activity in *aln-3* and Col-0 Arabidopsis.

4.2.3. Increased allantoin content triggers the activity of antioxidant enzymes in *aln-3* Arabidopsis.

The balance between ROS production and removal under normal or stress conditions is an important determining factor, regulating antioxidant expression and activity (Caverzan *et al*., 2016). Most of the enzymatic and non-enzymatic antioxidants are stimulated in response to a wide range of biotic and abiotic stresses in order to dispose of stress-imposed ROS generation and attenuate oxidative damage under this condition (Mittler, 2002; Mittler *et al*., 2004; Caverzan *et al*., 2016). In this study, the activity of three antioxidant enzymes, SOD, APX and CAT were quantified under control and Cd-treatment conditions and compared between Col-0 and *aln-3* samples. Activity of these antioxidant enzymes in two genotypes (*aln-3* and Col-0 Arabidopsis) and two plant tissues (shoot and root) exhibit similarities and differences which are inclusively discussed.

a) Similarities between antioxidant enzyme activity in *aln-3* and Col-0 shoots

In Col-0 and mutant leaves, an increased activity of three antioxidant enzymes is observed along with increasing Cd concentration, implying that Cd triggers antioxidants activity. Stress-derived stimulation of antioxidant enzymes has been reported in wheat (Wang *et al*., 2008; Devi *et al*., 2012), rice (Passaia *et al*., 2013 and 2014; Caverzan *et al*., 2014) and Arabidopsis (Mittler, 2004). My results also indicate that the enzymes SOD and APX are more active in *aln-3* mutant leaves than in Col-0 samples exposed to the same Cd concentration. This

difference in enzyme activity is also observed in the absence of Cd, at Control treatment, implying that *aln-3* mutants are pre-equipped with an activated antioxidant mechanism, which in turn helps them to combat stress more effectively and confers stress-tolerance to these plants. Highly active antioxidant enzymes, specifically SOD, in *aln-3* mutants also explains the lower concentration of superoxide radicals in Cd-treated mutants. In addition to these three antioxidants, relatively higher proline content of *aln-3* leaves may also be attributed to the better function and improved growth of these mutants in response to Cd treatment. Approximately equal proline concentration of both genotypes at control treatment indicates that proline does not prompt stress response or predispose plants to decreased ROS accumulation in the absence of stress. Whereas, proline content of Col-0 and *aln-3* leaves increases along with Cd increment, intimating that proline accumulation is an adaptive response to Cd treatment with the purpose of protecting plant from Cd-induced stress damage. Proline is suggested to act as metal chelator that minimizes metal toxicity, causing stress resistance in plants (Bao *et al*., 2011). Moreover, engagement of proline in quenching free radicals, singlet oxygen ($^1O_2$) and hydroxy radicals ($OH^\bullet$), is beneficial for plants upon stress exposure via diminishing oxidative damage (Matysik *et al*., 2002).

I conclude the better plant growth and higher Cd-tolerance observed in *aln-3* mutants relies on enhanced allantoin concentration in these mutants that boosts the activity of antioxidant enzymes (SOD, APX and CAT), leading to increased detoxification of Cd-induced ROS. This mechanism alleviates Cd toxicity and minimizes the negative effects of stress, resulting in Cd-tolerance of *aln-3* mutants. Decreased ROS generation and stress tolerance of *aln-3* mutants is also reported by Irani and Todd (2016) in response to drought and NaCl treatment. It is also demonstrated that in *aln* mutants, abscisic acid (ABA) and jasmonic acid (JA) is more accumulated than in wild-type Arabidopsis. Exogenous application of allantoin show that this increased ABA/JA production is contributed to enhanced allantoin content of *aln* mutants, leading to the activation of ABA/JA signalling pathways (Watanabe *et al*., 2014; Takagi *et al*., 2016). ABA and JA accumulate in response to many developmental processes, as well as environmental stresses, initiating signal transduction pathways that manipulate molecular mechanisms. This ABA- and JA-dependent change in gene expression causes different cellular responses leading to stress adaptation in plants (Finkelstein *et al*., 2008; Kim *et al*., 2010; Ng *et al*., 2014; Sah *et al*., 2016). It is reported that accumulated ABA and JA in *aln* mutants

modulate stress responsive genes, modifies plant function under stress condition and improves stress tolerance in these mutants (Watanabe *et al*., 2014; Takagi *et al*., 2016).

Involvement of allantoin in ABA/JA accumulation, as discussed above, together with increased activity of antioxidant enzymes obtained from my experiments support this conclusion that allantoin accumulation in *aln-3* mutants regulates plant stress response to lower Cd-induced ROS generation and consequently oxidative damage, causing a Cd-tolerance phenotype in these mutants. However, what is still not demonstrated is whether stimulatory effect of allantoin on the activity of antioxidant enzymes is linked with ABA production and is mediated through ABA signalling pathway or not.

b) Different antioxidant enzyme activity in *aln-3* and Col-0 roots

Consistent with *aln-3* shoot, activity of three antioxidant enzymes increases in response to Cd treatment in *aln-3* roots. However, Col-0 roots show a different pattern of antioxidant activity than that of shoot. Activity of SOD and CAT in Col-0 roots decreased significantly in response to higher Cd concentrations (1000 and 1500 µM $CdCl_2$). Furthermore, despite approximately similar Cd content of Col-0 and mutant at 500 µM $CdCl_2$, this Cd treatment did not influence SOD and CAT activity in *aln-3* roots, while it had a stimulatory effect on Col-0 roots where Col-0 CAT is more active than in *aln-3* roots. Considering these findings together with decreased allantoin content and gene downregulation in Col-0 roots at high Cd concentrations, it supports this understanding that 1000 and 1500 µM $CdCl_2$ treatments imposed are too toxic for Col-0 roots, impairing their molecular, metabolic and defence mechanisms, and resulting in decreased antioxidant function and decreased root growth under this condition. On the contrary, these results establish this conclusion that allantoin-derived activation of antioxidants in *aln-3* mutants mitigates the severity of Cd stress, at least at lower concentrations, restricts stress damage and postpones stress response in their roots.

c) Different antioxidant enzyme activity of roots and shoots

My results also indicate that, besides genotype (*aln-3* vs. Col-0), plant tissue (shoot vs. root) is another important factor determining dominant antioxidant activity in response to Cd treatment. Statistical analyses for the activity of antioxidant enzymes in root and shoot of both genotypes show that enhanced SOD and APX activity in *aln-3* shoots is responsible for their

lower superoxide content in respect with Col-0 shoots, whereas in *aln-3* roots elevated SOD and CAT activity play the significant role following Cd treatment. Considering that both APX and CAT catalyze the same reaction, decomposition of H_2O_2, it seems a reasonable conclusion that cellular localization of these two enzymes together with different structure of root and shoot influence their activities in these two tissues. Disruption in the structure or function of photosynthetic and respiratory electron transport chains (ETC) and their dependent components are mostly the main cause of ROS generation and oxidative damage in leaves exposed to Cd stress (Yamamoto *et al*., 2002; Seth *et al*., 2011). In aboveground tissues Cd impinges on oxygen evolving complex (Geiken *et al*., 1998; Parmar *et al*., 2013), interferes with the function of electron accepter/donor in PSII (Sigfridsson *et al*., 2004) and damages iron-sulfur centers in PSI (Michel and Pistorius, 2004). Therefore, malfunction of photosynthetic machinery leads to the accumulation of ROS and impose oxidative stress on Cd-exposed plants (Parmar *et al*., 2013). In the absence of chloroplasts in root tissue, the ETC in mitochondria is likely the primary target of Cd toxicity and main source of oxidative damage (Heyno *et al*., 2008). Cd is known to prevent mitochondrial electron transport from semiquinone to cytochrome b in complex III (cytochrome bc1), leading to the accumulation of semiquinone radicals which in turn generates O_2^- (Miccadei and Floridi, 1993; Heyno *et al*., 2008). It is also reported that Cd interferes with deprotonation mechanism of this complex in mitochondria which is required to maintain the proton gradient across inner mitochondrial membrane, deriving ATP synthase complex (Giachini *et al*., 2007). Functional disorder of mitochondrial ETR leads to increased ROS generation and oxidative stress upon Cd exposure. Thus, I conclude that different structure of root and shoot as well as different sources of stress perception and consequently stress response in these two tissues affect antioxidant activity in this experiment.

4.3. Exogenous application of allantoin protects Col-0 Arabidopsis from harmful effects of Cd treatment.

Allantoin is primarily known as an important nitrogen-rich compound involving in nitrogen transport and storage in plants. However, protective properties of allantoin have been demonstrated in other systems, implying that allantoin participates in plant responses to abiotic stresses, protecting plants from harmful effects of stress. Employing *aln-3* mutants in my

experiments revealed that these mutants benefit from more active antioxidant enzymes that restrict Cd-induced ROS accumulation, conferring Cd-resistance to this mutant. It also underlines this conclusion that high allantoin content of *aln-3* Arabidopsis, when compared with Col-0 samples, is coupled with improved plant growth and increased stress tolerance in these plants. These findings set the stage to propose this hypothesis if Cd-resistance phenotype observed in *aln-3* mutants relies on their increased amount of allantoin, exogenous administration of allantoin has the similar protective effect on Col-0 Arabidopsis and improves their seed germination and seedling growth in response to Cd treatment that is likely associated with promoted antioxidant activity at this condition.

