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Exploring the role of the thioredoxin system, peroxiredoxins and glutaredoxins in aluminum and cadmium tolerance in yeast and *Arabidopsis thaliana*

by

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Abstract

Aluminum (Al) and cadmium (Cd) are non-redox active metal ions of agricultural importance. Both are able to induce oxidative damage as a mechanism of toxicity. To increase our understanding of the mechanisms of Al and Cd toxicity and tolerance in plants, the potential role of antioxidant enzymes of the thioredoxin system, peroxiredoxins and glutaredoxins in Al and Cd tolerance was investigated in yeast (*Saccharomyces cerevisiae*) and *Arabidopsis thaliana*. Single and multiple mutants defective in genes of the thioredoxin system, peroxiredoxins and glutaredoxins in yeast were used to identify relevant genes in Al and Cd tolerance. A mutant defective in cytoplasmic thioredoxin reductase (*TRR1*) showed the most hypersensitive phenotype and increased levels of lipid peroxidation upon Al and Cd exposure. Transcript levels of *TRR1* increased with increasing concentrations of Al and Cd. Complementation of the *trr1*Δ mutant with the wild-type *TRR1* gene was able to restore growth to WT levels in the presence of Al and Cd. These results suggest that thioredoxin reductases are relevant genes in Al and Cd tolerance in yeast and that these genes might be also relevant in Al and Cd tolerance in plants.

The role of NADPH-thioredoxin reductases (NTRs) in Al and Cd tolerance was further investigated in *Arabidopsis thaliana*. Among the mutants studied in *Arabidopsis,* only lines defective in NTRs (*NTRA* and *NTRB*) showed reduced growth compared to WT upon Al and Cd exposure. *NTRA* and *NTRB* mutants also showed increased levels of Al- and Cd-induced lipid peroxidation compared to WT. However, *NTRA* and *NTRB* transcript levels did not show a clear induction with increasing concentrations of Al and

Cd. *NTRA* and *NTRB* were overexpressed in *Arabidopsis* to test whether increased expression of NTRs confers increased tolerance to Al and Cd. Only lines overexpressing *NTRA* showed increased transcript, protein and enzyme activity levels. However, these lines did not show increased tolerance to Al and Cd. My results suggest that NTRs might not be important ROS scavengers upon Al and Cd exposure, but they may play a role as a signal transducer and modulate other antioxidant enzymes.

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Dedication

A mi padre, Jorge López Origel

y

a mi hija, Isabel

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Al,	aluminum
ABRC,	Arabidopsis Biological Resource Center
APX,	ascorbate peroxidase
bp,	base pairs
^{o}C,	degrees Celsius
CaMV,	cauliflower mosaic virus
CAT,	catalase
Cd,	cadmium
CDS,	coding sequence
cDNA,	complementary DNA
Cu,	copper
Cys,	cysteine
d,	day (s)
DNA,	deoxyribonucleic acid
DTT**,**	dithiothreitol
EDTA,	ethylene diamine tetra acetic acid
Fe,	iron
g; mg; µg,	gram, milligram, microgram
GPX,	glutathione peroxidase
GRX,	glutaredoxin
GSH,	tripeptide glutathione

h,	hour (s)
Hg,	mercury
Kb,	kilo base pair (s)
KD,	kilo Dalton
L; ml; µl	liter, milliliter, microliter
LB,	left border
MDHAR,	monodehydroascorbate reductase
M; mM; µM,	molar, millimolar, micromolar
mol; µmol; nmol	mole, micromole, nanomole
mRNA,	messenger RNA
MS,	Murashige-Skoog
NADP(H),	nicotinamide adenine dinucleotide phosphate
NTR,	NADP(H)-thioredoxin reductase
Ni,	nickel
ORF,	open reading frame
%,	percent
PAGE,	polyacrylamide gel electrophoresis
Pb,	lead
PCR,	polymerase chain reaction
PMSF,	phenylmethylsulfonyl fluoride
PRX,	peroxiredoxin (thioredoxin peroxidase)
PVPP,	polyvinylpolypyrrolidone
QRT-PCR,	quantitative reverse transcription PCR

RB,	right border
RNA,	ribonucleic acid
ROS,	reactive oxygen species
rpm,	revolutions per minute
s,	second (s)
SE,	standard error of the mean
SOD,	superoxide dismutase
-SH,	sulfydryl group (s)
TCA,	tricarboxylic acid
T-DNA,	transfer-DNA
TRR,	thioredoxin reductase
TRX,	thioredoxin
UBC,	ubiquitin-conjugating enzyme
UTR,	un-translated region
v/v; v/w; w/v,	volume per volume, volume per weight, weight per volume
WT,	wild type
YPD,	yeast peptone dextrose

1. General Introduction

1.1. Understanding the mechanisms of metal toxicity and tolerance in plants: the case of aluminum and cadmium

1.1.1. Aluminum

Aluminum (Al) phytotoxicity is one of the major factors limiting plant growth on acid soils (pH < 5). The trivalent Al species (Al^{3+}), a major phytotoxic form of Al, becomes available at low pH and causes rapid inhibition of root elongation. Approximately 30-40% of the world's arable lands are acidic (von Uexküll and Mutert, 1995). Soil acidification occurs naturally due to leaching of minerals, but certain agricultural practices, such as the use of ammonium fertilizers, promote further soil acidification (Rasmussen and Rohde, 1989). A common strategy used to overcome this problem is the application of lime (calcium carbonate) to increase soil pH. This is often an uneconomical practice since large areas of soil usually need amendment. Even though Al is the metal ion best studied in the field of metal-induced stress in plants, a better understanding of the mechanisms of Al toxicity and tolerance would aid in identifying Al-tolerant germplasm and in the development of Al-tolerant transgenic plants.

1.1.1.1. Aluminum phytotoxicity

The first visible symptom of Al toxicity is the rapid inhibition of root elongation, which occurs within minutes after exposure to Al (Llugany *et al*., 1995; Delhaize and Ryan, 1995). The root apex has been found to be the primary site of Al toxicity. In fact, the terminal 2-3 mm of maize and pea roots, which includes the meristem and root cap, is

the only area that needs to be exposed to Al to cause inhibition of root growth. When the entire root except the root apex is exposed to Al, root growth is unaffected (Ryan *et al*., 1993; Matsumoto *et al*., 1996). The root apex also accumulates more Al than other portions of the root. After a short exposure to Al, Delhaize *et al*., (1993) stained wheat roots with hematoxylin (a compound that binds Al to form a colored complex) and the root apices showed the most intense staining. Furthermore, staining was less intense in Al-tolerant wheat (*Triticum aestivum*) lines than in Al-sensitive wheat lines, which has been interpreted to suggest that exclusion of Al from the root apices is one of the mechanisms of Al tolerance in these lines (Delhaize *et al*., 1993; Sasaki *et al*., 1997). Research on the fundamental mechanisms of Al toxicity and tolerance has consequently been directed to Al interactions within the root apex.

The rapid inhibition of root elongation induced by Al has been correlated with a reduction of cell division in root meristems (Clarkson, 1965). Accumulation of Al has been detected in the nuclei of root hair cells prepared from Al-treated pea and soybean (Matsumoto *et al*. 1976; Silva *et al*., 2000), and 73% of the total Al in nuclei was recovered in the chromatin fraction (Matsumoto *et a*l., 1977). The interaction of Al with nuclear constituents suggests that the toxicity of Al may be associated with inhibition of DNA replication. Furthermore, Sivaguru *et al*., (1999) observed that disintegration of spindle microtubules occurs after 24 h of Al treatment, which might block cell division directly at metaphase. However, since mitotic activity decreases after several hours of Al exposure, Al-induced inhibition of cell division cannot be the initial cause of root growth inhibition (Clarkson, 1965; Votrubová *et al*., 1997). Accordingly, inhibition of cell elongation is considered the initial event responsible for the rapid inhibition of root

growth after Al treatment. A reduction in length of root cells of barley, wheat and maize, accompanied by their radial expansion, suggests that Al affects orientation of cellular growth (Nichol and Oliveira, 1995; Sasaki *et al*., 1997; Budiková 1999; Votrubová *et al*., 1997). Recently, Abdel-Basset *et al*. (2010) found that Al inhibits sucrose uptake within 3 h of exposure, which contributes to the inhibition of cell elongation, but does not account for cell death.

The plasma membrane has often been considered a primary site of Al toxicity. Aluminum (Al), being a "Class A" metal, tends to form electrostatic bonds preferentially with oxygen donor ligands, therefore showing a strong affinity for the plasma membrane where it can bind either to carboxylate or phosphate groups (Nieboer and Richardson, 1980; Akeson *et al*., 1989). Aluminum bound to phosphate groups causes structural and functional changes in the plasma membrane. For instance, aluminum alters the architecture of membrane lipids, which affects membrane permeability (Vierstra and Haug, 1978; Chen *et al*., 1991). Moreover, Al binding to the plasma membrane creates a positively charged layer at the membrane surface, which alters its electrophysiological properties. This positively charged layer inhibits the movement of cations to the plasma membrane (Sasaki, *et al*., 1994; Miyasaka *et al*., 1989).

Aluminum also affects the movement of ions through the plasma membrane by binding directly to transport proteins such as K^+ and Ca^{2+} channels and H^+-ATPases (Dill *et al*., 1987; Huang *et al*., 1996; Ahn *et al*., 2001). Since Al-induced inhibition of root apical Ca^{2+} influx correlated well with Al-induced inhibition of root growth, the effect of Al on calcium transport has consequently been studied intensively. It has, however, been

shown that root growth inhibition occurs at low Al concentrations without affecting Ca^{2+} uptake (Ryan *et al*., 1997).

The aluminum phytotoxic response comprises a multitude of symptoms and alterations of physiological processes making it difficult to pinpoint a primary cause for inhibition of root growth. One of the processes affected by Al is respiration. Mitochondrial activity and oxygen uptake is repressed by Al in wheat roots and cultured tobacco cells (de Lima and Copeland, 1994; Yamamoto *et al*., 2002). In addition, respiration rates declined in *Phaseolus vulgaris* (cv Romano) over a 72 h Al treatment (Cumming *et al*., 1992). Treatment with Al also affects carbon metabolism. In Al-treated wheat (*Triticum aestivum* cv Vulcan) roots, the activities of alcohol dehydrogenase, sucrose synthase and lactate dehydrogenase increased, suggesting that a shift from aerobic to anaerobic metabolism takes place soon after exposure to Al (Copeland and de Lima, 1992). Similarly, the activity of two enzymes of the pentose phosphate pathway increased in an Al-resistant wheat cultivar after Al exposure, while no changes were observed in the activity of these enzymes in an Al-tolerant cultivar (Slaski *et al*., 1996).

Aluminum exposure has been observed to elicit the formation of reactive oxygen species (ROS) and to promote peroxidation of phospholipids and proteins in cell membranes (Cakmak and Horst, 1991; Yamamoto *et al*., 2001). It has been suggested that the interaction of Al with membranes facilitates the generation of ROS via redox-active (capable of catalyzing reduction-oxidation reactions) metals such as iron (Fe) (Oteiza, 1994; Yamamoto *et al*., 1997). Another proposed mechanism for Al-induced oxidative stress involves a disturbance in the balance of redox reactions that increase the

prooxidant activity of phenolic compounds (Sakihama and Yamasaki, 2002). Aluminum-induced oxidative damage does not seem to be a primary event that leads to root growth inhibition, but it is part of the overall Al-phytotoxic response. Currently, we do not have a clear understanding of the link between Al toxicity and oxidative stress. This thesis will endeavor to clarify the role that enzymes that protect against oxidative stress play in minimizing Al and Cd-induced injury of plants.

1.1.1.2. Mechanisms of aluminum tolerance

Mechanisms of Al tolerance have been classified into two categories: mechanisms that facilitate the exclusion of Al from the root symplasm (Al-exclusion mechanisms), and mechanisms that confer an ability to tolerate Al once it has entered the plant symplasm (internal tolerance mechanisms) (Taylor, 1991). Some of these mechanisms may be controlled by one or more major genes as well as several minor genes that may play a role in modulating the major Al tolerance genes. Some of the evidence that supports the hypothesis that multiple tolerance mechanisms operate in Al resistant plants come from analyses of the genetic complexity of Al tolerance in rice, wheat, maize and *Arabidopsis* (Ma *et al*., 2002; Papernik *et al*., 2001; Giaveno *et al*., 2001; Hoekenga *et al*., 2003).

The most extensively characterized mechanism of Al tolerance is the exclusion of Al from the root apex that is mediated by an Al-induced release of organic anions. Organic anions such as malate, citrate or oxalate form complexes with Al that do not appear to be transported across membranes. Evidence for this Al-tolerance mechanism

was initially found in studies focusing on Al-tolerant genotypes of snapbean and wheat that exhibited a strong Al-activated release of organic anions (Miyasaka *et al*., 1991; Delhaize *et al*., 1993). Currently, several lines of evidence show a strong correlation between Al-induced exudation of organic anions and Al resistance in numerous plant species (for review, see Ryan *et al*., 2001; Panda and Matsumoto, 2007; Singh and Chauhan, 2011).

A transport mechanism that could mediate Al-induced exudation of organic anions is the activation of an anion channel located in the plasma membrane (Piñeros and Kochian, 2001; Ryan *et al*., 1997; Zhang *et al*., 2001). Sasaki *et al*. (2004) isolated a wheat gene that encodes an Al-activated malate transporter and expression of this gene (*ALMT1*) conferred Al-activated malate exudation in *Xenopus oocytes*, transgenic rice seedlings and tobacco suspension cells. Furthermore, the expression of *ALMT1* in tobacco suspension cells increased Al tolerance. These findings suggest that *ALMT1* may be one of the first major genes identified that control Al tolerance in plants.

Another mechanism of Al exclusion that has been proposed relies on root-mediated increases in rhizosphere pH. However, some reports support this hypothesis whereas others contradict it. For instance, Degenhardt *et al*. (1998) observed a correlation between Al tolerance and Al-activated root apical H^+ influx in the Al-tolerant *Arabidopsis* mutant (*alr*-104). This H^+ influx resulted in an increase in root surface pH, presumably decreasing the activity of the toxic Al^{3+} species and improving root growth. In contrast, a lack of correlation between pH changes and Al tolerance was observed in wheat (Taylor and Foy, 1985; Miyasaka *et al*., 1989).

Secretion of mucilage is another mechanism that may play a role in Al exclusion. Mucilage is mainly formed of polysaccharides and has a high Al-binding capacity. Horst *et al*. (1982) found that root cap mucilage binds Al in cowpea and that the removal of mucilage increased Al sensitivity. Although mucilage from maize roots was also shown to bind Al, it did not protect these roots from Al (Li *et al*., 2000). Other mechanisms of Al exclusion such as a localized excretion of phosphate at the root apex to precipitate Al (Pellet *et al*., 1995), binding of Al by secreted proteins (Basu *et al*., 1999), and Al-induced exudation of phenolic compounds (Kidd *et al*., 2001) have also been proposed, but require further research.