Supporting my hypothesis, results show that exogenous allantoin improves seedling growth when exposed to Cd treatment. The stimulatory effect of allantoin on seed germination and seedling growth has been reported in a few studies. For example, it has been demonstrated that allantoin induces seed germination in *Lactuca saliva* (Grassi *et al*., 2005) and improves biomass in wheat (*Triticum aestivum*) (Gajic, 1966; Mallik and Williams, 2005). Although the mechanism underlying stimulatory effect of allantoin is not quite clear, allantoin is proposed to influence plant-plant (Grassi *et al*., 2005; Wang *et al*., 2012) and plant-microorganism (Wang *et al*., 2010) interaction, functioning as a biochemical agent with allelopathic properties (Wang *et al*., 2007b). My results also indicate that exposure to allantoin before seed germination and at early stages of seedling growth increased seed germination, root length and seedling growth that can likely be attributed to increased antioxidant capacity of seeds after allantoin treatment. In rice (*Oryza sativa*), cultivars with higher allantoin content exhibit increased seedling survival under low temperature and water deficit conditions, suggesting a protective role for allantoin under undesirable environmental conditions (Wang *et al*., 2012). Interestingly, traditional Chinese farmers soak rice seeds in allantoin and its derivative compounds (called guangzengsu) to improve their germination and protect them from chilling and water limitation in early spring (Xu, 1997; Xu *et al*., 1999). Wang *et al*. (2012) indicated that the positive correlation between exogenous allantoin and increased biomass, accumulated proline and improved microbial profile of soil is likely associated with increased seed germination and seedling growth under stress conditions (Wang *et al*., 2007b, 2010 and 2012). Therefore, these data provide another evidence that increased allantoin content (both *in vivo* and *in vitro*) is involved in stress resistance supporting plant growth at this condition.

4.4. Overexpression of *ALN* causes a sensitive phenotype in response to Cd.

The primary objective of constructing *ALNox* lines was to induce *ALN* gene expression constitutively, with the purpose of reducing allantoin content of transgenic plants and comparing stress response of these mutants with Col-0 Arabidopsis as well as *aln-3* mutants. Considering that enhanced allantoin concentration (in *aln-3* mutants or as exogenous allantoin-treatment) protects plants from Cd toxicity and is associated with Cd resistance in these samples, my hypothesis was that increased transcription of *ALN* is expected to generate the mutants that respond to stress in an opposite manner than *aln-3*. qRT-PCR analysis and measurement of enzyme activity showed that allantoinase transcript levels and enzyme activity levels was greater, when compared with its corresponding wild-type plant. This resulted in a lack of allantoin in *ALNox* lines at control treatment. Comparing between root length of Col-0 and overexpressed lines under Cd treatment implies that these Arabidopsis lines are more Cd-sensitive than wild-type plants which is likely related to the absence of allantoin in these plants. Similarly, *RD29A::ALN* transgenic plants were utilized by Lescano *et al*. (2016) to study the effect of allantoin content on stress response upon NaCl treatment. In their experiment *ALN* was expressed under the control of salt stress-inducible promoter of *RD29A* which increased *ALN* expression in response to NaCl treatment, causing a considerable decline in their allantoin content under this condition. Reduction of allantoin concentration in *RD29A::ALN* lead to decreased plan growth, lower fresh weight and chlorophyll content in comparison with wild-type and allantoinase-negative (*aln*) mutants (Lescano *et al*., 2016). Thus, it is reasonable to conclude that there is a positive correlation between allantoin content and stress tolerance in plants. Increasing amounts of allantoin confers stress-tolerance to plants, decreased amount or lack of allantoin imposes stress susceptibility.

Interestingly, once *ALNox* lines were fed with exogenous allantoin their root length didn't improve. Evaluation of allantoin content in Col-0 and *ALNox* lines at this condition showed that both genotypes contained increased amount of allantoin in their seedlings when grew on media plates containing allantoin (MS+Aln). However, along with increasing allantoin concentration a considerable increase is also observed in allantoinase activity of both genotypes with higher activity seen in overexpressed lines. Taken together, these results suggest that elevated amounts of allantoin induce ALN activity in both genotypes with highest

activity in *ALNox* lines due to their already enhanced basal ALN activity. My proposed conclusion in this experiment is attributed to subcellular localization of the ureide allantoin and enzyme allantoinase in wild-type Arabidopsis at a normal condition.

In plant cells allantoin is produced in peroxisome and transferred to the ER, where allantoinase degrades it to allantoate (Werner and Witte, 2011). Likewise, previous experiments reported that the activity of allantoinase is detected in the ER (Hanks *et al*., 1981). From these reports, it can be extracted that at normal condition only the amount of allantoin residing inside the ER can be utilized by the enzyme allantoinase. Experimental procedure in this study revealed that total allantoin content of *ALNox* seedlings increases following exogenous allantoin treatment. However, these results do not provide any evidence regarding where in plant/cell allantoin accumulates at this treatment. Moreover, present data indicates raised ALN enzyme activity in *ALNox* seedlings without pointing to the subcellular localization of allantoinase in these plants. However, inefficient effect of exogenous allantoin on *ALNox* lines despite elevated amount of allantoin in these samples led me to conclude that part of the absorbed allantoin is likely located in extracellular spaces which is not accessible to plant cells and the major amount of allantoin that reaches inside *ALNox* cells are extensively degraded, due to elevated ALN enzyme activity. In other words, applied exogenous allantoin does not enter plant cell (extracellular localization) or does not accumulate inside the cell (immediate degradation by overexpressed ALN) to protect cells from negative effects of Cd toxicity and improve *ALNox* growth at Cd treatment.

4.5. Differential antioxidant responses of *abi* mutants to Cd and allantoin treatments.

My experiments on the shoot and root of Col-0 and *aln-3* mutants indicated that antioxidant enzymes APX (in shoot), CAT (in root) and SOD (in root and shoot) are more active in *aln-3* mutants in respect with Col-0 Arabidopsis, causing limited ROS accumulation and improved plant growth of *aln-3* under Cd treatment. Furthermore, allantoin treatment of Col-0 seeds induced SOD activity in seeds which is assumed to help the future seedling to overcome Cd toxicity efficiently. These findings together with allantoin-induced ABA accumulation reported by Watanabe *et al*. (2014) led me to question if stimulatory impact of allantoin on antioxidant enzymes is mediated through ABA-dependent pathway. Assuming that regulatory function of allantoin is ABA-dependent, I hypothesized that allantoin in growth culture would

not be able to play its protective role and enhance plant growth in *abi* mutants exposed to Cd. In other words, if the function of allantoin is ABA-dependent *abi* mutants are not able to employ allantoin to overcome Cd toxicity. To test this hypothesis, three *abi* mutants (*abi1*, *abi3* and *abi4*) and two wild-type Arabidopsis (Col-0 and Ler-0) employed to discover the potential link between the function of allantoin and ABA signalling pathway.

In my system, there is a significant difference between seed germination and root length of MS+Cd- and MS+Aln+Cd-treated wild-type Arabidopsis (both Col-0 and Ler-0), suggesting that exogenous application of allantoin can improve decreased seed germination and root length derived from Cd toxicity in Col-0 and Ler-0 seedlings. Likewise, in *abi* mutants seed germination of MS+Aln+Cd-treated samples is higher than that of MS+Cd treatment. However, such a positive effect of allantoin and correlation between MS+Cd and MS+Aln+Cd treatments are not detected in *abi* root length. These findings lead to this understanding that at germination stage function of allantoin is independent of ABA pathway, whereas positive effect of allantoin on root elongation, under Cd treatment requires the involvement of ABA-signalling pathway.

The effect of allantoin treatment, both solely (MS+Aln) and combined with Cd (MS+Aln+Cd), on transcript level of six antioxidant enzymes resulted in various patterns in *abi* mutants and wild-type Arabidopsis. For example, transcript level of *FSD1* in Col-0 and Ler-0 increased at MS+Aln+Cd treatment which is consistent with the same measurement in *abi1*, whereas it did not change in *abi3* and *abi4*. The opposite result is observed in the transcription level of *CSD1*; while this gene remained unchanged in MS+Aln+Cd-treated wild-type samples, this treatment enhanced the transcript level of *CSD1* in *abi* mutants when compared with MS+Cd treatment.

Different studies showed that regulation of a gene through ABA signalling pathway relies on the presence of ABA Responsive Element (ABRE) in the promoter region of ABA-inducible genes. ABRE is a cis regulatory element with an identified sequence (ACGTGGC) in *A. thaliana* (Jakoby *et al*., 2002; Nakashima *et al*., 2006). Expression of ABA-responsive genes is mediated by three transcription factors (TFs) participate in ABA signalling pathway: ABI3, belonging to the family of B3 TFs, ABI4, a member of apetala2 (AP2) family, and ABI5, a basic leucine zipper (bZIP) TF. Although each transcription factor can bind with their specific target motif, they are all shown to induce promoters containing ABRE, upregulating ABA-

responsive genes (Monke *et al*., 2004; Jakab *et al*., 2005). Additionally, ABI3 interacts with ABI5 via regulation of *ABI5* transcription as well as enhancing its activity specifically during seed germination (Lopez-Molina *et al*., 2002). Following observed variation in the transcription of antioxidant genes in response to Cd and allantoin in *abi* mutants, I analysed the promoter sequence of tested genes for the existence of ABRE motifs using Arabidopsis Gene Regulatory Information Server (AGRIS) online tool. Data showed that among six tested antioxidant genes, four contain predicted ABRE-like binding site motif as followed: *APX2* (CACGTGTC), *MSD1* (TACGTGTC), *CSD1* (GACGTGGC) and *CAT3* with two predicted ABRE motifs (TACGTGGC and CACGTGGA). Results obtained from AGRIS did not show ABRE or ABRE-like binding sites in *APX1* and *FSD1* promoters. These results demonstrate that these four genes can be induced by ABA accumulation, while do not assure that ABA-signalling is the only regulatory mechanism for their transcription. Studies on the effect of ABA accumulation and ABA signalling pathway on gene regulation at stress conditions illustrate the complexity of this mechanism and cross-talk between ABA-dependent and -independent gene induction.

Transcriptional analysis of *APX2* in Arabidopsis following high light exposure indicates that H_2O_2 accumulation derived from excess light exposure induces *APX2* transcription in wild-type samples, whereas high light-induced expression of *APX2* decreases in *abi1–1* and *abi2–1*, intimating the involvement of ABA signalling pathway in *APX2* gene regulation at this condition (Fryer *et al*., 2003). Despite this finding, transcript level of *APX2* did not show a significant difference in *abi* mutants (*abi1–1* and *abi2–1*), when compared with wild-type samples, in response to wounding. This data demonstrates that wound-induced *APX2* expression is not mediated through an ABA-signalling mechanism (Chang *et al*., 2004). Additionally, a study carried out on the effect of ABI1 and ABI2 (two distinct protein phosphatase 2C) mutation on ABA response and stomatal closure at water-deficit condition, reveals that ABI1 is necessary for ABA-induced ROS accumulation while ABI2 is required for ROS-induced activation of Ca channels and consequent stomatal closure (Murata *et al*., 2001). Therefore, not only stress type determines the gene expression reliance on ABA, but also decreased/lack of ROS accumulation due to *ABI1* mutation may compromise the signal transduction cascades derived by increased ROS, altering the expression pattern of stress-responsive genes at this condition.

Furthermore, in response to a single stress, such as water limitation, both ABA-dependent and -independent responsive pathways may participate to modulate the most effective stress response and tolerance in plants. For example, dehydration-responsive gene, *RD29A*, is expressed in the first few hours of water-deficit recognition. Although, the promoter of this gene contains both dehydration-responsive element (DRE; TACCGACAT) and ABRE cis elements, its expression at the beginning of dehydration perception is mediated through an ABA-independent pathway, while ABA regulates its expression at later stages of stress response (Furini *et al*., 1996; Niu *et al*., 1996; Bray, 1997). Conversely, it is shown that the homolog of this gene, *RD29B*, with ABRE sequence (and lack of DRE) is immediately upregulated by ABI3, implying that interaction between ABRE and other regulatory elements may alter expression of ABA-responsive genes (Nakashima *et al*., 2006). It is also worth noting that besides the presence of a specific regulatory sequence in a gene, structural condition of DNA which is not predictable by promoter sequence analysis, is another determinant factor influencing TF-gene bound and gene regulation. For example, chromatin structure and nucleosomal position of a gene alter the TF binding with regulatory sequence and affects gene expression (Niu *et al*., 1996; Bray, 1997, Shinozaki and Yamaguchi-Shinozaki, 1997).

From these reports and my results, it can be concluded that presence of ABRE sequence in the promoter does not necessarily mean that that gene depends on ABA for regulation at stress conditions. Additionally, although Watanabe *et al*. (2014) reported ABA accumulation in *aln* mutants and in response to external allantoin, whether allantoin functions as a stimulator for ABA production or it modifies gene expression and stress response through an ABA-dependent mechanism is still unknown. My understanding based on existing data lead me to conclude that there is not a constant model for the association between the function of allantoin and ABA-regulatory pathway in Arabidopsis in response to Cd treatment. It seems that the link between protective effect of allantoin on plant stress response and the engagement of ABA in this process is case-dependent. While the effect of allantoin on some stress-responsive events relies on ABA signalling pathway and requires the intermediates of this pathway, some other responses are mediated in an ABA-independence manner, with a possible crosstalk between these two pathways. Therefore, better understanding of the interaction between regulatory function of allantoin and ABA signalling pathway requires more investigations using double mutants (such as *aln/aba*, *aln/abi*, *ALNox/abi*) to perform ROS quantification and antioxidant

activity measurements. The potential difference between Col-0 and these mutants will be informative to propose a model for better understanding of the connection between allantoin and ABA as two stress-responsive regulatory systems in plants.

4.6. *aln-3* and *aah* mutants show a resistant phenotype in response to Li and Ni.

Lithium is an alkali metal that naturally exists in nature, also enters the soil through various industrial activities (Kszos and Steward, 2003; Aral *et al*., 2008; Hawrylak-Nowak *et al*., 2012). The effect of Li on plant is highly dose- and species-dependent (Hawrylak-Nowak *et al*., 2012). Low amounts of Li (5 mg Li dm^{-3}) are reported to have a stimulatory effect on plant growth, whereas increasing Li concentration of growth media (50 mg Li dm^{-3}) imposes metal toxicity and oxidative stress on plant growth and function (Hawrylak-Nowak *et al*., 2012; Shahzad *et al*., 2016). My data showed that Li decreased seed germination and root length of Col-0 and two ureides mutants, *aln-3* and *aah*. Li is shown to interfere with gravitropism of maize roots (Mulkey, 2005) and causes chlorosis and necrosis in lettuce leaves (Kalinowska *et al*., 2013). The inhibitory effect of Li on plant growth and development, specifically from photosynthetic aspects, is likely attributed to Li-induced pigment degradation, disruption in stomatal conductance and interference with nitrogen assimilation (Shahzad *et al*., 2016). Additionally, negative effects of Li on plant growth is also attributed to ROS accumulation and oxidative damage mediated through increased lipid peroxidation under Li treatments (Shahzad *et al*., 2016).

Nickle is an essential element, participating in the structure and is required for the function of enzyme urease. In the absence of Ni, urease is not functional to break urea, leading to its accumulation in plants. However, same as other essential nutrients excess amount of Ni induces toxicity, retarding seed germination and seedling growth (Eskew *et al*., 1983 and 1984; Walker *et al*., 1985; Polacco *et al*., 2013). Consistent with other metals tested in my experiments, Ni treatment caused a considerable decrease in seed germination and root length of three genotypes. Previous research indicates that Ni-induced germination decrease is associated with its negative effect on hydrolytic enzymes of seeds and interfering with cell elongation that impinges on radical emergence (Walker *et al*., 1985; Seregin and Kozhevnikova, 2006; Ahmad and Ashraf, 2011; Yusuf *et al*., 2011). Significant decline in seed germination was observed in *Zea mays* (Bhardwaj *et al*., 2007) and *Brassica juncea* (Sharma

et al., 2008) in response to different Ni concentrations. Ni shares similar physical properties with other elements, such as Ca, Zn, Fe and Cu, thus competing for their transporters and plant uptake (Chen *et al*., 2009). As a result, high amounts of available Ni induce nutrient deficiency (Ahmad *et al*., 2007), decreases chlorophyll content (due to its substitution for Mg) (Gajewska and Sklodowska, 2007) and interferes with the function and structure of intermediates of the electron transport chain in chloroplasts (Mohanty *et al*., 1989; El-Sheekh, 1993). All mentioned failures in physiological and biochemical functions of plant lead to a remarkable decrease in plant growth and reproduction under Ni exposure.

In addition to declined seed germination and plant growth under Li and Ni toxicity, applied concentrations of these two elements increased allantoin content in Col-0 and *aah* mutants, but not in *aln-3* mutants. Interestingly, Li induced allantoate accumulation in Col-0 seedlings. To my knowledge effect of metals on ureide metabolism, ureide accumulation and ureide-related mutants have not been investigated before and this is the first data in this field showing allantoin (and allantoate) accumulation upon metal exposure. Although ROS content of Ni/Li-exposed seedlings is not quantified in this experiment, I assume both tested metals prompt ROS generation and induce oxidative damage (Gajewska and Sklodowska, 2007; Shahzad *et al*., 2016). Therefore, oxidative stress initiated by Ni and Li likely stimulates allantoin and allantoate accumulation to serve as cell protectants following the mechanism described above (section 4.2.2).