Research in Al-accumulator plants such as hydrangea and buckwheat has increased our understanding of an important internal Al detoxification mechanism (Ma *et al*., 1997; Ma *et al*., 1998). This mechanism involves the chelation of Al by organic anions or phosphates in the cytosol and subsequent storage of the Al complex in the vacuole. The Al-chelating intracellular organic anions are mainly oxalate and citrate. With the neutral pH of the cytosol, Al forms a strong complex with organic anions such as citrate, thus protecting the cell from Al injury. It has been shown that Al undergoes a ligand exchange from oxalate to citrate when it is transported into the xylem, and exchanged back to oxalate when transported into the leaves (Ma and Hiradate, 2000).

Regulation of gene expression may be an important factor in the operation of Al tolerance mechanisms. For instance, glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase (Slaski *et al*., 1996) and vacuolar H^+ ATPase (Kasai *et al*., 1992, Hamilton *et al*., 2001) are gene products induced by Al that are thought to play

a role in the maintenance of cellular metabolism during Al stress. However, many of the gene products induced by Al have also been observed to be induced by other abiotic stresses and can be considered part of a general stress response. For instance, Al has been observed to increase the levels of expression of genes that also respond to heat shock (Ezaki *et al*., 1998), osmotic stress (Hamilton *et al*., 2002), phosphate starvation (Ezaki *et al*., 1995), pathogens (Hamel *et al*., 1998), oxidative stress (Richards *et al*., 1998; Basu *et al*., 2001) and also to other metal ions (Snowden *et al*., 1995; Sugimoto and Sakamoto, 1997).

The interaction between Al and components of signal transduction pathways has not been studied in detail, but appears to be important in the establishment of certain mechanisms of Al tolerance in plants. For instance, it has been suggested that the exudation of organic anions in wheat is activated as a result of the Al signal being perceived and transduced in the plasma membrane and cytosol (Osawa and Matsumoto, 2001; Sasaki *et al*., 2004). Although more research is needed in this area, the rapid activation of a protein kinase in cell suspension cultures of *Coffea arabica* L. and the expression of a cell wall-associated receptor kinase in *Arabidopsis*, by toxic concentrations of Al further supports the idea that the transduction of the Al signal could be an important step in the establishment of Al tolerance (Sivaguru *et al*., 2003; Arroyo-Serralta, 2005). More recently, it has been found that Al induces ethylene production, which is likely to act as a signal to alter auxin distribution in roots (Sun *et al*., 2010).

The antioxidant defense systems have been found to play a role in Al tolerance. The antioxidant systems may protect plants against Al-induced oxidative stress by

scavenging reactive oxygen species or repairing oxidatively damaged proteins. Several studies have reported elevated RNA and increased activities of various antioxidant enzymes in plants under Al stress (Richards *et al*., 1998; Boscolo *et al*., 2003; Panda *et al*., 2003). Not surprisingly, overexpression of peroxidases, such as a tobacco peroxidase gene (*NtPox*) and a peroxidase gene from *Arabidopsis* (*AtPox*), has been observed to increase Al tolerance in *Arabidopsis* (Ezaki *et al*., 2000). Similarly, overexpression of a mitochondrial manganese superoxide dismutase in *Brassica napus* resulted in increased resistance to Al (Basu *et al*., 2001). Recently, Yin *et al*. (2010) showed that overexpression of an *Arabidopsis* dehydroascorbate reductase in tobacco confers increased tolerance to Al in transgenic plants.

1.1.2. Cadmium

Cadmium (Cd), a widespread toxic metal with no described biological function, is considered an important environmental pollutant. Cadmium (Cd) is one of the metal ions that is most readily taken up by plant roots and it has by far a greater bioavailability than other toxic metal ions such as lead (Pb) or mercury (Hg) (Clemens, 2006). Unlike Al, Cd is not abundant in the earth's crust and it is found in a wide range of concentrations in natural soils. The main sources of cadmium contamination in soils come from human activities such as fertilization by phosphorous and nitrogen fertilizers as well as the addition of sewage sludge to farmlands (Alloway and Steinnes, 1999; Lambert *et al*., 2007; McBride, 2003). Contamination of soils with Cd is particularly important since it causes phytotoxicity even at low doses (Das *et al*., 1997; Chakravarty and Srivastava,

1994). Cadmium accumulates readily in plants and consumption of crops grown in agricultural soils constitutes the major source of dietary Cd for humans (Wagner, 1993).

The use of plants to clean up contaminated soils (phytoremediation) has gained increasing attention during the last decade as an environmental friendly and cost effective strategy (Kotrba *et al*., 2009). Some plant species such as *Thlaspi caerulescens* are capable of hyperaccumulating considerable concentrations of metal ions, but have a limited phytoremediation potential due to their low biomass and slow growth. Research aimed to elucidate the mechanisms of Cd phytotoxicity and tolerance could be helpful at engineering plants with an improved phytoremediation potential. This knowledge could also be applied in the development of cultivars that do not accumulate high concentrations of Cd, which could pose a risk for human consumption.

1.1.2.1. Cadmium phytotoxicity

High concentrations of Cd, which are not normally found in agricultural soils, may elicit a phytotoxic response in plants that includes visible morphological effects such as chlorosis, leaf roll, stunted growth and inhibition of stomatal opening (Wojcik and Tukendorf, 1999; Nocito *et al*., 2002; Sanità di Toppi and Gabbrielli, 1999; Laetitia *et al*., 2002). Leaf chlorosis appears to be a result of Cd-induced damage to the photosynthetic apparatus, in particular the light harvesting complex II and photosystems II and I (Siedlecka and Krupa, 1996; Baryla *et al*., 2001; Pagliano *et al*., 2006). Leaf roll and stunted growth occur partly due to the Cd-induced irreversible inhibition of the proton pump that is responsible for the elongation rate of cells (Aidid and Okamoto, 1993;

Sanità di Toppi and Gabbrielli, 1999). Cadmium affects water balance and causes the inhibition of stomatal by interfering with movements of K^+, Ca^{2+} and abscisic acid in guard cells (Barceló and Poschenrieder, 1990; Perfus-Barbeoch *et al*., 2002). Other symptoms of Cd toxicity at the cellular level include the inhibition of cell proliferation and cell division, degeneration of mitochondria, chromosomal aberrations and pycnosis (Siegel, 1977; Das *et al*., 1997).

Although the mechanisms of Cd toxicity are not well understood, it has been suggested that Cd may affect a number of metabolic enzymes. The chemical similarity between Cd^{2+} and Zn^{2+} means that Ca^{2+} can replace Zn^{2+} in Zn-binding molecules and thereby interfere with many Zn-dependent processes (Stohs and Bagchi, 1995). Similarly, Cd^{2+} ions compete with Ca^{2+} ions in binding Ca-binding proteins such as calmodulin, thereby affecting cellular signal cascades (Clemens, 2006). Finally, just like in the case of Al, Cd is also capable of causing oxidative stress in plants, even though Cd is not a redox-active metal ion. Symptoms of Cd-induced oxidative stress such as lipid peroxidation have been attributed mainly to glutathione (GSH) depletion and binding of Cd to thiols of several antioxidant enzymes (Schutzendubel and Polle, 2002).

1.1.2.2. Mechanisms of cadmium tolerance

The best studied mechanism of Cd detoxification in plants is binding to phytochelatins (PC). Phytochelatins are glutathione-derived peptides that possess a high-binding capacity to a variety of metal ions, and they are particularly activated by Cd (Sanità di Toppi and Gabbrielli, 1999). Phytochelatins are peptides that are synthesized

enzymatically from GSH in a transpeptidase reaction in response to exposure to metal ions and consist of the general structure $(\gamma\text{-Glu-Cys})_n\text{-Gly}$ (n = 2-11) (Clemens, 2006). The PC pathway consists of two main processes, metal-activated synthesis of the peptides and transport of the metal-PC complex into the vacuole. Phytochelatins start being synthesized by the enzyme phytochelatin synthase within minutes following exposure to Cd (Sanità di Toppi and Gabbrielli, 1999). Phytochelatins form a complex with Cd that is subsequently transported across the tonoplast and accumulated in the vacuole. Once in the vacuole, the PC-Cd complex dissociates due to the acidic pH and Cd ions can be complexed by vacuolar organic anions such as citrate, oxalate and malate (Krotz *et al*., 1989).

Other responses to Cd toxicity in plants include numerous changes in protein abundance, especially the upregulation of carbon, nitrogen and sulfur metabolism to supply precursors of PCs. Similarly, several stress proteins are induced by Cd in several species (Leita *et al*., 1991). Not surprisingly, several components of the antioxidant defense system have shown changes in their activities following Cd exposure (Shaw, 1995; Chaoui *et al*., 1997; Smeets *et al*., 2008). Many questions remain to be answered regarding the role of the various components of the antioxidant defense system in Cd tolerance. One of the objectives of this research is to query the role of the thioredoxin system, glutaredoxins and peroxiredoxins in Cd tolerance.

1.2. Metal-induced oxidative stress

Induction of oxidative stress is a well-known mechanism of toxicity for redox-active metal ions such as Fe and copper (Cu) (Weckx and Clijsters, 1996; Becana *et al*., 1998). These metals act as catalysts in the Fenton and Haber-Weiss reactions (Figure 1-1), producing highly reactive oxygen species (ROS) such as superoxide anion, hydrogen peroxide and hydroxyl radical (Stohs and Bagchi, 1995; Becana *et al*., 1998). Reactive oxygen species attack and modify many cellular constituents and easily decompose lipid peroxides to peroxyl and alkoxyl radicals (Bowler *et al*., 1992; Gurer and Ercal, 2000;

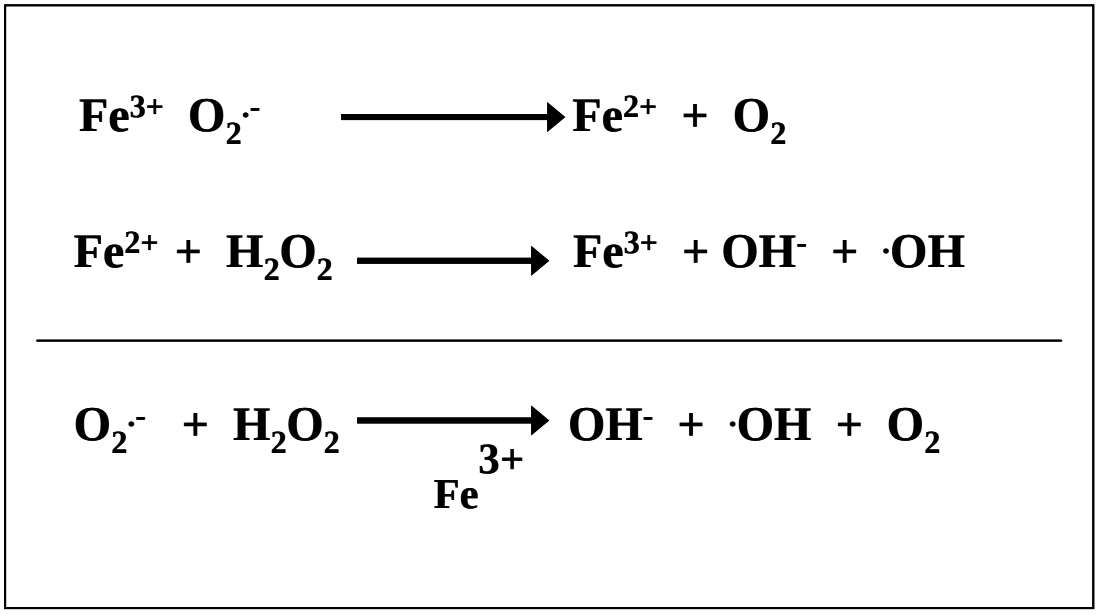

Figure 1-1. The Haber-Weiss and Fenton reactions. Reactive oxygen species are produced during these reactions that are catalyzed by redox active metal ions. Iron (Fe) is shown in these examples. $O_2^{\bullet -}$ (superoxide anion) OH^-, $^{\bullet}OH$ (hydroxyl radicals).

Zago and Oteiza, 2001). Lipid peroxidation has been related to increases in membrane fluidity, membrane permeabilization, and loss of cytosolic solutes, which ultimately leads to loss of plasma membrane integrity and cell death (Gurer and Ercal, 2000; Avery, 2001; Zago and Oteiza, 2001). In addition to lipid peroxidation, other effects of oxidative stress in cells include damage to DNA and proteins, altered calcium homeostasis, depletion of cellular antioxidant defense systems, and disruption of signal transduction pathways (Gurer and Ercal, 2000; Zago and Oteiza, 2001; Galaris and Evangelou, 2002).

Even though the Fenton and Haber-Weiss reactions are unlikely to be catalyzed by non-redox active metals, such as Al (Cakmak and Horst, 1991; Ezaki *et al*., 1996), Cd (Vitoria *et al*., 2001; Chien *et al*., 2001; Pereira *et al*., 2002; Iannelli *et al*., 2002; Fornazier *et al*., 2002), Hg (Cho and Park, 2000), and Pb (Gurer and Ercal, 2000; Malecka *et al*., 2001), there is evidence that these metal ions induce oxidative stress in plants through different mechanisms. For instance, as mentioned earlier in this chapter, Al-induced rearrangements of lipids in the plasma membrane are thought to promote lipid peroxidation by redox-active metal ions.

1.3. Aluminum-induced oxidative stress

Aluminum-induced lipid peroxidation has been observed in different species and tissues, including roots of soybean (*Glycine max*) (Cakmak and Horst, 1991), cultured cells of tobacco (*Nicotiana tabacum*) (Ono *et al*., 1995; Yamamoto *et al*., 1997) and roots of pea (*Pisum sativum*) (Yamamoto *et al*., 2001). This phenomenon, which leads to loss of plasma membrane integrity, has been proposed as a primary lesion that ultimately

leads to the loss of viability in tobacco cells exposed to Al (Yamamoto *et al*., 1997). More recently, Jones *et al*. (2006) observed that Al induces an immediate and sustained production of ROS and causes a significant rigidification of the cell wall. Furthermore, the application of synthetic antioxidants or glutathione (GSH) has been observed to decrease the accumulation of Al and Fe in tobacco cells, suggesting that accumulation of Al and Fe is a consequence of lipid peroxidation (Yamamoto *et al*., 1997; Yamaguchi *et al*., 1999). Yamamoto *et al*. (2001) observed that Al-enhanced lipid peroxidation in roots of pea (*Pisum sativum*) in the absence of an external supply of Fe was correlated with an increase in callose production (a physiological marker for Al stress), but not with a significant inhibition of root elongation. Similarly, Liu *et al*. (2008) observed that although Al induces oxidative stress in triticale, this was not the primary cause of root growth inhibition. These results suggest that lipid peroxidation is part of the overall expression of Al toxicity in plants, but it is not the primary cause of Al-induced root growth inhibition.