In agreement with Cd experiments, these data showed that *aln-3* mutants are more resistant to Li and Ni compared with Col-0 seedlings under the same treatment. Interestingly, in addition to *aln-3*, *aah* mutants also demonstrated Ni- and Li-tolerance, reflected in their higher seed germination and root length. Accumulation of allantoate, along with allantoin, under stress conditions was previously demonstrated in response to dark treatment. Moreover, exogenous application of allantoate diminished ROS accumulation in dark-treated plants (Brychkova *et al*., 2008). These results propose this hypothesis that allantoate might have a protective role under abiotic stressors induce oxidative damage. The potential protective role of allantoate may be mediated through induction of antioxidant systems, consistent with the stimulatory effect of allantoin, and/or direct effect of allantoate as cellular ROS scavenger. Furthermore, the molecular structure of allantoate exhibits the typical properties of a chelate agent

(containing three C═O, two ─NH_2 and one ─ OH chemical groups), making it as a candidate metal chelator that binds with toxic metals, minimizes metal toxicity and improves stress tolerance under this condition. Therefore, more investigations are required to address these questions and unravel the role ureides, in particular allantoate, plays in response to stress.

4.7. Conclusion

This study was designed relying on the basal hypothesis that ureide pathway is responsive to Cd treatment, a toxic metal that induces ROS accumulation and oxidative damage in plants. I started my project with this hypothesis that Cd treatment induces ureide pathway and causes ureide accumulation in Arabidopsis. Experiments and results showed that wild-type Arabidopsis accumulates allantoin in response to Cd treatments. Molecular analysis and biochemical assays demonstrated that the effect of Cd on the gene expression of two enzymes important for allantoin production (*UO*) and degradation (*ALN*), are the main causes of allantoin accumulation under Cd treatment. Enhanced transcription of *UO* accompanied with decreased transcript level and enzyme activity of *ALN* results in increased allantoin content of Cd-treated Arabidopsis. This first experiment and results lead to the second hypothesis that allantoinase-negative (*aln-3*) mutants, with constitutive accumulation of allantoin, are more resistant to Cd. Exposing Col-0 and *aln-3* Arabidopsis to Cd indicated that *aln-3* mutants grow more than Col-0 samples under Cd treatment which is reflected in their higher seed germination, longer roots and more biomass at this condition. Considering that Cd toxicity is associated with increased ROS generation and consequently oxidative damage in plants, better plant growth of *aln-3* mutants in respect with Col-0 samples following Cd treatment raised this question whether these mutants contain lower amounts of ROS than Col-0 Arabidopsis. To answer this question ROS content of Col-0 and mutant shoots and roots were measured. Quantification of H_2O_2 and O_2^- indicated that in spite of Cd-induced ROS accumulation in both genotypes, concentration of O_2^- in *aln-3* mutants is relatively lower than that of Col-0 plants. However, it was important to know how *aln-3* mutants limited their ROS content under Cd treatment. The first possible mechanism that has been suggested for ROS detoxification was about direct involvement of allantoin in ROS scavenging and its function as an antioxidant. This hypothesis has been tested in rice (*Oryza sativa* L) and ruled out. It has been indicated that although exogenous allantoin has a stimulatory effect on rice seedlings growth and their

proline content, it didn't show any antioxidant activity for scavenging free radicals (Wang *et al*., 2012). Therefore, I proposed another hypothesis regarding the potential effect of allantoin on antioxidant system. Considering lower O_2^- level of *aln-3* Arabidopsis at Cd treatment, I hypothesized that antioxidant enzymes are more active in *aln-3* mutants, decomposing Cd-induced ROS generation. Results obtained from antioxidant assay for *aln-3* and Col-0 shoots and roots supported my hypothesis, showing that in *aln-3* leaves superoxide dismutase (SOD) and ascorbate peroxidase (APX), and in *aln-3* roots SOD and catalase (CAT) are more active than those of Col-0 Arabidopsis exposed to the same Cd treatment. Interestingly, the difference between the activities of these enzymes in Col-0 and *aln-3* in the absence of Cd implies that *aln-3* mutants are equipped with a highly active antioxidant enzyme that protect the plant from Cd-derived oxidative damage and confer Cd-resistance to them.

Assuming that elevated allantoin content in *aln-3* mutants is associated with more active antioxidant enzymes, restricted ROS accumulation and consequently improved plant growth in these mutants, I hypothesized that external application of allantoin exhibits the similar protective effect on Col-0 Arabidopsis at Cd treatment. To test this hypothesis, Col-0 Arabidopsis seeds were exposed to Cd and exogenous allantoin and their plant growth were compared with seedlings grown in the absence of external allantoin. Results indicated that wild-type seedlings that have access to allantoin in their growth culture show higher seed germination and longer roots in comparison with those grown in the absence of allantoin. Allantoin treatment of Col-0 seeds before seed germination also improved their growth under Cd exposure, inducing the activity of antioxidant enzymes, specifically SOD, that prepares the plant to face stress and overcome Cd toxicity more effectively. These data showed that there is a link between enhanced allantoin content and stress tolerance in Arabidopsis. They also raised this question whether lower amount of allantoin causes a stress-sensitive response. To answer this question *ALN*-overexpressed (*ALNox*) Arabidopsis lines were generated and treated with different Cd concentrations.

In *ALNox* Arabidopsis, *ALN* is highly expressed and shows more enzyme activity than Col-0 samples. Overexpression of *ALN* decreased allantoin concentration, imposing Cd-sensitivity in these lines. *ALNox* has lower seed germination and shorter roots under Cd treatment in comparison with Col-0 plants exposed to same Cd treatment. Contrary to wild-type

Arabidopsis, feeding *ALNox* lines with exogenous allantoin didn't improve plant growth under Cd treatment and didn't rescue them from Cd toxicity. Quantification of allantoin in Col-0 and *ALNox* Arabidopsis indicated that in spite of same amount of allantoin in both genotypes grown in the presence of exogenous allantoin, more activity of the enzyme allantoinase in *ALNox* lines induced increased degradation of cellular allantoin. However, most of allantoin absorbed by plant was probably located in intercellular spaces and/or sequestrated in other cellular compartments and was not accessible for allantoinase.

Results obtained from Cd treatments led to similar measurements using other metals such as lithium (Li) and nickel (Ni) with the purpose of evaluating ureides metabolism at this condition. Treating *aln-3* and Col-0 Arabidopsis with Li and Ni showed that allantoin accumulates in Li- and Ni-treated wild-type samples and *aln-3* mutants are relatively more resistant than Col-0 samples in response to these elements. Interestingly, my results indicated that allantoate amidohydrolase-negative mutants (*aah*), with concentrated amount of allantoate, are also more resistant to Li and Ni when compared with-type samples that proposes a protective role for allantoate, in a similar or different way than allantoin. According to data available here and considering my primary experiments on *abi* mutants and their results, I proposed a model indicating the working mechanism of allantoin and its potential role in stress response under Cd treatment (Fig. 4.1). I assume involvement of one or more of these pathways mediates the protective role allantoin plays in Cd toxicity, and in general under stress conditions.

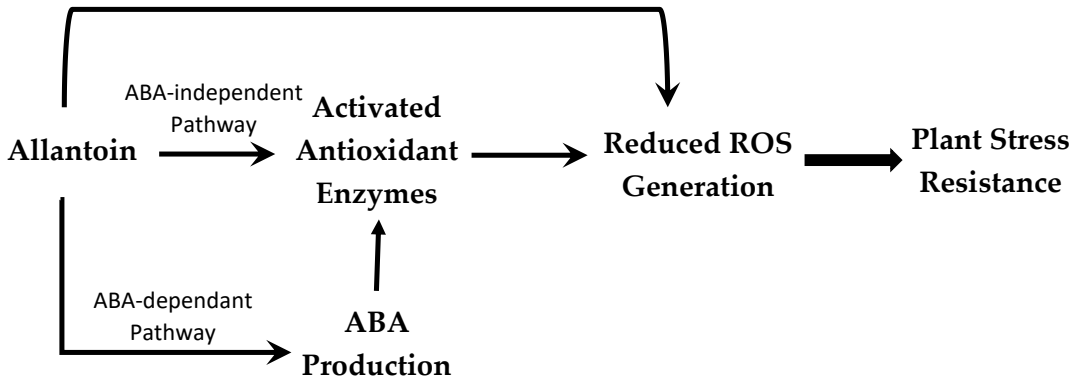

Figure 4.1. Proposed model for the regulatory function of allantoin in plant under stress conditions.