1.4. Cadmium-induced oxidative stress

Cadmium is another non-redox active metal ion capable of inducing oxidative stress in plants. Aluminum (Al) and Cd induce oxidative stress in plants through different mechanisms. Cadmium (Cd), unlike Al, is a metal ion with a strong class “B” character showing a high affinity for sulfhydryl (-SH) groups (Nieboer and Richardson, 1980). Sulfhydryl groups have a broad range of roles in cells. The redox status of sulfhydryl groups in cysteine (Cys) residues can affect the structure and function of many enzymes, receptors and transcription factors. For instance, the sulfhydryl group in the tripeptide

glutathione (γ-glutamyl-cysteinyl-glycine; GSH) is responsible for the crucial role of this molecule in cellular redox homeostasis, since GSH is used as a co-substrate by a number of antioxidant enzymes such as GSH-peroxidase and ascorbate peroxidase. Depletion of GSH observed during stress resulting from exposure to non-redox active metal ions has also been correlated with the synthesis of phytochelatins, which are molecules rich in –SH groups. Thus, depletion of free -SH groups and ROS scavengers seems to be a likely mechanism for class "B" metal-induced oxidative stress (De Vos *et al*., 1992; Chrestensen *et al*., 2000; Schutzendubel and Polle, 2002). Not surprisingly, a number of studies have found that Cd can deplete GSH and protein-bound -SH groups during stress conditions, which ultimately leads to an increase in intracellular ROS (Stohs and Bagchi, 1995). The comparative work with Cd in this study was done in an attempt to determine whether a unique response to Al stress exists versus a general response to oxidative stress induced by other non-redox active metal ions.

1.5. The antioxidant defense systems

Plants possess complex antioxidant defense systems that play a key role in protecting cells from oxidative injury. However, ROS also play important roles in defense against pathogens, in developmental processes, and as intermediate signaling molecules to regulate the expression of genes (Schutzendubel and Polle, 2002; Mittler *et al*., 2011). Thus, tight control of ROS in plants is maintained by complex antioxidant systems.

The plant's antioxidant systems (Figure 1-2) include enzymatic scavengers of ROS such as superoxide dismutases (SOD; EC 1.15.1.1), catalases (CAT; EC 1.11.1.6), peroxidases (such as GSH-peroxidase, GPX; EC 1.11.1.9), and the ascorbate-glutathione cycle, which involves the enzymes ascorbate peroxidase (APX; EC 1.11.1.11) and GSH-reductase (GR; EC 1.6.4.2). It also includes metabolites such as GSH and ascorbate (Bowler *et al*., 1992; Noctor and Foyer, 1998; Malecka *et al*., 2001). Although the primary function of the antioxidant system is the regulation of cellular concentrations of ROS produced during normal conditions, many of these enzymes are up-regulated during metal-induced oxidative stress. The response of the antioxidant system observed during Al and Cd stress in plants further supports the idea that oxidative stress is an important mechanism leading to Al and Cd toxicity (Cakmak and Horst, 1991; Ezaki *et al*., 1996; Richards *et al*., 1998; Ezaki *et al*., 2001; Shickler and Caspi, 1999; Balestrasse *et al*., 2001; Markvska *et al*., 2009).

Overexpression of some antioxidant enzymes such as Mn-SOD, glutathione S-transferase and *Nt*Pox (tobacco peroxidase) have been correlated with increased tolerance to Al (Basu *et al*., 2001; Ezaki *et al*., 2000; Ezaki *et al*., 2001). However, not all components of the antioxidant system are well positioned to respond effectively against metal-induced oxidative injury, which is primarily a root-related phenomenon (Cakmak and Horst, 1991; Delhaize and Ryan, 1995; Wekcx and Clijsters, 1996; Schutzendubel *et al*., 2001). For instance the ascorbate-glutathione cycle is a highly efficient radical scavenger system, but since it is located in the chloroplast is not well positioned to respond against metal-induced oxidative stress.

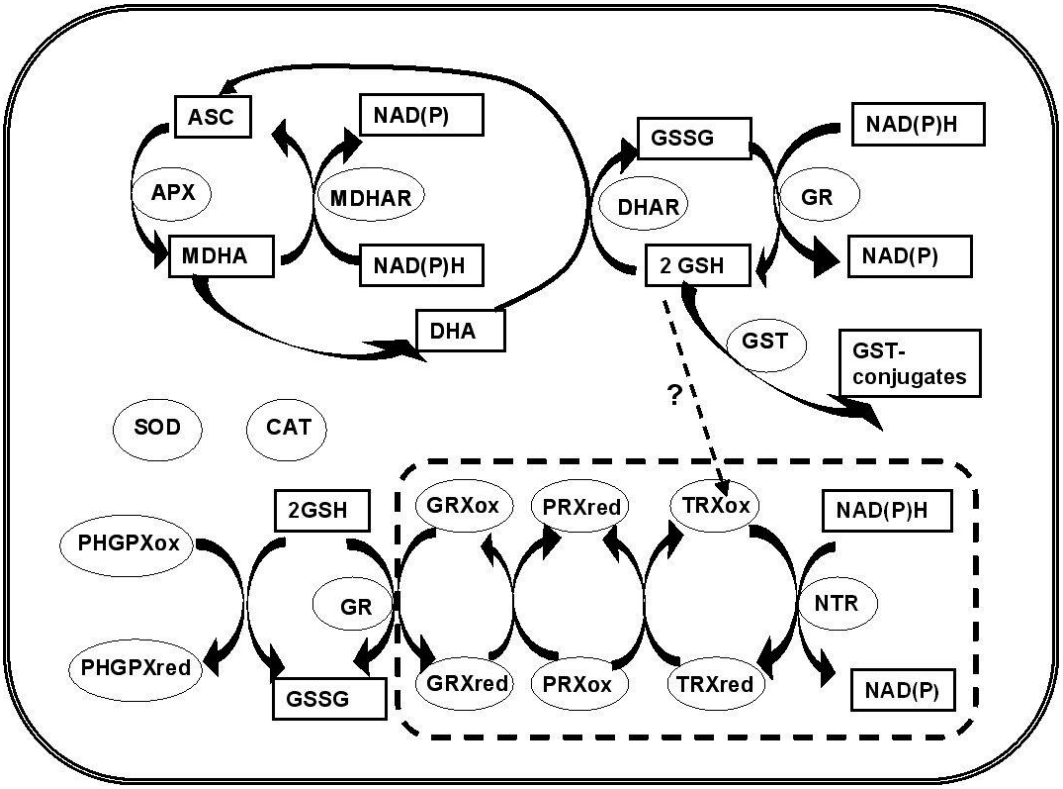

Figure 1-2. Schematic diagram illustrating the interactions between the various components of the antioxidant defense system in plants. Arrows indicate the direction of redox reactions. Antioxidant enzymes that constitute the focus of this study are shown within the dashed box. These include GRXs, glutaredoxins; PRXs, peroxiredoxins; TRXs, thioredoxins and NTR, thioredoxin reductases. Oxidized glutaredoxins (GRXox) are reduced by glutathione reductase (GR), which uses glutathione (GSH) as a source of electrons. Oxidized thioredoxins (TRXox) are reduced by NADPH-dependent thioredoxin reductases (NTR). Oxidized peroxiredoxins (PRXox) can be reduced by TRX or GRX. Abbreviations: ASC, ascorbate; APX, ascorbate peroxidase; MDHA, monodehydroascorbate; MDHAR, monodehydroascorbate reductase; DHA, dehydroascorbate; GSSG, glutathione oxidized; DHAR, dehydroascorbate reductase; GST, glutathione transferase; SOD, superoxide dismutase; CAT, catalase; PHGPX, glutathione peroxidase.

1.5.1. Thiol-disulfide oxidoreductases

Although detailed knowledge of antioxidant enzymes such as SOD, CAT, peroxidases and those involved in the ascorbate-glutathione cycle is currently available (for review, see Van Breusegem *et al*., 2004; Shao *et al*., 2008), the entire network of antioxidant enzymes is still far from being fully understood. A number of reports have highlighted the key role played by sulfhydryl groups (-SH) in response to oxidative stress (Carmel-Harel and Storz, 2000; Grant 2001). Thiol-disulfide oxidoreductases (TDORs) contain a -Cys-X-X-Cys- motif as part of their active site. Examples of TDORs include glutaredoxin, thioredoxin, glutathione reductase, thioredoxin reductase and peroxiredoxin.

1.5.2. Glutaredoxins and the thioredoxin system

Thioredoxins and glutaredoxins are ubiquitous, small (~12 KD), structurally-related, redox-active proteins characterized by highly reactive disulfide groups on the two conserved cysteine residues in their active site (Cys-X-X-Cys). The major function of these proteins is to reduce disulfide bridges on other proteins (Holmgren, 1989). Thus, Trx and Grx have been proposed to function in various cellular processes, including synthesis of deoxyribonucleotides, repair of oxidatively-damaged proteins, protein folding, and sulfur metabolism (Grant, 2001). Thioredoxin, in concert with the glutathione/glutaredoxin system, also maintains the low redox potential and high free -SH levels within cells.

In plants, NADPH-dependent thioredoxin reductases (NTRs) are key regulatory enzymes determining the redox state of the thioredoxin system. Together with thioredoxins, NTRs constitute the NADPH-dependent thioredoxin system in plants (NTS). NADPH-dependent thioredoxin reductases reduce thioredoxins using reducing equivalents provided by NADPH. Thioredoxins are subsequently able to provide reducing power to numerous target proteins like peroxidases or reductases. They also play signalling roles through structural modifications of target proteins, such as several Calvin cycle enzymes (Ruelland and Miginiac-Maslow, 1999).

Glutaredoxins and thioredoxins have been observed to play an important role in protecting yeast from oxidative stress (Luikenhuis *et al*., 1998; Collinson *et al*., 2002; Ocon-Garrido and Grant, 2002). Both Grx1 and Grx2 are active as glutathione-dependent oxidoreductases and their overexpression increases resistance to hydroperoxides (Collinson *et al*., 2002). This Grx-mediated resistance to hydroperoxides is dependent on the presence of an intact glutathione system, but does not require the activity of phospholipid hydroperoxide glutathione peroxidases (PHGPX1–3) (Collinson *et al*., 2002). Hydroperoxides are reduced by Grx1 and Grx2 directly in a catalytic manner, requiring GSH, GSH-reductase and reducing power provided by NADPH. Collinson *et al*. (2002) proposed a model in which the glutathione peroxidase activity of Grx converts hydroperoxides to their corresponding alcohols and these can be conjugated to GSH by glutathione-*S*-transferases (GSTs) and transported into the vacuole. Moreover, Lee *et al*. (2002) showed that a glutaredoxin (OsGRX) in rice (*Oryza sativa*) also functions as a GSH-dependent peroxidase, supporting the important role that these enzymes may potentially play under oxidative stress conditions in plants.

Overexpression of either *TRX1* or *TRX2* also leads to increased resistance to hydroperoxides in yeast (Ocon-Garrido and Grant, 2002). In yeast, *TRX1* and *TRX2* participate in regulation of redox status and levels of protein-bound GSH (Ocon-Garrido and Grant, 2002) and seem to act as cofactors for peroxiredoxin *TSA1*. Accordingly, Mouaheb *et al*. (1998) showed that disruption of two Trx genes in yeast leads to hypersensitivity to H_2O_2, and heterologous expression of an *Arabidopsis* Trx (AtTrx3) complemented this mutant. Recently, Li *et al*. (2010) reported that overexpression of a thioredoxin gene (*PTrx*) in barley confers increased tolerance to Al.

1.5.3. Peroxiredoxins

Peroxiredoxins (Prx) are ubiquitous proteins from archaebacteria to mammals, ranging in size from 150 to 220 amino acids in their mature form (Rouhier and Jacquot, 2002). Peroxiredoxins play a role in combating oxidative stress by reducing either H_2O_2 or various alkyl hydroperoxides to water and the corresponding alcohol (Rouhier *et al*., 2001). The unique feature of peroxiredoxins is that they are nonheme-containing peroxidases that rely on an external electron donor to compensate for the lack of a prosthetic group. This electron donor is often reduced thioredoxin, thus peroxiredoxins are frequently referred to as thioredoxin peroxidases. Peroxiredoxins can also be reduced by glutaredoxins in certain organisms. For instance, while peroxiredoxin's electron donor in yeast is limited to Trx, *in vitro* studies show that the plant Prx Pt-Prx type II (*Populus trichocarpa*) is regenerated by both Grx and Trx (Rouhier *et al*., 2001).

The active site of Prxs consists of one or two conserved N-terminal catalytic Cys residues that are converted into sulfenic acid (Cys-SOH) and regenerated by Trx or Grx (Rouhier *et al*., 2001). Mutating the putative functional Cys residues in these enzymes completely inactivates their antioxidant activity (Grant, 2001). The lack of a metal cofactor in Prxs can be advantageous in maintaining these enzymes in an active form during strong oxidative stress. Metal cofactors are often released during strong oxidative stress, rendering metal cofactor-dependent antioxidant enzymes such as CAT, SOD, ascorbate peroxidase, and guaiacol peroxidase inactive (Casano *et al*., 1997). Peroxiredoxins would presumably remain active during strong oxidative stress as long as their electron donor is available.

To date, the most widely studied plant Prx isoforms are those involved in scavenging ROS at the level of the chloroplastic electron transfer chain. Although these Prx isoforms are not well positioned to play a role in metal tolerance, recent studies have demonstrated the existence of non-chloroplastic isoforms. The *Arabidopsis* genome encodes 10 open reading frames (ORFs) for Prxs, two of which are type II Prxs (Prx II B and Prx II C) that do not contain an apparent signal peptide and may thus remain in the cytosol (Dietz, 2003). High rates of H_2O_2 reduction have been reported for Prx II C, which was also found to be induced under saline stress (Horling *et al*., 2002; Horling *et al*., 2003). Choi *et al*. (1999) found that a chinese cabbage (*Brassica campestris*) Bc-PrxII (*CPrxII*) is expressed predominantly in root tissue and showed a H_2O_2 reduction activity 6-fold higher than a chloroplastic 2-Cys Prx of the same plant.

1.6. Thiol-disulfide based antioxidant enzymes and metal stress

Not surprisingly a number of studies have shown a direct link between thiol-disulfide-based antioxidants and tolerance to several metal ions. Induction of mRNA encoding cytoplasmic Trx h and an increase in the abundance of this protein in the alga *Chlamydomonas reinhardtii* was observed after exposure to Cd and Hg (Lemaire *et al*., 1999). Nguyen-nhu and Knoops (2002) observed a higher sensitivity of a yeast mutant deficient in the cytoplasmic Prx AHP1 compared to the wild-type under stress induced by various metals such as Cu, cobalt (Co), chromium (Cr), arsenite (As^{3+}), arsenate (As^{5+}), Hg, Zn as well as diethyl maleate, a glutathione depleting agent. It has also been suggested that Cu resistance conferred by Trx and Trx-like genes in *Escherichia coli* was achieved via repairing proteins damaged during exposure to this metal ion (Gupta *et al*., 1997). Most of the metal ions used in the studies mentioned above are either Class B metal ions or ions that possess a strong Class B character with a high affinity for sulfhydryl groups. Thus, the –SH groups of TDORs and Prxs cysteines may act similarly to the Cys-rich ligands of phytochelatins in metal chelation by reducing the concentration of free metal ions and their toxicity.