4.8. Future Directions

The main purpose of this project has been to evaluate the link between ureide pathway and Cd as an abiotic stressor and clarify the role allantoin plays under this condition. Employing molecular tools, biochemical techniques and mutational analysis (*aln-3* and *ALNox*), I showed the positive correlation between allantoin content of plants and their Cd-resistance, also elucidated the role of allantoin in plant response and protection under stress condition. However, there are still unknown aspects of ureide pathway that needs to be addressed in future works.

Effect of plant growth stages on ureide pathway: Most studies on the ureide pathway investigate the effect of environmental stresses on this pathway and little in known about the potential impact of different stages of plant development on this pathway. Considering that ureide pathway and its intermediates are recognized as one of the major routs of nitrogen recycling, it is hypothesized that regulation of this pathway and concentration of ureides is extensively influenced by different stages of plant growth, in order to provide required nitrogen for the proper growth and development.

Distribution of ureides in plant/cell in response to stress: Ureides accumulation in response to abiotic stresses have been reported in many studies but it is not quit clear which organ of the

plant and which organelle of cell is the main location for ureides build-up. Ureide pathway in general is an organelle-dependent pathway, taking place in cytosol, peroxisome and ER. Additionally, involvement of this pathway in ROS scavenging reinforces this idea that subcellular localization of ureides, specifically allantoin, is probably related to that of ROS accumulation. To gain a better understanding of where ureides accumulate in plant or inside the cell, promoter-reporter constructs are required to tag ureide enzymes with Green Fluorescent Protein (GFP) and assess the difference between their locations at control treatment and under stress conditions. Merging this data with results from ROS distribution obtained from staining techniques will help us to explain the possible link between ureide and ROS accumulation and their interaction in response to stress.

Post-translational modification of ureides genes: In addition to gene expression and enzyme activity which I focused on in this project, post translational modification (PTM) of ureides enzymes is another aspect of ureide pathway that requires more investigations. My data on allantoinase demonstrated that this gene is down-regulated at transcription level in response to Cd. Enzyme activity of allantoinase also showed a significant decline under Cd treatment. However, it is still unknown whether decreased activity of the enzyme allantoinase is due to its decreased gene expression and consequently decreased translation or Cd interfered with proper function of allantoinase causing its lower activity, while it didn't change allantoinase concentration in the cell. PTM studies about allantoinase, and other enzymes participate in this pathway, will fill this gap providing us with new insights into the regulation of ureide pathway in response to abiotic stresses at translational and post-translational levels.

5. Appendices

Appendix A: Supplementary figures and tables

Table A1. List of Primers employed to confirm T-DNA insertions in *ALN* and *AAH*.

Gene Name	Allantoinase (At4g04955)	Allantoate amidohydrolase (At4g20070)
T-DNA Inserted Line	SAIL-810-E12	SALK-112631
Forward Primer	GCTGAATAAAGCACGTAAGCG	CATGCAATGGACAATAC CATG
Reverse Primer	AATATCTCACCACAACCGTCG	TGGAAAGTGTTTGCAAA AACC
Left T-DNA border primer	TAGCATCTGAATTTCATAACCAA TCTCGATACAC	ATTTTGCCGATTTCGGAA C

Table A2. List of *abi* mutants

Gene Name	**Mutant name**	**Stock number**	**Background**
ABA Insensitive1 (At4g26080)	*abi1-1*	CS22	Ler-0
ABA Insensitive3 (At3g24650)	*abi3*	CS6130	Ler-0
ABA Insensitive4 (At2g40220)	*abi4-1*	CS8104	Col-0

Table A3. Primer pairs designed to amplify *ALN* coding sequence. Each primer contains a "CACACA" overhangs and restriction site of either NCoI or BstEII (letters in red).

Gene	Allantoinase (*ALN*) (At4g04955) Coding sequence
Forward Primer (NCoI Restriction Site)	CACACACCATGGTGGAGAGAACTTTGCTTCAATGG
Reverse Primer (BstEII Restriction Site)	CACACAGGTCACCTTGCAACATGAGAGCAACTCTT
Product Size	1603 bp

Table A4. *ALNox* lines and calculated ratios.

	Lines	Hyg-R	Hyg-S	Total	Ratio	Expected Hyg-R	Expected Hyg-S	Chi-Square (X^2)
Transformed Plants (T0)	T0 - 1	20	180	200	0.11111			
	T0 - 2	12	188	200	0.06383			
	T0 - 3	4	196	200	0.02041			
	T0 - 4	7	193	200	0.03627			
T1 (from T0 - 1)	T1 - 11	125	58	183	2.15517	137.25	45.75	4.373406193
	T1 - 12	103	72	175	1.43056	131.25	43.75	24.32190476
	T1 - 13	190	35	225	5.42857	168.75	56.25	10.7037037
T1 (from T0 - 2)	T1 - 21	64	38	102	1.68421	76.5	25.5	8.169934641
	T1 - 22	45	25	70	1.8	52.5	17.5	4.285714286
	T1 - 23	65	6	71	10.8333	53.25	17.75	10.37089202
T1 (from T0 - 3)	T1 -31	15	71	86	0.21127	64.5	21.5	151.9534884
	T1 -32	56	30	86	1.86667	64.5	21.5	4.480620155
	T1 -33	72	44	116	1.63636	87	29	10.34482759
T2 (from T1 - 11)	T2 - 111	62	13	75	4.76923	56.25	18.75	2.351111111
	T2 - 112	130	60	190	2.16667	142.5	47.5	4.385964912
	T2 - 113	46	30	76	1.53333	57	19	8.49122807
T3 (from T2 - 111)	T3 - 111-1	41	14	55	2.92857	41.25	13.75	0.006060606
	T3 - 111-2	20	35	55	0.57143	41.25	13.75	43.78787879
	T3 - 111-3	16	39	55	0.41026	41.25	13.75	61.82424242
	T3 - 111-4	25	30	55	0.83333	41.25	13.75	25.60606061

Table A5. Chi-Square (X^2) significance table. 0.05 probability level at df = 1 was considered to validate calculated X^2. df = n-1. As df, degree of freedom; n, number of tested factors. Table is taken from Rana and Singhal (2015).

	Probability level (alpha)					
df	0.5	0.10	0.05	0.02	0.01	0.001
1	0.455	2.706	3.841	5.412	6.635	10.827
2	1.386	4.605	5.991	7.824	9.210	13.815
3	2.366	6.251	7.815	9.837	11.345	16.268
4	3.357	7.779	9.488	11.668	13.277	18.465
5	4.351	9.236	11.070	13.388	15.086	20.517

Table A6. List of Primers used in RT-PCR.

Gene Name	Abbreviation	Forward Primer	Reverse Primer	Size
Actin 7 (At5g09810)	*ACT7*	GATATTCAGCCACTTGTCTGTGAC	CATGTTCGATTGGATACTTCAGAG	187
Allantoate amidohydrolase (At4g20070)	*AAH*	AAGGGACGAAGCTGTAGCAA	TAGCACTTGGCCATGTTGAG	872
Allantoinase (At4g04955)	*ALN*	GAACTAAGGCTGCTGCTGCT	TTCAGGTTCCCACACAACAA	977
Allantoin synthase (At5g58220)	*ALNS*	AATTCGCGAAGCAGATGTCTA	AGTGGGACATGGAAATGCTC	880
Uricase (At2g26230)	*UO*	GAAAGCCAAGGAATGTGGAG	TGAAGGGTTTTCCTTCGTTG	662
Xanthine dehydrogenase 1 (At4g34890)	*XDH1*	CGCGGATACACATGAAAATG	TTTCCAGAGCTGATGCAATG	904

Table A7. List of Primers employed in qRT-PCR analysis.