1.7. The present study

Several lines of evidence suggest that oxidative damage is one of the mechanisms of Al and Cd toxicity in plants and that the antioxidant defense system plays a role in Al and Cd tolerance. However, many questions remain to be answered in order to better understand the biology of Al and Cd toxicity and tolerance in plants as it relates to

oxidative stress. Can all components of the antioxidant defense system protect plants against Al and Cd injury? Which antioxidant enzymes play a key role in Al and Cd tolerance? Can we manipulate the levels of key antioxidant enzymes to improve Al and Cd tolerance in plants? Do these enzymes play a role that is specific to Al-induced oxidative injury or do they also respond against oxidative damage induced by other non-redox active metal ions such as Cd?

As summarized in Table 1-1 and Table 1-2, the response of various components of the antioxidant defense system to Al and Cd stress has been studied in a variety of plant species. These studies, however, have been limited to a few antioxidant enzymes such as superoxide dismutase, catalase and peroxidase. In order to address the questions stated above, I have focused my research on several potentially important groups of antioxidant enzymes that have not been previously studied in this context. These enzymes include the thioredoxin system, glutaredoxins and peroxiredoxins. My research is a continuation of a large-scale project designed to systematically query the role of every antioxidant enzyme during Al stress. Previously, Basu *et al*. (2001) found that overexpression of a mitochondrial manganese superoxide dismutase in *Brassica napus* plants conferred increased resistance to Al. As part of this large scale project, Basu *et al*. (2004) also investigated the role of phospholipid glutathione peroxidases (*PHGPX*), glutathione reductases and various genes of the glutathione metabolic pathway in response to Al in yeast (*Saccharomyces cerevisiae*). A triple mutant defective in phospholipid glutathione peroxidases was found to be hypersensitive to Al, which suggests that the *PHGPX* genes may collectively contribute to Al resistance in yeast.

Table 1-1. Summary of studies that indicate a link between various components of the antioxidant defense system and Al stress in different plant species.

Antioxidant enzymes	Plant species (Common name)	Reference
Superoxide dismutase	*Brassica napus* (canola)	Basu *et al*., 2001
Peroxidase, superoxide dismutase	*Zea mays* L. (corn)	Boscolo *et al*., 2003
Superoxide dismutase, peroxidase	*Glycine max* (soybean)	Cakmak and Horst, 1991
Glutathione transferase	*Zea mays* L. (corn)	Cancado *et al*., 2005
Anionic peroxidase	*Nicotiana tabacum* (tobacco)	Ezaki *et al*., 1996
Superoxide dismutase	*Hordeum vulgare* L. (barley)	Guo *et al*., 2004
Dehydroascorbate reductase	*Nicotiana tabacum* (tobacco)	Yin *et al*., 2010
Peroxidase, glutathione transferase	*Arabidopsis thaliana* (mouseear cress)	Richards *et al*., 1998
Guaiacol peroxidase	*Hordeum vulgare* L. (barley)	Tamas *et al*., 2003

Table 1-2. Summary of studies that indicate a link between various components of the antioxidant defense system and Cd stress in different plant species.

Antioxidant enzymes	**Plant species (Common name)**	**Reference**
L-ascorbate peroxidase, Dehydroascorbate reductase, Glutathione reductase	*Glycine max* L. (soybean)	Balestrasse *et al*., 2001
Ascorbate peroxidase, Glutathione reductase	*Phaseolus vulgaris* L. (bean)	Chaoui *et al*., 1997
Superoxide dismutase, Catalase, ascorbate Peroxidase, glutathione reductase	*Pisum sativum* L. (pea)	Dixit *et al*., 2001
Catalase	*Saccharum officinarum* L. (sugar cane)	Fornazier *et al*., 2002
Ascorbate peroxidase, Catalase, Superoxide dismutase	*Zea mays* L. (corn)	Kumar *et al*., 2008
Ascorbate peroxidase, monodehydroascorbate reductase, glutathione reductase	*Brassica juncea* L. (Indian mustard)	Markovska *et al*., 2009
Superoxide dismutase	*Glycine max* L. (soybean)	Pawlak *et al*., 2009
Superoxide dismutase	*Alyssum maritimum*	Shickler and Caspi, 1999

In order to address the question of whether the response of the thioredoxin system, glutaredoxins and peroxiredoxins would be specific to Al, experiments in this study were also performed with Cd, which as described earlier, is another non-redox active metal ion that induces oxidative stress in plants. The working hypothesis of this study is that glutaredoxins, the thioredoxin system and peroxiredoxins confer varying degrees of protection against Al toxicity and Cd toxicity, by ameliorating the oxidative damage induced by these metal ions.

The specific objectives of this study were the following:

1. To use yeast (*Saccharomyces cerevisiae*) as a model system to identify candidate genes encoding glutaredoxins, thioredoxins, thioredoxin reductases and peroxiredoxins that are likely to play the greatest role in Al and Cd tolerance in plants.
2. To identify candidate genes that do play a key role in Al and Cd tolerance in plants by screening T-DNA mutant lines of *Arabidopsis*.
3. To determine if overexpression of these genes can confer enhanced Al and Cd tolerance in plants by developing *Arabidopsis* transgenic lines.

In Chapter 2, I describe the use of the yeast *S. cerevisiae* as a model system to identify candidate antioxidant enzymes that could play a role in Al and Cd tolerance in plants. By systematically screening a collection of yeast disruption mutants for hypersensitivity to Al, I was able to rapidly identify enzymes within the glutaredoxins, thioredoxin system and peroxiredoxins that could potentially play a role in Al tolerance in plants. The rapidity of the yeast system also allowed me to perform parallel experiments with Cd, with the purpose of querying whether any of these candidate genes

play a specific role in response to Al, as opposed to a more general response to oxidative stress. The list of candidate genes was further refined based on RNA expression patterns observed for these genes in the wild-type (parental) yeast under conditions of Al and Cd stress and on the amount of lipid peroxidation observed in the mutants under these conditions. Several mutant strains defective in thioredoxin peroxidases, glutaredoxins and cytoplasmic thioredoxin reductase were hypersensitive to both Al and Cd, but the most dramatic hypersentive phenotype was observed for the yeast strain defective in cytoplasmic thioredoxin reductase (*TRR1*) under both Al and Cd stress.

In Chapter 3, I describe the use of *Arabidopsis* T-DNA mutant lines to test whether the antioxidant genes selected from my studies with yeast also play a role in Al and Cd tolerance in plants. The complete genomic sequence of *Arabidopsis* and an array of bioinformatic tools facilitated the identification of plant homologues of the yeast candidate genes. *Arabidopsis* lines homozygous for the T-DNA inserts were selected and decreased RNA expression levels were confirmed for the genes of interest. The phenotypes of these lines were then tested under conditions of Al and Cd stress. Levels of lipid peroxidation in hypersensitive mutants, and RNA expression levels of genes selected from these sensitivity studies were determined to refine the selection of candidate genes whose overexpression might have a significant impact on Al and Cd tolerance. Similar to the results of my studies in yeast, lines of *Arabidopsis* that showed the greatest hypersensitivity to Al and Cd were the lines defective in thioredoxin reductase.

In Chapter 4, I describe the development and testing of *Arabidopsis* transgenic lines overexpressing the major cytosolic (NTRA) and mitochondrial (NTRB) isoforms of NADPH- thioredoxin reductase. These transgenic lines were used to develop overexpression lines under the control of a constitutive promoter (CaMV 35S). *Arabidopsis* plants homozygous for the overexpression construct were selected and increased expression of RNA and higher levels of protein abundance were confirmed. The overexpression lines selected with increased transcript levels of both thioredoxin reductases showed no visible differences in growth from the wild-type in normal conditions. These overexpression lines also showed a phenotype similar to the wild-type under conditions of Al and Cd stress. These results suggest that elevated levels of NTRA or NTRB in *Arabidopsis* are not enough to increase the overall tolerance to Al or Cd. In the final chapter, I discuss why the lack of a phenotype with increased tolerance for these overexpressing lines does not completely negate a role for these antioxidant enzymes in Al and Cd tolerance in plants.

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2. Exploring the role of the thioredoxin system, peroxiredoxins and glutaredoxins in aluminum and cadmium tolerance in yeast (*Saccharomyces cerevisiae*)

2.1. Introduction

A large body of literature suggests that Al and Cd cause oxidative stress, which acts as a mechanism of toxicity in plants. Not surprisingly, several components of antioxidant systems, including catalases, superoxide dismutases, glutathione peroxidases, and glutathione S-transferases, have been shown to play a role in Al and Cd tolerance (Cakmak and Horst, 1991; Chaoui *et al*., 1997; Richards *et al*., 1998; Boscolo *et al*., 2003; Smeets *et al*., 2008; Markovska *et al*., 2009). However, the groups of antioxidant enzymes comprising the thioredoxin system, peroxiredoxins and glutaredoxins have not been studied in the context of Al and Cd stress before. The thioredoxin system, peroxiredoxins and glutaredoxins not only are able to scavenge reactive oxygen species and lipid hydroperoxides, but they are also capable of repairing oxidatively damaged proteins (Herrero *et al*., 2008). Thus, the thioredoxin system, glutaredoxins and peroxiredoxins may play an important role in ameliorating the symptoms of Al- and Cd-induced oxidative stress in plants.

The main objective of the work described in this chapter was to use yeast (*Saccharomyces cerevisae*) as a model organism to rapidly identify candidate genes to study further in the plant system *Arabidopsis thaliana*. Many characteristics make yeast an ideal experimental organism for this study; a sequenced genome, availability of yeast

strains containing deletions for the genes of interest, its short life cycle, and the fact that yeast contains fewer isozymes of the various components of the antioxidant system compared to plants. Yeast has been previously used as a model organism to elucidate the role of various genes in metal tolerance. For instance, Ezaki *et al*. (1999) were able to rapidly test whether a group of eleven Al-induced plant genes play a protective role against Al stress by expressing these genes in yeast. Furthermore, investigating several putative genes responsible for Al tolerance, such as genes involved in synthesis of organic anions, ATPases, genes of the glutathione biosynthetic pathway and glutathione peroxidases, has been facilitated by the use of the readily available collection of mutant strains in yeast (Hamilton *et al*, 2001; Anoop *et al*., 2003; Basu *et al*, 2004).

As described in Chapter 1, thioredoxins and glutaredoxins are small oxidoreductases with two conserved cysteine residues in their active site that are reduced respectively by thioredoxin reductase, using NADPH directly as the source of electrons, or by glutathione reductase, using GSH as the source of reducing power. The redox state of the thioredoxin system, however, seems to be maintained independently of the glutathione system (Trotter and Grant, 2003). Peroxiredoxins are commonly reduced by thioredoxins, although some peroxiredoxins have been found to be reduced by glutaredoxins (Figure 2-1). Thioredoxins and glutaredoxins can regulate many metabolic enzymes that form disulfide bridges during their catalytic cycle (Holmgren, 1989). Thioredoxins are also required for protection against reactive oxygen species (ROS), and they provide reducing power to peroxiredoxins, also known as thioredoxin peroxidases (Park *et al*., 2000). On the other hand, yeast glutaredoxins seem to act directly as antioxidants, since they have been found to possess glutathione peroxidase activity

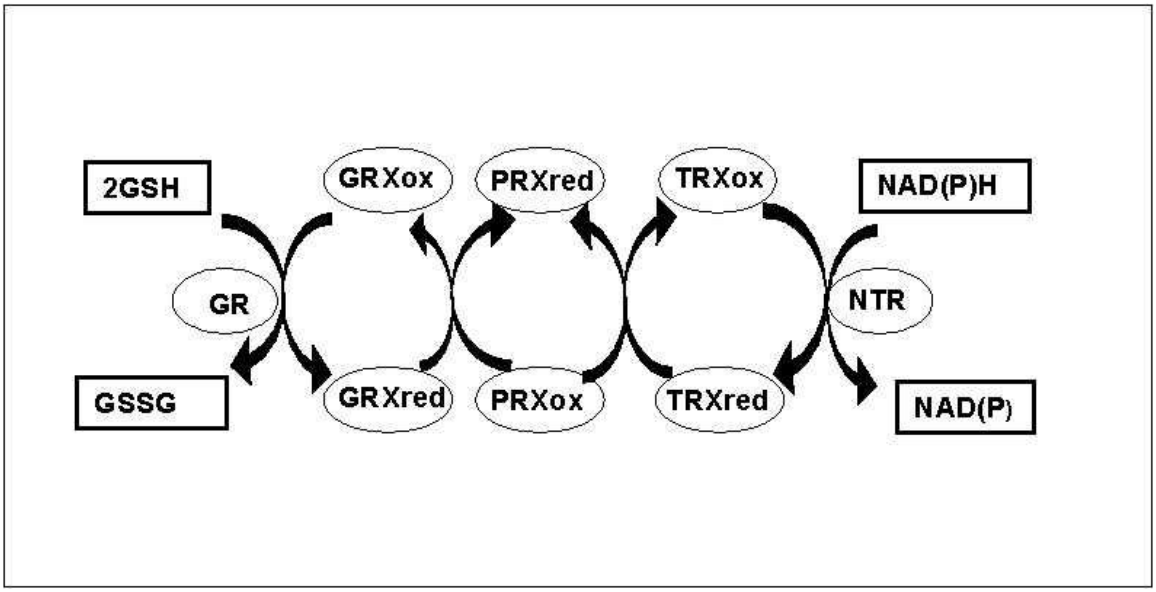

Figure 2-1. Interactions between glutaredoxins (Grx), peroxiredoxins (Prx) and the thioredoxin system: thioredoxin (Trx) and thioredoxin reductase (NTR). Glutaredoxins are reduced by glutathione reductase (GR) using glutathione (GSH) as a source of electrons, whereas thioredoxins are reduced by thioredoxin reductase (NTR) using NADPH directly. Peroxiredoxins are reduced by thioredoxin and by glutaredoxins in some cases.

(Luikenhuis *et al*., 1998; Collinson et *al*., 2002). The first peroxiredoxin identified was a thiol-specific antioxidant protein (TSA) in yeast that did not appear to contain any of the known prosthetic groups (Kim *et al*., 1988). Peroxiredoxins can detoxify various peroxidase substrates using one or two cysteine residues as their catalytic center, and these cysteine residues were found to be specifically reduced by thioredoxin for the yeast TSA peroxiredoxin (Chae *et al*., 1994).

Although the role of the thioredoxin system, glutaredoxins and peroxiredoxins has not been clearly elucidated, several lines of evidence have shown that these antioxidant

enzymes play an important protective role against strong oxidative stress conditions (Luikenhuis *et al*., 1998; Rodriguez-Manzaneque *et al*., 1998; Grant, 2001; Herrero and Ros, 2002; Ocon-Garrido and Grant, 2002). These findings suggest that these antioxidant enzymes may also play an important role in tolerance to Al and Cd. The work described in this chapter was designed to test this hypothesis. The use of yeast as a model system was particularly useful in this study, since yeast possess fewer isozymes of the antioxidant ezymes of interest compared to plants (Table 2-1). For instance, the thioredoxin system in yeast is encoded by a total of five genes that include a mitochondrial system composed of a thioredoxin reductase (*TRR2*) and a thioredoxin (*TRX3*), and also a cytoplasmic system composed of a thioredoxin reductase (*TRR1*) and two thioredoxins (*TRX1*, *TRX2*), whereas the thioredoxin system in *Arabidopsis*, including cytosolic and mitochondrial components, involves approximately 23 genes. This redundancy observed in plants makes it difficult to characterize these genes, since many of the gene products show overlapping functions. Using yeast as a model system, I was able to screen a large collection of mutants defective in the various genes of the thioredoxin system, glutaredoxins and peroxiredoxins for their sensitivity to Al and Cd. The levels of lipid peroxidation in hypersensitive mutants were also determined to better understand the role of these genes in Al and Cd tolerance, and the RNA expression levels of selected genes was also determined to find out whether they are induced by Al or Cd.

Table 2-1. Number of genes encoding isoforms of the thioredoxin system, glutaredoxins and peroxiredoxins in yeast (*Saccharomyces cerevisiae*) and *Arabidopsis*.

Antioxidant enzymes	**Number of genes (known and predicted) encoding these enzymes in:**	
	Yeast (*S. cerevisiae*)	***Arabidopsis***
Thioredoxins and thioredoxin reductases	5	23
Glutaredoxins	5	30
Peroxiredoxins	5	17

2.2. Materials and Methods

2.2.1. Yeast strains and growth conditions

2.2.1.1. Single mutants

Yeast (*Saccharomyces cerevisiae*) strains harbouring single mutations in genes of the thioredoxin pathway, glutaredoxins and peroxiredoxins (Table 2-2), as well as the wild-type parental strain (BY4741), were obtained from Euroscarf (European *Saccharomyces cerevisiae* archive for functional analysis, Frankfurt, Germany).

Table 2-2. Yeast single mutants defective in genes of the thioredoxin pathway, glutaredoxins and peroxiredoxins used in this study. Strains were obtained from Euroscarf. ***** The gene deleted in this strain has not yet been given a standard yeast genetic name.

Strain	Gene mutated	Genotype
BY4741	parental strain	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*
*trr1*Δ	Thioredoxin reductase cytoplasmic (*TRR1*)	*YDR353w::kanMX4*
*trr2*Δ	Thioredoxin reductase mitochondrial (*TRR2*)	*YHR106w::kanMX4*
*trx1*Δ	Thioredoxin 1 (*TRX1*)	*YLR043c::kanMX4*
*trx2*Δ	Thioredoxin 2 (*TRX2*)	*YGR209c::kanMX4*
*trx3*Δ	Thioredoxin 3 (*TRX3*)	*YCR083w::kanMX4*
*ahp1*Δ	Peroxiredoxin (*AHP1*)	*YLR109w::kanMX4*
*dot5*Δ	Peroxiredoxin (*DOT5*)	*YIL010w::kanMX4*
*YBL064c*Δ*	Peroxiredoxin mitochondrial (*YBL064c*)	*YBL064c::kanMX4*
*tsa1*Δ	Peroxiredoxin (*TSA1*)	*YML028w::kanMX4*
*tsa2*Δ	Peroxiredoxin (*TSA2*)	*YDR453c::kanMX4*
*grx1*Δ	Glutaredoxin (*GRX1*)	*YCL035c::kanMX4*
*grx2*Δ	Glutaredoxin (*GRX2*)	*YDR513w::kanMX4*
*grx3*Δ	Glutaredoxin (*GRX3*)	*YDR098c::kanMX4*
*grx4*Δ	Glutaredoxin (*GRX4*)	*YER174c::kanMX4*
*grx5*Δ	Glutaredoxin (*GRX5*)	*YPL059w::kanMX*

These single mutant strains were generated using a PCR-based gene deletion strategy in which the open reading frame (ORF) of the gene of interest was replaced by *kanMX4*, a kanamycin resistance gene mediating G418 sulfate (geniticin) resistance to the yeast strain. Deletion mutants were selected in rich medium (YPD; 1% w/v yeast extract, 2% w/v Bacto-peptone, 2% w/v glucose) containing 200 µg ml^{-1} geniticin (G418 sulfate). Media were solidified by the addition of 2% w/v agar.

2.2.1.2. Multiple mutants

Yeast strains harbouring multiple mutations in genes of the thioredoxin system and glutaredoxins (Table 2-3) were kindly provided by Dr. Chris Grant, University of Manchester, England. These strains are all isogenic derivatives of CY4 (MATa ura3-52 leu2-3 leu2-112 trp1-1 ade2-1 his3-11 can1-100) (Grant *et al*., 1996). To develop these strains, single mutant strains were created first by deleting the ORF of the gene of interest in strain CY4 using a one-step polymerase chain reaction (PCR) amplification protocol that replaced the entire ORF with a selectable marker. Then, these single mutant strains were crossed to generate double and triple mutants as described by Draculic *et al*. (2000). Since these strains are derivatives of an *ade2* (defective in adenine biosynthesis) mutant strain (CY4), the multiple mutant strains were purified upon arrival in order to avoid contamination from white *ade* suppressors generated during shipping. The multiple mutant strains were grown in a medium containing a suboptimal amount of adenine (YED; 1% w/v yeast extract, 2% w/v glucose, 2% w/v agar) for 2d at 30^{o}C.

Table 2-3. Multiple mutants defective in genes of the thioredoxin pathway and glutaredoxins used in this study (provided by Dr. Chris Grant, University of Manchester).

Strain	Genes mutated	Genotype
CY4	parental strain	*MATa ura3-52 leu2-3 112 trp1-1 ade2-1 his3-11 can1-100*
Y522	*TRR1, TRX1*	*trr1::HIS3 trx1::TRP1*
Y523	*TRR1, TRX2*	*trr1::HIS3 trx2::URA3*
Y524	*TRR1, TRX1, TRX2*	*trr1::HIS3 trx1::TRP1 trx2::URA3*
Y302	*TRX1, TRX2*	*trx1::TRP1 trx2::URA3*
Y117	*GRX1, GRX2*	*grx1::LEU2 grx2::HIS3*
Y323	*GRX1, GRX2, TRX1*	*grx1::LEU2 grx2::HIS3 trx1::TRP1*
Y325	*GRX1, GRX2, TRX2*	*grx1::LEU2 grx2::HIS3 trx2::URA3*
Y320	*GRX1, TRX1, TRX2*	*grx1::LEU2 trx1::TRP1 trx2::URA3*
Y321	*GRX2, TRX1, TRX2*	*grx2::HIS3 trx1::TRP1 trx2::URA3*

Yeast cells defective in adenine biosynthesis accumulate a red pigment when the concentration of adenine in the medium is low and the yeast attempt to synthesize their own adenine. Thus, cells from red colonies only were used to make glycerol stocks for each of the multiple mutant strains. Purified strains were grown in minimal SD media (0.17% w/v yeast nitrogen base without amino acids, 5% w/v ammonium sulfate, 2% w/v glucose) supplemented with appropriate amino acids and bases: 2 mM leucine, 4mM isoleucine, 1 mM valine, 0.3 mM histidine, 0.4 mM tryptophan, 1mM lysine, 0.15 mM adenine and 0.2 mM uracil. Media were solidified by the addition of 2% w/v agar.

2.2.2. Screening yeast mutants for hypersensitivity to Al and Cd

A modified low-pH, low-phosphate (LPP) medium was used to screen for mutants hypersensitive to Al. This medium has been optimized to maintain solubility of Al, and it is a synthetic complete (SC) minimal medium containing 0.67% yeast nitrogen base (with ammonium sulfate, without amino acids, without phosphate; without dextrose), 2% glucose, 78 µM K_2HPO_4, 1.1 mM KH_2PO_4, 3.2 mM KCl, and pH reduced to 3.5 (Schott and Gardner, 1997; Basu *et al*., 2004). The medium used to screen for mutants hypersensitive to Cd was a synthetic complete (SC) medium (pH 4.0). Seed cultures of the wild-type strains (BY4741 or CY4) and mutant strains were prepared in 3 ml of YPD medium and grown overnight at 30^{o} C (20 h, 225 rpm). The cells were harvested by centrifugation at 1,300 x g for 2 min, washed three times with sterile water (< 18 mΩ) and suspended in 1 ml of sterile water. This cell suspension was used to inoculate triplicate 3-ml aliquots of LPP medium (pH 3.5) containing 0-300 µM $AlCl_3$ or SC medium containing 0-30 µM $CdCl_2$ to a starting OD_{600} of 0.05. Cultures were incubated

at 30°C for 16 h (225 rpm) and the final OD_{600} was measured in a 96-well plate using a plate reader (μQuant, Biotek Instruments, Vermont, USA). The relative growth of each strain in the presence of Al or Cd was expressed as percentage of control for that strain.

2.2.3. Aluminum and cadmium-induced changes in transcript levels

Total RNA was isolated from the wild-type strain BY4741 exposed for 16 h to varying concentrations of Al or Cd as described above. Approximately 5 X 10^7 cells were used for RNA isolations using the QIAGEN RNeasy mini kit (QIAGEN Inc., Canada). The RNA was separated by electrophoresis on agarose formaldehyde denaturing gels and transferred to nitrocellulose membranes following the manufacturer's recommendations (Genescreen, NEN Research Products). Briefly, a capillary blot was set up using 10X SSPE buffer (1.5 M NaCl, 100 mM NaH_2PO_4, Na_2EDTA) and blotted overnight. The membrane was then rinsed in 2X SSPE and baked at 80° C for 2 h. Probes used for hybridization were amplified by PCR from *S. cerevisiae* genomic DNA using gene specific primers (Table 2-4) and confirmed by sequencing. Hybridization probes were radioactively labeled with [^{32}P] dCTP using using the Ready·To·Go DNA Labeling Beads (Amersham Biosciences-GE Healthcare, Ontario, Canada). Approximately 150-200 ng of denatured DNA was added to the labeling reaction with 5 μl of [^{32}P] dCTP in a total volume of 50 μl, and incubated at 37 °C for 30 min. Radioactive-labeled probes were separated from the unincorporated ^{32}P labeled nucleotides using the Sephadex G-50TM DNA grade NICK column (Amersham Biosciences-GE Healthcare). Membranes were prehybridized, hybridized and washed under standard stringent conditions recommended by Genescreen. Briefly, membranes were prehybridized with 1 μg denatured salmon sperm DNA at 42° C

in 20 ml of prehybridization solution overnight (For 100 ml prehybridization/hybridization solution: 10 g dextran sulfate, 15 ml water, 25 ml 20X SSPE, 5 ml 20% SDS, 5 ml Denhardt's solution, 50 ml deionized formamide). Membranes were then hybridized in 20 ml of new hybridization solution at 42^{o} C for 20 h with 200 µl of denatured radioactively-labeled probe. After hybridization, membranes were washed three times: first in 2X SSPE at room temperature for 15 min, a second wash in 2X SSPE and 2% SDS at 65^{o} C for 15 min and a third wash in 0.1X SSPE at room temperature for 15 min. Membranes were then wrapped in plastic film (Saran wrap) and exposed to KODAK BioMax MR autoradiography films (Marketlink Scientific, Burlington, Ontario) for 6 h and 24 h at -80^{o} C. All the x-ray films were developed by the Kodak X-OMAT 2000 processor in a dark room.

Table 2-4. List of primers used for amplification of probes for Northern analyses.

Gene	Primer sequence	Probe size
DOT5 (peroxiredoxin)	LP 5' GGAAGAGTCCAAACTGGCCC 3'	291 bp
	RP 5' TTGTCACGAA ATCCACAGGC 3'	
GRX3 (glutaredoxin)	LP 5' GCCGGCGACAAGTTAATCG 3'	600 bp
	RP 5' GTTGAGGGAAAGTTGGCCATT 3'	
TRR1 (thioredoxin reductase)	LP 5' TTGGTTCAGGTCCAGCTGC 3'	913 bp
	RP 5' TTCTCAGCATCCAAAGCGG 3'	
YBL064c (peroxiredoxin)	LP 5' TTGTAGCGCTCAATTAAAGAG 3'	768 bp
	RP 5' TTCGACTTGGTGAATCTTAAA 3'	

2.2.4. Complementation of the *trr1*Δ yeast mutant

For complementation of the *trr1*Δ mutant a ~ 1.7 kb fragment containing the coding sequence of the *TRR1* gene, including its endogenous promoter, was amplified from *S. cerevisiae* genomic DNA with sense (5' ACAATGCACTCTGCTCTTGGG 3') and antisense (5' GTCGGTGTATAGGCAACCACG 3') primers using a Taq/Pfu enzyme mixture (Fermentas). The 1704 bp PCR product (510 bp 5' UTR + 960 bp *TRR1* CDS + 234 bp 3' UTR) was sequenced and TA-cloned into pBluescript (Stratagene). The pBluescript/*TRR1* construct was then sequenced and the fragment containing the *TRR1*

gene was gel- purified (QIAGEN gel extraction kit) and subcloned into the yeast vector pRS426 using the restriction enzymes *Kpn*I and *Sac*I (Amersham Biosciences) and T4 DNA ligase (Invitrogen). The pRS426-*TRR1* construct was sequenced, and this plasmid was used to transform the *trr1* mutant strain following the lithium acetate method (Gietz *et al*., 1995). As a control, the *trr1* mutant strain was also transformed with the empty yeast vector. Other controls used were the WT strain transformed with the construct pRS426-*TRR1* and the WT strain transformed with the empty vector.

2.2.5. Analysis of lipid peroxidation

Lipid peroxidation was determined using the thiobarbituric acid (TBA)-reactive substances (TBARS) method adapted from Buege and Aust (1978). This assay is based on the reaction of malondialdehyde, a product of lipid peroxidation, with thiobarbituric acid that results in the formation of a red species with maximal absorbance at 535 nm. Yeast cultures (50 ml) were exposed to $AlCl_3$ (0 µM, 250 µM) or $CdCl_2$ (0 µM, 10 µM for 16 h (30 ^{o}C, 225 rpm). Cells were harvested by centrifugation (5 m, 2000 x g), washed twice with Tris-HCl buffer (pH 7.4), and the pellets were resuspended in 600 µL of the same buffer. The cells were disrupted in the presence of glass beads by vortexing for six periods of 20 s followed by 1 min on ice, and 50 µL of the extracts were set aside for a Bradford assay to calculate the amount of protein in the samples for normalization purposes. The cell lysates (500 µL) were combined with 1 ml of TCA-TBA-HCl reagent (15% w/v trichloroacetic acid; 0.375% w/v thiobarbituric acid; 0.25 N hydrochloric acid) and heated for 15 min in a boiling water bath. After cooling, the samples were centrifuged for 10 min (1,000 g) to remove any precipitate. The absorbance of the

samples was determined at 532 nm, and the amount of MDA-TBA complex was calculated using an extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}.