Gene Name	Abbreviation	Forward Primer	Reverse Primer
Actin2 (At3g18780)	*ACT2*	TTGTGCTGGATTCTGGTGATGG	CCGCTCTGCTGTTGTGTG
Allantoinase (At4g04955)	*ALN*	CCTGGTCTCATTGATGTGCATGTTC	TGTTTTTCGCAGCTTCAATCTTGAGT
Allantoate amidohydrolase (At4g20070)	*AAH*	CTTTTGTGCTCCATTGAACGAAAGC	AACAACATTCCCACCTTGGTTAAGTGT
Uricase (At2g26230)	*UO*	CACTGTTTATGTGAAAGCCAAGGAATG	CCCAAGCTTAAAACCATGTAAATGTGG
Ascorbate peroxidase1 (At1g07890)	*APX1*	CCACTCGCATTTCTCCAGAT	TCGAAAGTTCCAGCAGAGTG
Ascorbate peroxidase2 (At3g09640)	*APX2*	ACCCGCTCATTTTTGACAAC	TTCGAAGAAGGCATCCTCAT
Mn-Superoxide dismutase1 (At3g10920)	*MSD1*	CCTTACGATTATGGCGCATT	CCTTCACTGGAAGGAGCAAG
Cu-Superoxide dismutase1 (At1g08830)	*CSD1*	TGGCGAAAGGAGTTGCAGTT	TGGCAATCAGTGATTGTGAAG
Fe-Superoxide dismutase1 (At4g25100)	*FSD1*	CATCAGGAGAGCTTCTTGCTTT	ACAGCTTCCCAAGACACAAGAT
Catalase3 (At1g20620)	*CAT3*	GACTACATGTCCCACTTGCC	TCTTGATCCCACAAGTTGGT

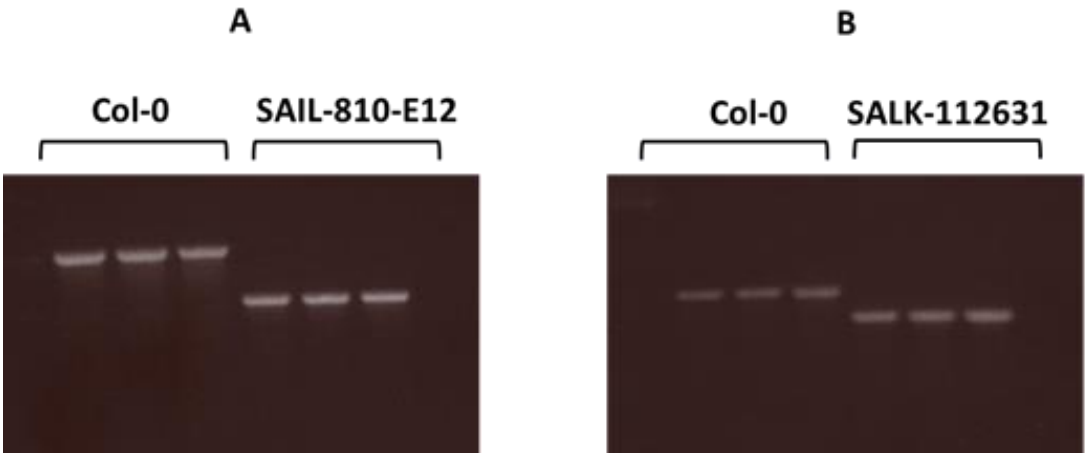

Figure A1. Screening T-DNA insertion in homozygous (A) SAIL-810-E12 and (B) SALK-112631 mutants using three-primer PCR. As described in (http://signal.salk.edu/tdnaprimers) amplification of wild-type gDNA (containing no T-DNA insertion) with three primers results in a PCR product of ~900 bp, showing the amplified DNA sequence between forward and reverse primers. Whereas, PCR product of homozygous mutant lines (containing T-DNA insertion in both chromosomes) is ~400 bp. Picture shows PCR products of three independent wild-type and mutant lines. SAIL-810-E12, allantoinase-negative mutant (*aln-3*); SALK-112631, allantoate amidohydrolase-negative mutant (*aah*).

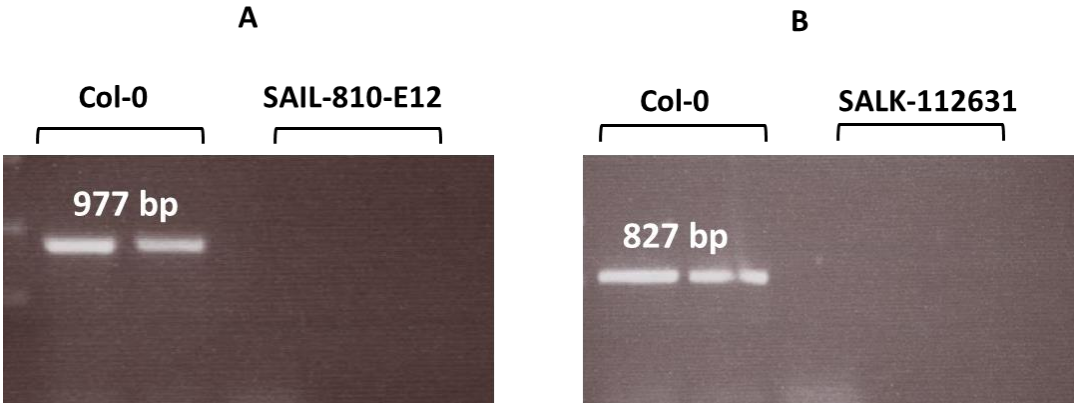

Figure A2. Absence of (A) *ALN* transcripts in *SAIL-810-E12* mutants and (B) *AAH* transcript in *SALK-112631* mutants. PCR was carried out employing RT-PCR primers in Table A6. *ALN*, allantoinase gene; *AAH*, allantoate amidohydrolase gene; SAIL-810-E12, allantoinase-negative mutant (*aln-3*); SALK-112631, allantoate amidohydrolase-negative mutant (*aah*).

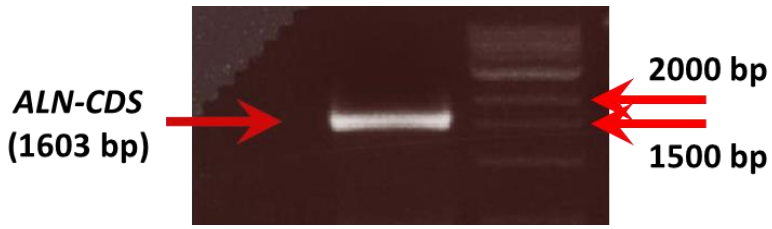

Figure A3. Double digestion of *ALN-CDS* using two restriction enzymes NCoI and BstEII**.** *ALN-CDS*, allantoinase coding sequence.

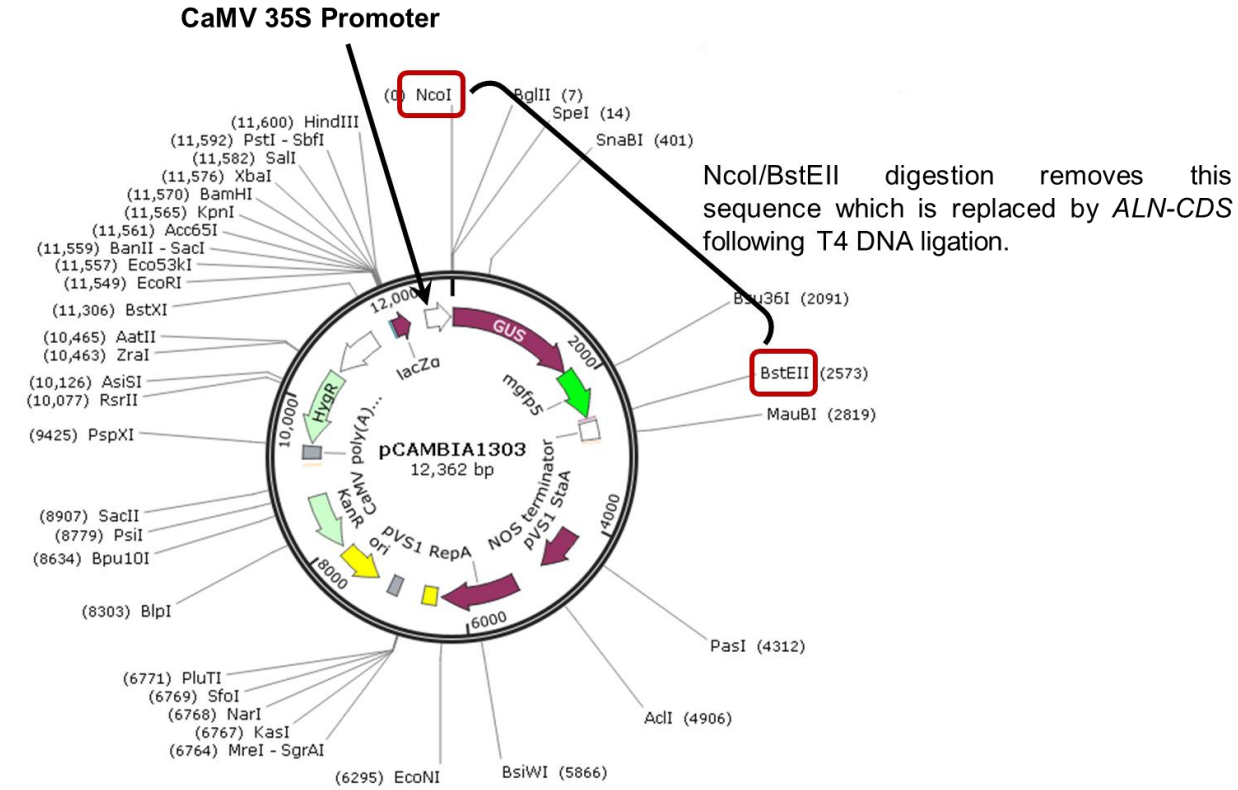

Figure A4. pCAMBIA 1303 construct used to generate allantoinase-overexpressed (*ALNox*) lines. Restriction sites of NcoI and BstEII are shown as red boxes. Employing these two restriction enzymes removes reporter genes, GUS+mgfp5, allowing allantoinase coding sequence (*ALN-CDS*) to express under the control of CaMV 35S promoter. This picture is taken from (https://www.novoprolabs.com/vector/V10891).