2.2.6. Experimental design and statistical analyses

All experiments include three independent replicates and results are expressed as mean ± standard error (SE). Statistical analyses were performed using the program SigmaStat Version 3.5 (2006, Systat Software, Inc.). Student's t-test was used to determine significant differences between treatment and control. Experiments were repeated at least three times to ensure reproducibility of results.

2.3. Results

2.3.1. Sensitivity of single disruption mutants defective in thioredoxins, thioredoxin reductases, glutaredoxins and peroxiredoxins to aluminum

To determine whether thioredoxins, thioredoxin reductases, glutaredoxins or peroxiredoxins are involved in Al tolerance, single yeast mutants (Table 2-2) were tested for hypersensitivity in LPP medium with varying concentrations of Al (0-300 µM). The wild-type and mutant strains showed a reduction in growth in a dose-dependent manner. The growth of the wild-type yeast, BY4741, was reduced by 27% in the presence of 300 µM Al (from OD_{600} 0.826 ± 0.017 at 0 µM to OD_{600} 0.601 ± 0.027 at 300 µM) (Figure 2-2A). All the mutant strains of the thioredoxin system showed enhanced sensitivity to Al compared to the WT (between 29% and 57% at 300 µM Al compared to their respective controls). Disruption of the cytosolic thioredoxin reductase (*trr1*Δ) caused the most

dramatic reduction in growth (57%) at 300 µM Al compared to a 27% growth reduction in the WT at the same Al concentration. The OD_{600} of *trr1*Δ grown without Al was 0.804 ± 0.019 and decreased to 0.457 ± 0.049 at 300 µM Al (Figure 2-2A). Single disruption mutants lacking *AHP1*and *TSA2* peroxiredoxin genes showed similar growth inhibition to the WT in the presence of Al (23% and 22%, respectively, in the presence of 300 µM Al), whereas the mutants lacking *DOT5*, *TSA1* and *YBL064c* peroxiredoxin genes showed enhanced sensitivity to Al compared to the WT (39%, 35% and 33%, respectively, at 300 µM Al) (Figure 2-2B). In regards to the glutaredoxin system, only *grx3*Δ and *grx5*Δ mutant strains showed enhanced sensitivity to Al compared to the WT (39% and 41% at 300 µM Al, respectively) (Figure 2-2C).

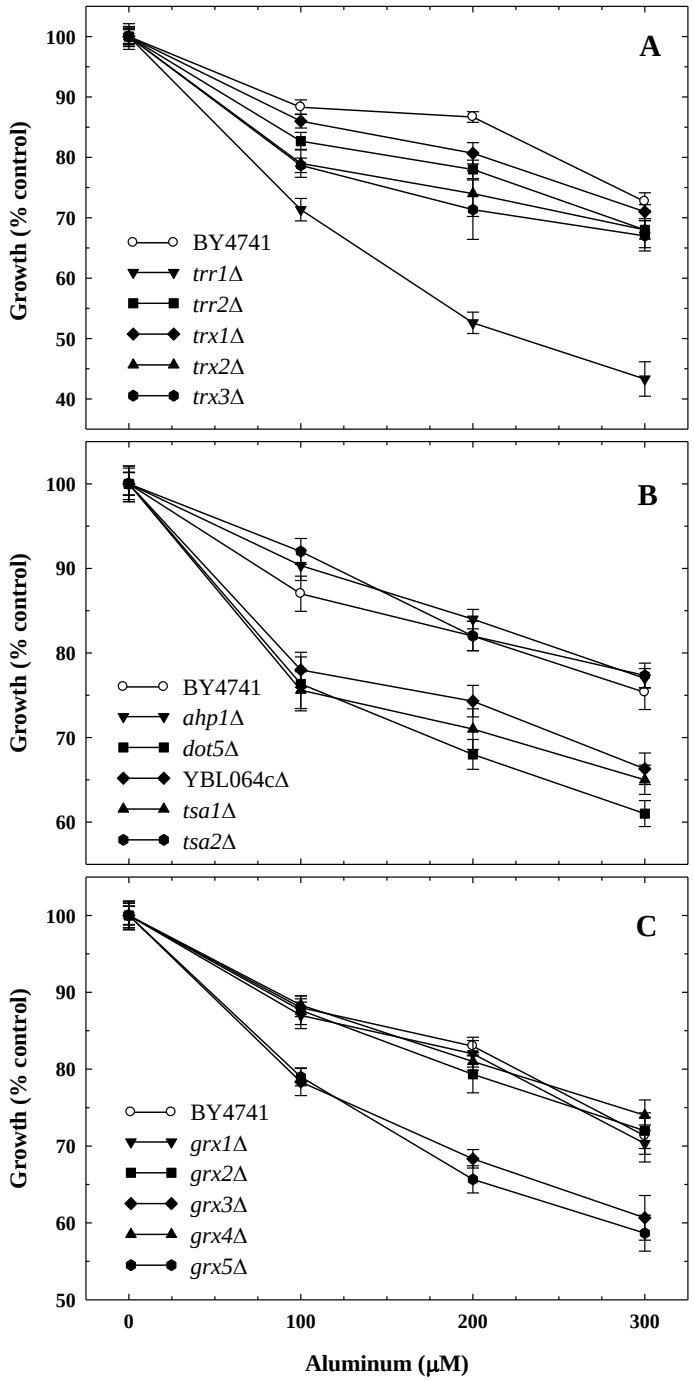

Figure 2-2. Effect of Al on growth (percentage of control) of yeast single disruption mutants compared to the wild-type parent strain (BY4741). (A) Thioredoxin system (*trr1*Δ, *trr2*Δ, *trx1*Δ, *trx2*Δ and *trx3*Δ). (B) Thioredoxin peroxidases (*ahp1*Δ, *dot5*Δ, *YBL064c*Δ, *tsa1*Δ and *tsa2*Δ). (C) Glutaredoxins (*grx1*Δ, *grx2*Δ, *grx3*Δ, *grx4*Δ and *grx5*Δ). Cells were exposed to Al in LPP medium for 16 h at 30°C. Total growth (OD_{600}) was measured and compared to the control (0 $AlCl_3$) for each strain. Vertical bars represent standard error (n=3). Results shown are representative of three independent experiments.

2.3.2. Sensitivity of single disruption mutants defective in thioredoxins, thioredoxin reductases, glutaredoxins and peroxiredoxins to cadmium

In order to determine whether thioredoxins, thioredoxin reductases, peroxiredoxins and glutaredoxins play a role in Cd tolerance, single yeast mutant strains (Table 2-2) were screened for Cd hypersensitivity in SC medium containing 0-30 µM $CdCl_2$. The wild-type yeast strain, BY4741, showed a progressive reduction in growth with increasing concentrations of Cd reaching a maximum inhibition of growth of 42% at 30 µM Cd (from OD_{600} 0.712 ± 0.016 at 0 µM Cd to OD_{600} 0.412 ± 0.021 at 30 µM Cd). Interestingly, just like in the case of Al, the mutant strain lacking the cytoplasmic thioredoxin reductase (*trr1*Δ) showed the highest hypersensitivity to Cd (79% growth inhibition) among all the antioxidant enzymes of the thioredoxin system. The OD_{600} of *trr1*Δ at 0 µM Cd was 0.709 ± 0.02 and decreased to OD_{600} 0.146 ± 0.032 at 30 µM Cd (Figure 2-3A). Single disruption mutants defective in *TRR2*, *TRX1* and *TRX3* showed similar inhibition on their growth (51%, 54% and 52%, respectively, at 30 µM Cd), whereas the *trx2*Δ mutant showed the next greatest reduction in growth (73%) at 30 µM Cd (Figure 2-3A). All single disruption mutants lacking peroxiredoxins showed enhanced sensitivity to Cd compared to the WT strain, however, mutants lacking *DOT5*, *YBL064c* and *TSA1* peroxiredoxins showed the most hypersensitive phenotype from this group of antioxidant enzymes (79%, 78% and 77%, respectively, at 30 µM Cd) (Figure 2-3B). Among the mutant strains defective in glutaredoxins, *grx3*Δ and *grx5*Δ showed the most hypersensitive phenotype at 30 µM Cd with a growth reduction of 64% and 77%, respectively (Figure 2-3C).

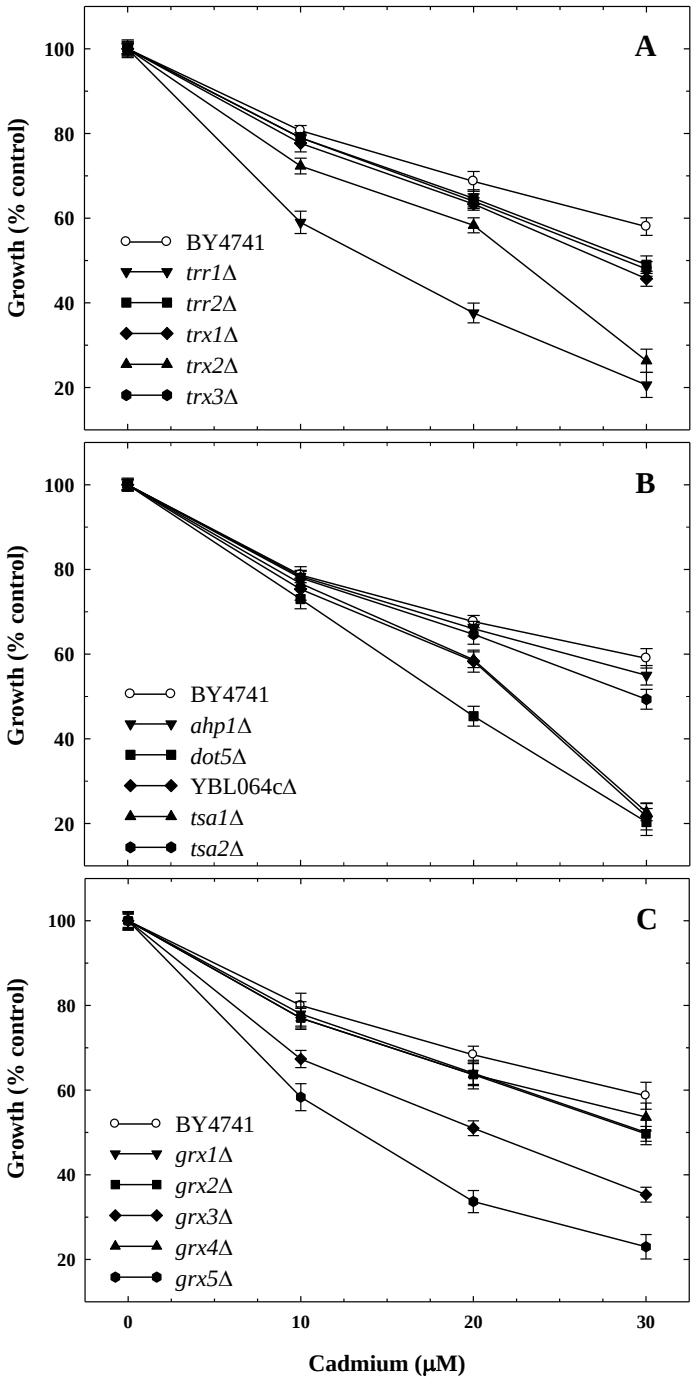

Figure 2-3. Effect of Cd on growth (percentage of control) of yeast single disruption mutants compared to the wild-type parent strain (BY4741). (A) Thioredoxin system (*trr1*Δ, *trr2*Δ, *trx1*Δ, *trx2*Δ and *trx3*Δ). (B) Thioredoxin peroxidases (*ahp1*Δ, *dot5*Δ, *YBL064c*Δ, *tsa1*Δ and *tsa2*Δ). (C) Glutaredoxins (*grx1*Δ, *grx2*Δ, *grx3*Δ, *grx4*Δ and *grx5*Δ). Cells were exposed to Cd in SC medium for 16 h at 30°C. Total growth (OD_{600}) was measured and compared to the control (0 $CdCl_2$) for each strain. Vertical bars represent standard error (n=3). Results shown are representative of three independent experiments.

2.3.3. Sensitivity of multiple mutants defective in thioredoxins, thioredoxin reductases and glutaredoxins to aluminum

Viable yeast mutant strains harboring multiple mutations in thioredoxin, thioredoxin reductase, and glutaredoxin genes (Table 2-3) were exposed to varying concentrations of Al to test whether these mutants show increased sensitivity to Al compared to yeast strains harboring single mutations in these genes. The wild-type strain (CY4) showed a progressive reduction in growth with increasing concentrations of Al and a maximum of 28% reduction in growth at the highest Al concentration (from OD_{600} of 0.705 ± 0.015 at 0 µM Al to OD_{600} of 0.504 ± 0.019 at 300 µM Al) (Figure 2-4A). The triple mutant, *trr1*Δ *trx1*Δ *trx2*Δ, was more sensitive to Al than any other of the multiple mutants or the parental strain with a maximum reduction in growth of 80% at 300 µM Al (from OD_{600} of 0.484 ± 0.021 at 0 µM Al to OD_{600} of 0.096 ± 0.028 at 300 µM Al) (Figure 2-4A). The double mutants, *trr1*Δ *trx1*Δ, *trr1*Δ *trx2*Δ and *trx1*Δ *trx2*Δ, were more sensitive than the parental strain and showed a growth reduction of 50%, 63% and 62%, respectively, at 300 µM Al (Figure 2-4A). The triple mutant defective in *GRX1*, *GRX2* and *TRX1* genes showed a dramatic reduction in growth of 78% at 300 µM Al (from OD_{600} of 0.502 ± 0.024 at 0 µM Al to OD_{600} of 0.110 ± 0.022 at 300 µM Al) (Figure 2-4B). Other multiple mutant strains defective in glutaredoxin and thioredoxin genes, *grx1*Δ *grx2*Δ, *grx1*Δ *grx2*Δ *trx2*Δ, *grx1*Δ *trx1*Δ *trx2*Δ and *grx2*Δ *trx1*Δ *trx2*Δ, also showed increased sensitivity to Al than the parental strain with a growth reduction of 64%, 57%, 65% and 64%, respectively (Figure 2-4B).

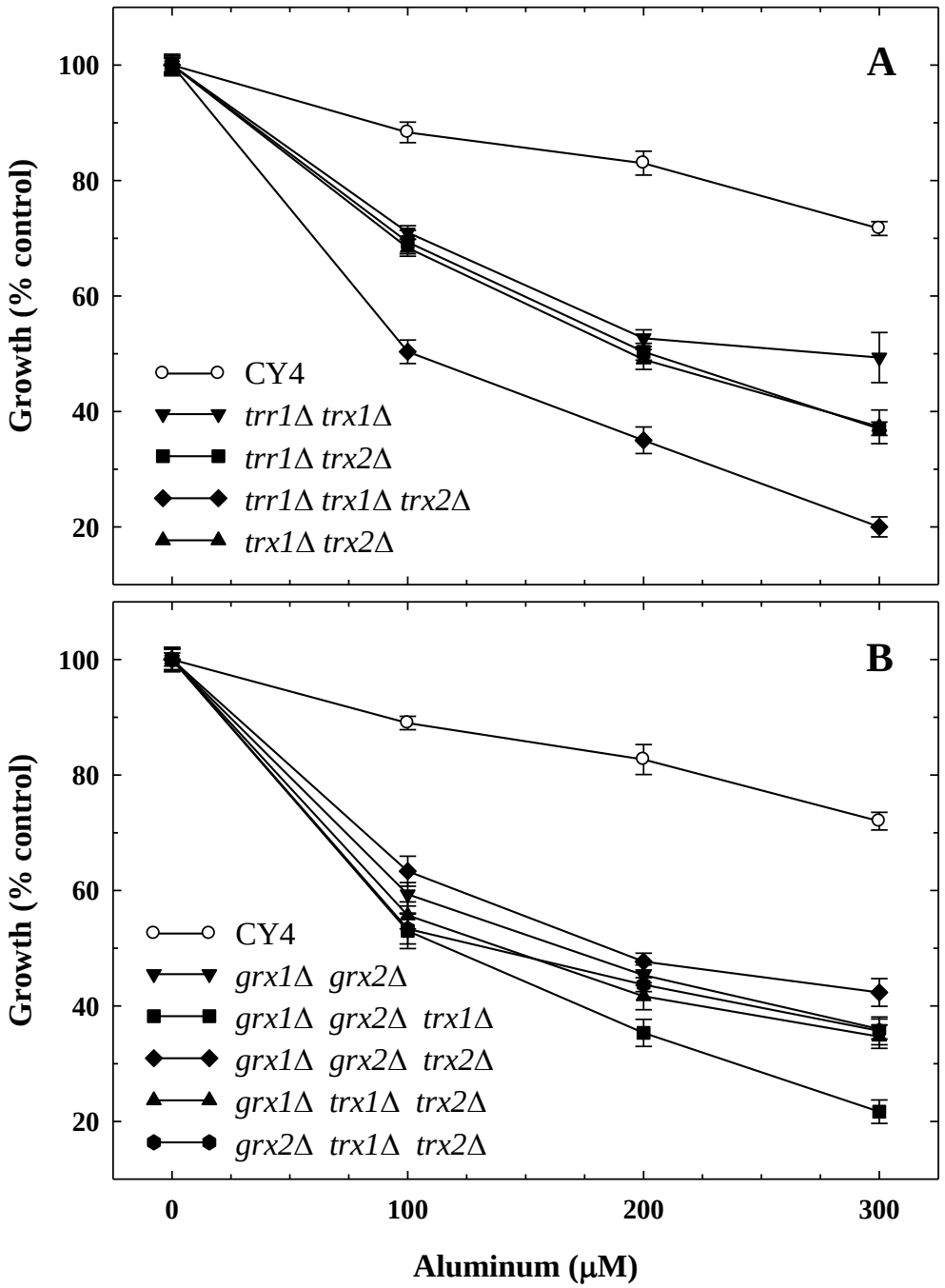

Figure 2-4. Effect of Al on growth (percentage of control) of yeast multiple disruption mutants compared to the wild-type parent strain (CY4). (A) Thioredoxin system (*trr1*Δ *trx1*Δ, *trr1*Δ *trx2*Δ, trr1Δ *trx1*Δ *trx2*Δ and *trx1*Δ *trx2*Δ). (B) Thioredoxin system and glutaredoxins (*grx1*Δ grx2Δ, *grx1*Δ grx2Δ *trx1*Δ, *grx1*Δ *grx2*Δ *trx2*Δ, *grx1*Δ *trx1*Δ *trx2*Δ, *grx2*Δ *trx1*Δ *trx2*Δ). Cells were exposed to Al in LPP medium for 16 h at 30°C. Total growth (OD_{600}) was measured and compared to the control (0 $AlCl_3$) for each strain. Vertical bars represent standard error (n=3). Results shown are representative of three independent experiments.

2.3.4. Sensitivity of multiple mutants defective in thioredoxins, thioredoxin reductases and glutaredoxins to cadmium

Yeast strains with multiple mutations in thioredoxin, thioredoxin reductase, and glutaredoxin genes (Table 2-3) were screened for sensitivity to varying concentrations of Cd in order to test whether these mutants are more sensitive to Cd compared to single mutants. The first experiments exposing these yeast multiple mutant strains to Cd showed that they were extremely hypersensitive to the Cd concentrations previously studied in the experiments with single mutants (data not shown), thus, in order to accurately determine OD_{600} values the Cd concentrations used with these multiple mutants were reduced to 5, 10 and 15 µM $CdCl_2$. At the highest Cd concentration tested (15 µM), the growth of the wild-type strain (CY4) was reduced by 15% (from OD_{600} of 0.529 ± 0.013 at 0 µM Cd to OD_{600} of 0.396 ± 0.015 at 15 µM Cd) (Figure 2-5A). Similar to the experiments with Al, the triple mutant defective in *TRR1*, *TRX1* and *TRX2* also showed the most hypersensitive phenotype to Cd among all multiple mutants with a growth reduction of 75% at 15 µM Cd. The OD_{600} of the *trr1*Δ *trx1*Δ *trx2*Δ strain at 0 µM Cd was 0.475 ± 0.029 and declined to OD_{600} of 0.071 ± 0.038 at 15 µM Cd) (Figure 2-5A). The double mutants *trr1*Δ *trx1*Δ, *trr1*Δ *trx2*Δ and *trx1*Δ *trx2*Δ, were also hypersensitive to Cd and showed a growth reduction of 68%, 65%, and 72% at 15 µM Cd, respectively (Figure 2-5B). Among the multiple mutants defective in glutaredoxin and thioredoxin genes, *grx1*Δ *trx1*Δ *trx2*Δ and *grx2*Δ *trx1*Δ *trx2*Δ, showed the most hypersensitive phenotype to Cd (66% and 65% reduction in growth, respectively) (Figure 2-5B). At 15

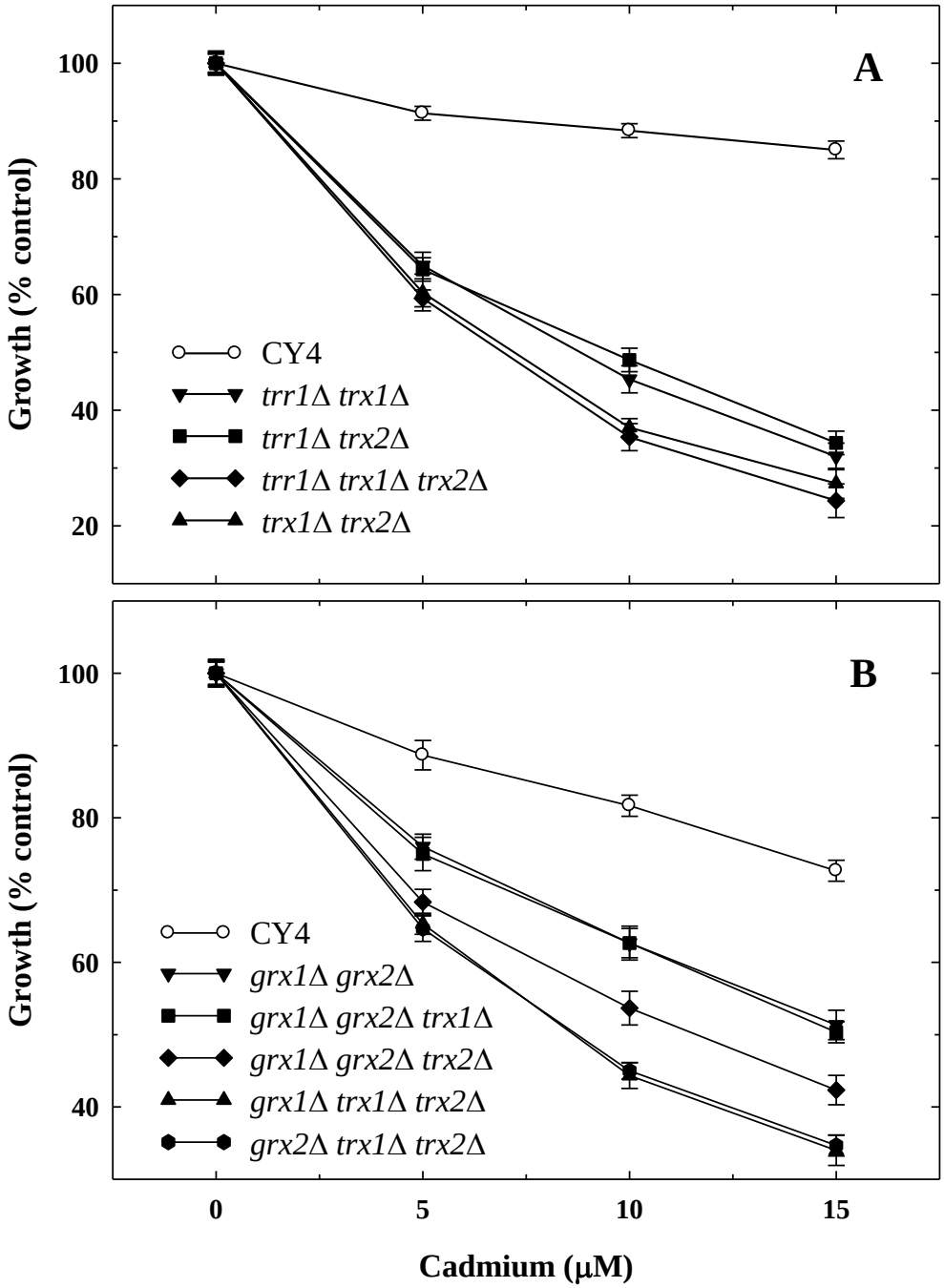

Figure 2-5. Effect of Cd on growth (percentage of control) of yeast multiple disruption mutants compared to the wild-type parent strain (CY4). (A) Thioredoxin system (*trr1*Δ *trx1*Δ, *trr1*Δ *trx2*Δ, trr1Δ *trx1*Δ *trx2*Δ and *trx1*Δ *trx2*Δ). (B) Thioredoxin system and glutaredoxins (*grx1*Δ grx2Δ, *grx1*Δ grx2Δ *trx1*Δ, *grx1*Δ *grx2*Δ *trx2*Δ, *grx1*Δ *trx1*Δ *trx2*Δ, *grx2*Δ *trx1*Δ *trx2*Δ). Cells were exposed to Cd in SC medium for 16 h at 30°C. Total growth (OD_{600}) was measured and compared to the control (0 $CdCl_2$) for each strain. Vertical bars represent standard error (n=3). Results shown are representative of three independent experiments.

μM Cd the multiple mutants *grx1*Δ *grx21*Δ *trx2*Δ, *grx1*Δ *grx2*Δ *trx1*Δ and *grx1*Δ *grx2*Δ showed a growth reduction of 57%, 49%, and 48%, respectively (Figure 2-5B).

2.3.5. Effect of Al on transcript abundance of *TRR1, DOT5, YBL064c* and *GRX3* genes in BY4741

Northern analysis was performed in the parental strain (BY4741) to determine whether the levels of transcripts of thioredoxin, thioredoxin reductase, peroxiredoxin or glutaredoxin genes change in response to Al. The genes used as probes were selected based on the hypersensitivity to Al observed in the single disruption mutants. Interestingly, the levels of cytoplasmic thioredoxin reductase transcript (*TRR1*), showed the greatest induction by Al and Cd among the genes studied. The level of *TRR1* transcript increased in a dose-dependent manner by 40% at 100 μM Al, 110% at 200 μM Al and 130% at 300 μM Al (Figure 2-6). Transcript levels of *TRR1* induced by higher concentrations of Al, however, appear to be underestimated by the quantification method used (spot densitometry). The peroxiredoxin gene *DOT5* showed the next greatest induction in response to Al by 34% of its control, however, at the highest Al concentration (300 μM Al) practically no increase in transcript levels were observed (2%) for this gene (Figure 2-6). The transcript levels of the second peroxiredoxin gene studied (*YBL064c*) remained nearly constant over the range of Al concentrations tested, with only a slight increase of 14% of control at 200 μM Al (Figure 2-6). Similarly, the levels of the glutaredoxin gene selected (*GRX3*) remained nearly constant over the range of Al concentrations, but showing a slight increase of 25% at 100 μM Al (Figure 2-6).

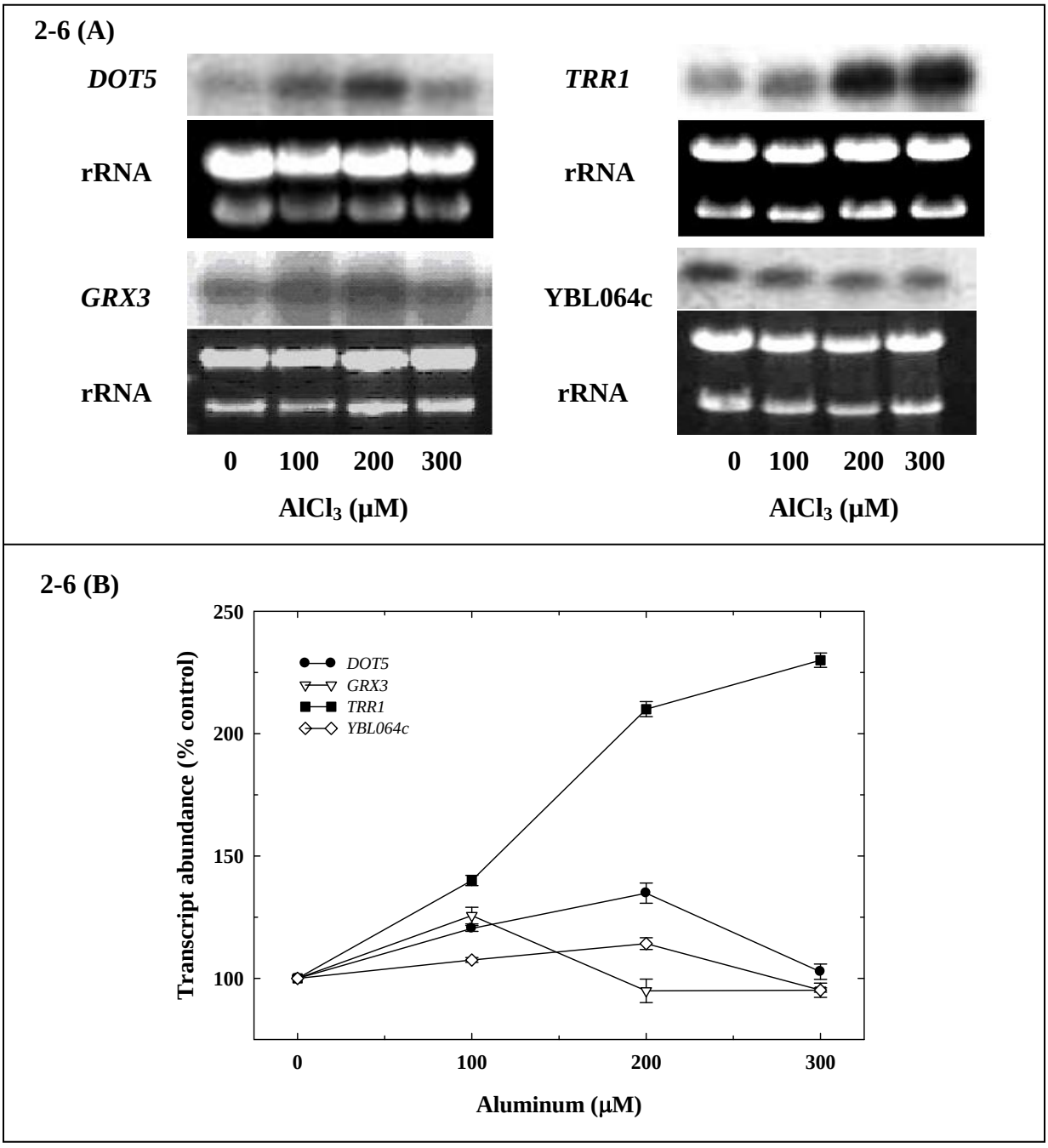

Figure 2-6. Effect of aluminum on transcription of thioredoxin peroxidases (*DOT5*, YBL064c), glutaredoxin (*GRX3*), and thioredoxin reductase (*TRR1*) in yeast. Northern analysis was done using total RNA (8 µg) extracted from BY4741 cells exposed to Al (0-300 µM) for 16 h. Blots were hybridized with ^{32}P-labelled DNA probes. The experiment was repeated at least two times and pictures of representative blots are shown **(A)** Transcript abundance was quantified by calculating the intensity ratio between the entire band of the gene of interest and the ethidium bromide stained rRNA band **(B)** All values given are the means of at least two independent experiments.