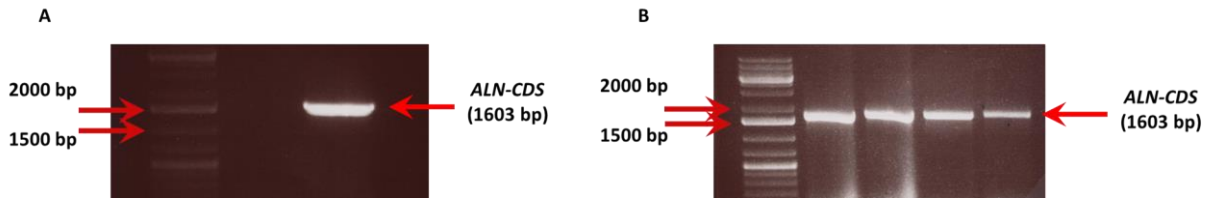

Figure A5. PCR product obtained from Colony PCR of (A) Kanamycin-resistant *E. coli* and (B) Kanamycin-resistant *A. tumefaciens*.

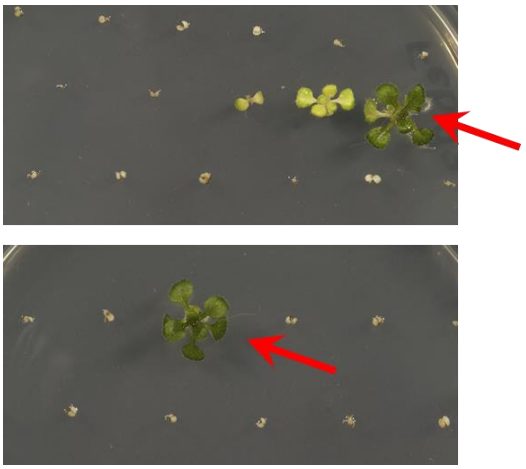

Figure A6. Seeds obtained from Col-0 Arabidopsis plants transformed by *A.tumefaciens* germinated on 25 µg ml^{-1} Hygromycin (Hyg) plates. Only truly transformed seeds were able to develop root and leaf and stayed green (red arrows) on Hyg.

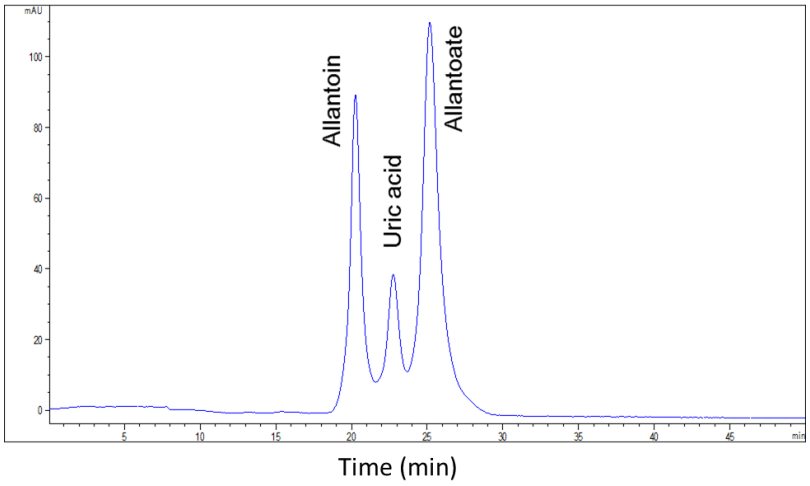

Figure A7. Typical chromatogram obtained from pure allantoin, uric acid and allantoate.

Appendix B: Effect of allantoin content on photosynthetic features of Col-0 and *aln-3* Arabidopsis

B1. Introduction

Photosynthetic electron transport chain composes of two light reaction centers (photosystems I and II), plastoquinone (Q), b_6-f complex ($Cytb_6f$), plastocyanin (Pc), ferredoxin (Fd), ATP synthase and NADP reductase locating in thylakoid membrane (Rochaix, 2011). Electron transfer starts with the exciting energy obtained from sunlight that moves one electron from PSII to the first electron accepter, Q (Joliot and Joliot, 2006). This electron transfer is coupled with a water splitting reaction mediated by PSII in the luminal side of thylakoid to release molecular oxygen and one electron from water that compensates the removed light-energized electron from PSII (Seelert *et al*., 2000). Q passes the electron to $Cytb_6f$ that functions as a proton (H^+) pump, transferring one H^+ from stroma to thylakoid lumen while accepting electron from Q (Page *et al*., 2004). Therefore, the function of $Cytb_6f$ causes a H^+ gradient across thylakoid membrane that drives ATP synthesis in chloroplast stroma. This process that is catalyzed by the enzyme ATP synthase is called photophosphorylation (Finazzi *et al*., 1999). Once energy of sunlight is absorbed by PSI, excited electron moves from PSI to ferredoxin (Fd) that is finally consumed by NADP reductase to generate NADPH (Hase *et al*., 2006). The lost electron in PSI is replaced by the electron from $Cytb_6f$ which is carried by PC. ATP and NADPH produced from light reactions (Hill reaction) are then employed by Calvin cycle to fix CO_2 (Hill and Bendall, 1960; Rosso *et al*., 2006).

In response to the captured energy from sunlight, three possible ways initiate in chloroplast. The balance between these three processes determine the quantum yield of photosynthesis which is an important indicator of plant response (tolerance or sensitivity) under stress conditions (Derks *et al*., 2015). Excitation energy from sunlight may pass through photochemical reactions (PSI and II) that leads to the generation of ATP and NADPH which are eventually recruited in photosynthesis as described above. It is called photochemical quenching (PQ). The excess energy, which is not used via photochemistry, is dissipated as heat which is known as non-photochemical quenching (NPQ) or is released as fluorescence (Schreiber, 2004; Gerotto *et al*., 2013; Endo *et al*., 2014). The quantum yield (Y) of photosynthesis is defined as the relative proportion of PQ to NPQ (Y = 1 – NPQ). Therefore, this measurement illustrates the effective function of photosynthetic machinery

in a plant, specifically when exposed to stress condition (Salehian and Bruce, 1992). In this experiment the effect of Cd treatment on electron transport rate (ETR) and photosynthetic yield (Y) of PSI and II has been evaluated in *aln-3* mutants and Col-0 Arabidopsis. This experiment has been carried out to study the influence of Cd on photosynthesis, also to clarify the potential effect of allantoin on ETR and Y.

B2. Material and Methods

aln-3 mutants and Col-0 Arabidopsis grew as explained in section 2.1.4. ETR (I) and (II) and Y (I) and (II) were measured for intact leaves on the plant employing Dual-PAM-100 Measuring System after 5 min dark treatment.

B3. Results

The effect of different Cd concentrations on the electron transport rate (ETR) in photosystem I and II, ETR (I) and ETR (II), are shown in Fig. B1. Results indicated that Cd had a negative effect on ETR of PS I and II in both genotypes. ETR (II) showed a value of 12-16 µmol / m^2s under control condition and decreased to 6-8 µmol / m^2s at the highest Cd concentration (1500 µM $CdCl_2$). Similarly, ETR (I) of Col-0 and *aln-3* Arabidopsis showed a significant decreasing trend from 20-25 µmol / m^2s at control to 10-15 µmol / m^2s in 1500 µM $CdCl_2$-treated samples. Although *aln-3* mutants constantly showed higher values in comparison with Col-0 Arabidopsis, the difference between these two genotypes was not statistically significant.

As shown in Fig. B2, quantum yeild of PS I and II decreased in response to increasing Cd concentration in wild-type and *aln-3* mutants. Consistent with the data shown for ETR, yeild of PS I and II in *aln-3* mutants demonstrated relatively higher numbers in respect with Col-0 Arabidopsis but the difference between these two genotypes is not statistically significant.

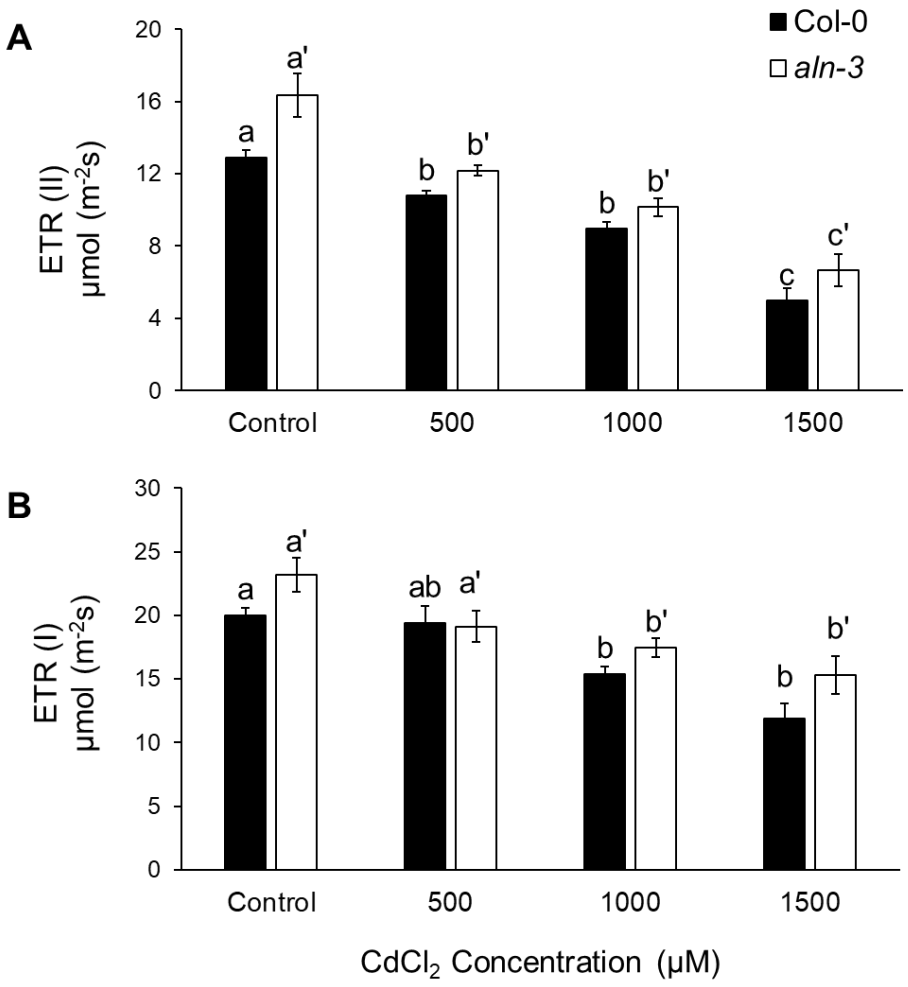

Figure B1. Effect of Cd treatment on (A) Electron Transport Rate in PSII, ETR (II) and (B) Electron Transport Rate in PSI, ETR (I) in Col-0 and *aln-3* Arabidopsis. Values shown are the mean of five independent replicates ± SE. Different letters (a,b,c in Col-0 and a',b',c' in *aln-3* samples) show significant differences in each genotype ($P \leq 0.05$).

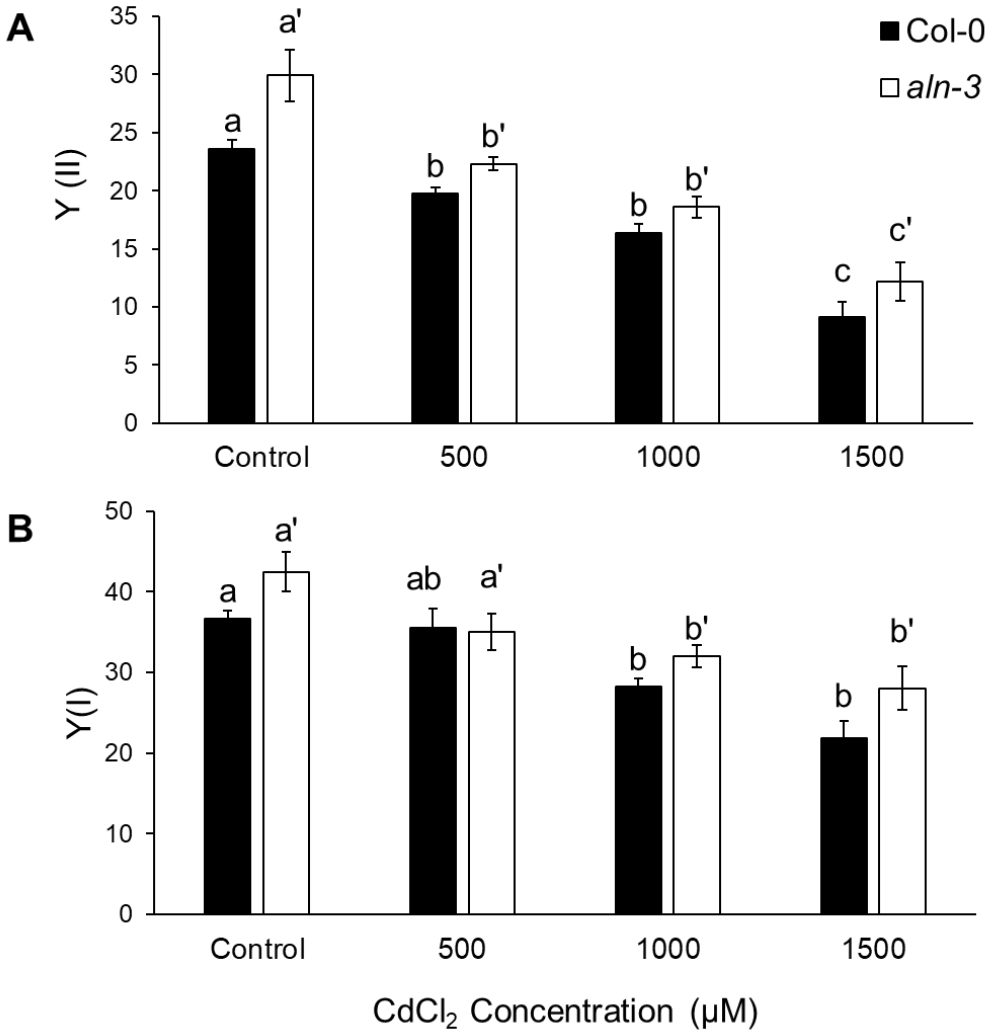

Figure B2. Effect of Cd treatment on (A) quantum yields of PSII, Y (II) and (B) quantum yields of PSI, Y (I) in Col-0 and *aln-3* Arabidopsis. Values shown are the mean of five independent replicates ± SE. Different letters (a,b,c in Col-0 and a',b',c' in *aln-3* samples) show significant differences in each genotype ($P \leq 0.05$).

B4. Discussion

The effect of Cd on photosynthesis has been reported in different studies, showing that Cd has both a direct inhibitory impact on photosynthesis and an indirect effect via interference with other stress response mechanisms such as antioxidant systems (DalCorso *et al*., 2010). Direct inhibitory effect of Cd is associated with its similarity with other cations such as Mg^{2+}, Zn^{2+} and Fe^{2+} (Parmar *et al*., 2013). Cd is replaced by Mg^{2+} in the structure of chlorophyll that causes a significant decrease in chlorophyll content of the cell (Gillet *et al*., 2006). Moreover, substitution of Cd for Ca^{2+} and Mn^{2+} in the cofactor of oxygen evolving complex decreases the kinetic of Hill reaction (Faller *et al*., 2005; Pagliano *et al*., 2006). It was also shown that Cd impinges on electron transport rate via preventing electron transfer between Q_A and Q_B (Geiken *et al*., 1998). Additionally, once the plastoquinone pool is in a highly reduced state due to a block of forward electron transport, excitation energy gives rise to the generation of triplet chlorophyll. If not quenched by carotens in PSII reaction centre, triplet chlorophyll can react with oxygen and forms singlet oxygen ($^1O_2$). Generated $^1O_2$ may be detoxified by carotenoids, otherwise it immediately interacts with proteins in the reaction centre (Krieger-Liszkay, 2004). Therefore, Cd can disrupt the function of photosynthetic electron transport chain, decrease photosynthetic quantum yield and interrupt with CO_2 fixation process (DalCorso *et al*., 2010; Pietrini *et al*., 2010). My results showed that both measured factors, ETR and Y in PSI and II, are negatively influenced by Cd. However, ETR and Y of *aln-3* mutants show relatively higher values in comparison with Col-0 samples. It has been demonstrated that chlorophyll content of *aln* mutants exposed to NaCl treatment and drought shock was more than that of wild-type Arabidopsis. Irani and Todd (2016) also reported a higher chlorophyll content and maximal quantum yield of PSII photochemistry (F_v/F_m ratio) in *aln-3* mutants exposed to water limitation when compared with Col-0 Arabidopsis. Taken together, these studies suggest that allantoin is likely associated with chlorophyll biosynthesis and the efficiency of photosynthetic machinery under stress condition. It also indicates that positive effect of allantoin on chlorophyll concentration and F_v/F_m value results in improved plant growth of *aln-3* mutants in response to stress. Nonetheless, to my knowledge the direct impact of allantoin on the function and structure of chlorophyll biosynthesis procedure, electron transport chain in chloroplast and photosynthetic apparatus in response to stress hasn't been studied yet. Therefore, more investigations are required in this field to explain whether allantoin improves the function and/or

configuration of photosynthetic components or it protects this complex from damaging effect of stress-induced ROS that leads to the better efficiency of photosynthesis in *aln-3* mutants.

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