2.3.6. Effect of Cd on transcript abundance of *TRR1*, *DOT5*, *YBL064c* and *GRX3* genes in BY4741

Northern analysis was also performed with Cd to determine whether the levels of the transcripts of the genes studied (thioredoxin system, peroxiredoxins and glutaredoxins) change in response to stress induced by this metal ion. Levels of the transcript encoding *TRR1* increased over the range of Cd concentrations tested by 13%, 56%, and 75% at 5, 15 and 30 µM Cd, respectively (Figure 2-7). The peroxiredoxin gene (*DOT5*) also showed a progressive increase in transcript levels in response to increasing concentrations of Cd by 12%, 34% and 39% of control at 5, 15 and 30 µM Cd, respectively (Figure 2-7). Levels of *YBL064c* transcript increased by 14%, 16 and 16% above control in the presence of 5, 15 and 30 µM Cd, respectively. Levels of *GRX3* transcript did not show induction and remained nearly constant over the range of Cd concentrations tested (Figure 2-7).

2.3.7. Complementation of *trr1*Δ mutant

Since the yeast strain defective in *TRR1* showed the most hypersensitive phenotype, and *TRR1* also showed the greatest induction of transcript levels in the presence of Al and Cd, a complementation test was performed to test whether transforming the mutant strain *trr1*Δ with a plasmid containing the functional *TRR1* gene (pRS426-*TRR1*) would restore normal growth in the presence of Al and Cd. As controls, the parental strain, BY4741, was transformed with the empty vector (pRS426) and the pRS426-*TRR1* plasmid and the *trr1*Δ mutant was also transformed with the empty vector.

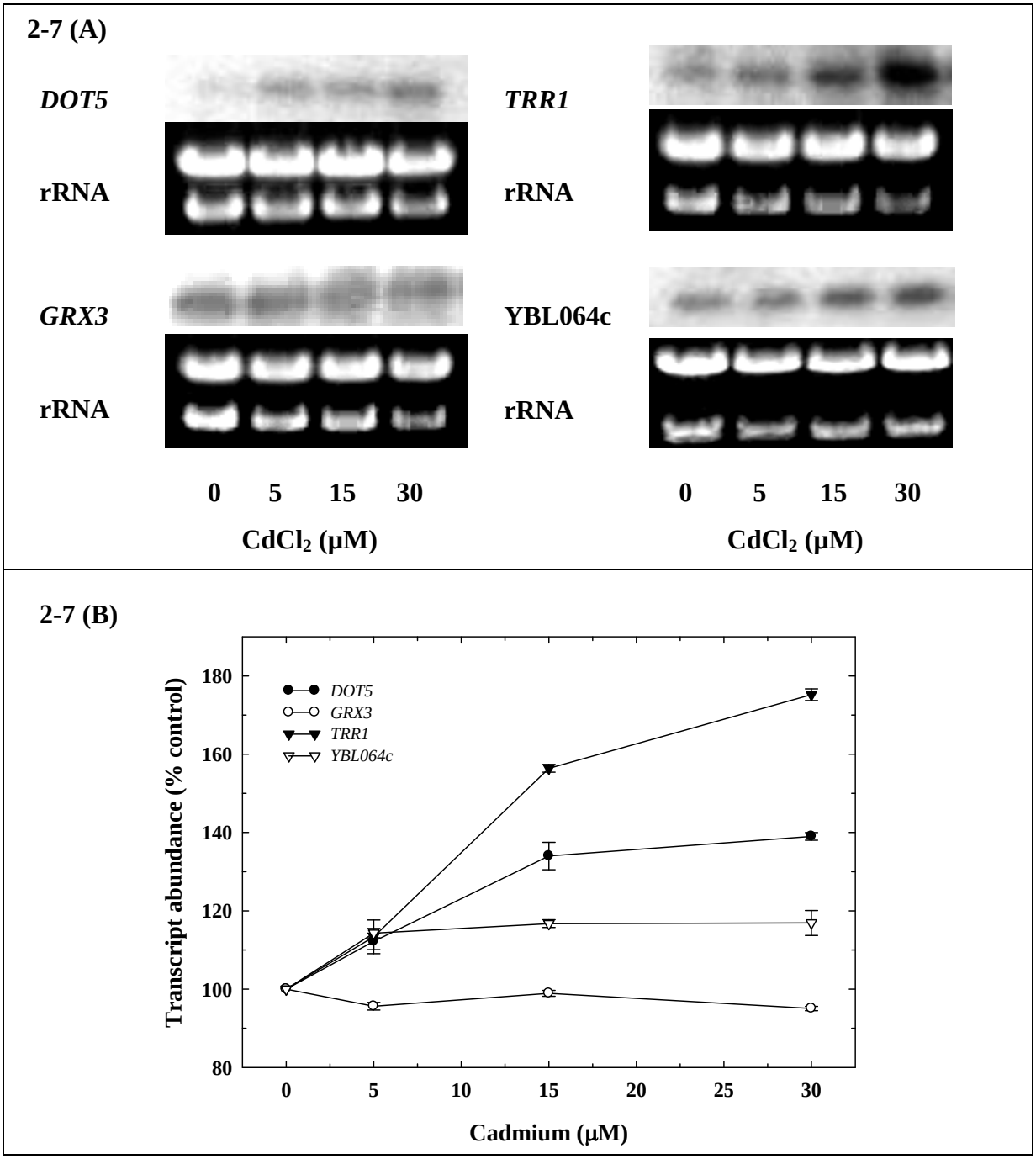

Figure 2-7. Effect of cadmium on transcription of thioredoxin peroxidases (*DOT5*, YBL064c), glutaredoxin (*GRX3*), and thioredoxin reductase (*TRR1*) in yeast. Northern analysis was done using total RNA (8 µg) extracted from BY4741 cells exposed to Cd (0-30 µM) for 16 h. Blots were hybridized with ^{32}P-labelled DNA probes **(A)** Transcript abundance was quantified by calculating the intensity ratio between the entire band of the gene of interest and the ethidium bromide stained rRNA band **(B)** All values given are the means of at least two independent experiments.

At 300 µM Al, the parental strain (BY4741) transformed with the empty vector (BY4741/pRS426) and the parental strain transformed with the plasmid (BY4741/pRS426-TRR1) showed similar growth to the untransformed parental strain (22%, 32% and 32%, respectively) (Figure 2-8A). Similarly, the mutant strain (*trr1*Δ) transformed with the complementation construct (*trr1*Δ/pRs426-*TRR1*), showed a reduction in growth of 30% in the presence of 300 µM Al. The *trr1*Δ mutant strain transformed with the empty vector (*trr1*Δ/pRs426) and the untransformed *trr1Δ* mutant strain (*trr1*Δ) showed a reduction in growth of 54% and 54%, respectively, at 300 µM Al (Figure 2-8A).

In the presence of 30 µM Cd, BY4741 showed a reduction in growth of 45% compared to its control (Figure 2-8B). Similarly, the parental strain transformed with the empty vector (BY4741/pRS426), and the parental strain transformed with the pRs426-*TRR1* construct (BY4741/pRS426-TRR1) showed a 43% and 47% inhibition of growth respectively, at 30 µM Cd (Figure 2-8B). The *trr1*Δ mutant strain complemented by the pRs426-*TRR1* vector (*trr1*Δ/pRs426-*TRR1*) showed a growth pattern similar to that of the parental strain with a 43% inhibition of growth at 30 µM Cd (Figure 2-8B). The *trr1*Δ mutant strain and the *trr1*Δ mutant transformed with the empty vector (*trr1*Δ/pRs426) showed a 74% and 72% growth inhibition in the presence of 30 µM Cd (Figure 2-8B).

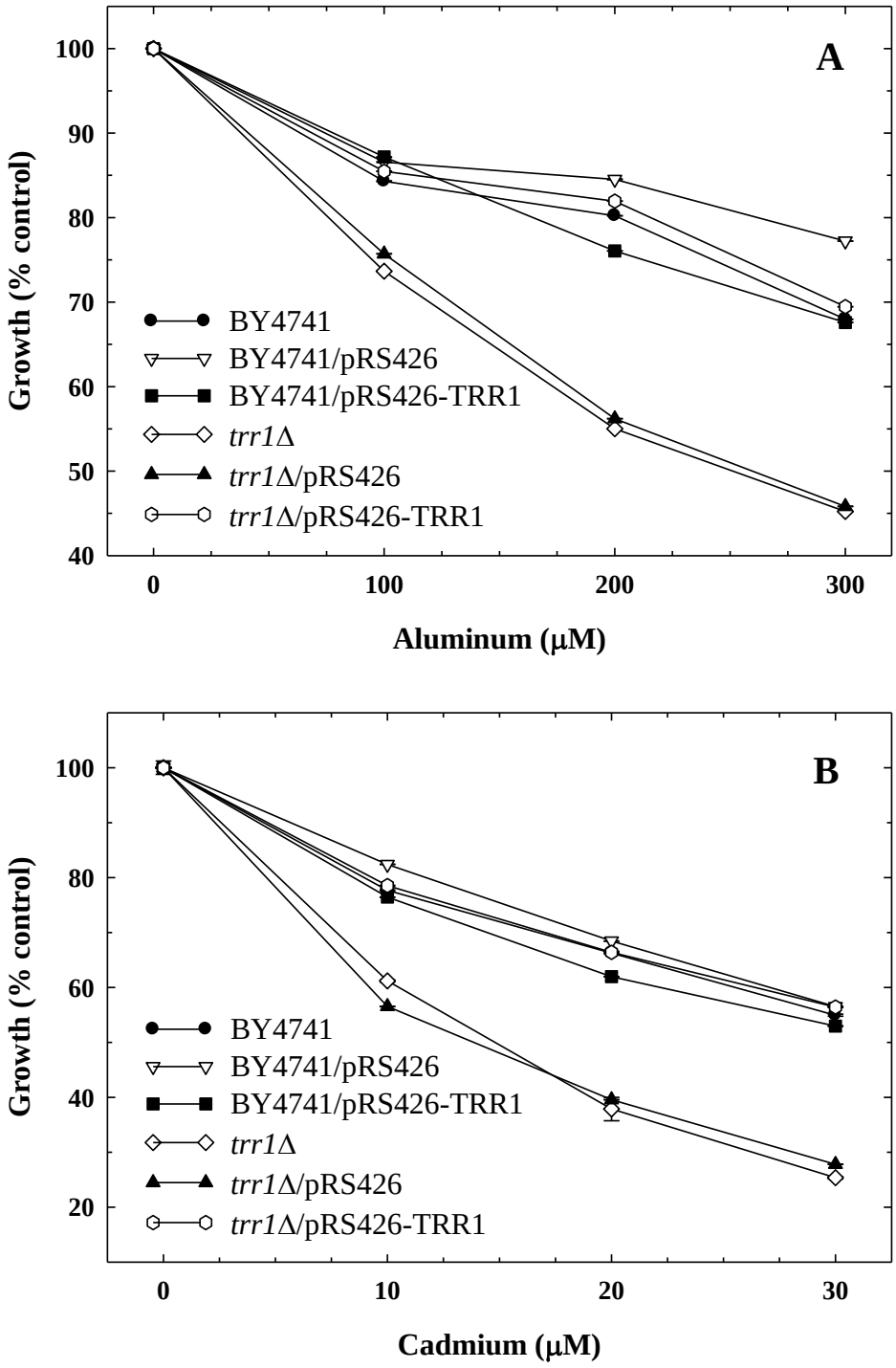

Figure 2-8. Effect of aluminum (A) and cadmium (B) on growth of *trr1*Δ complemented with pRS426-*TRR1*. Cells were exposed to aluminum (A) or cadmium (B) in SC medium for 16 h at 30°C. Total growth (OD_{600}) was measured and compared to the control for each strain. Vertical bars represent standard error (n=3). Results shown are representative of three independent experiments.

2.3.8. Lipid peroxidation in single mutants under aluminum and cadmium stress

Malondialdehyde (MDA) levels were determined to investigate whether single disruption mutants hypersensitive to Al and Cd show increased levels of lipid peroxidation relative to the parental strain. The MDA levels in the parental strain, BY4741, increased by 54 nmol MDA mg^{-1} protein above its control (Figure 2-9). Basal MDA levels for BY4741 were 86 nmol MDA mg^{-1} protein ± 4.2 at 0 µM Al and increased to 140.1 nmol MDA mg^{-1} protein ± 4.2 in the presence of 250 µM Al (Figure 2-9). The MDA levels in the mutant strains *trr1*Δ, *dot5*Δ and *grx5*Δ increased by 66, 52 and 53 nmol MDA mg^{-1} protein, respectively, at 250 µM Al compared to their respective controls (Figure 2-9). The basal MDA levels for *trr1*Δ were 122.4 ± 3.8 nmol MDA mg^{-1} protein at 0 µM Al and increased to 189.3 ± 4.0 nmol MDA mg^{-1} protein at 250 µM Al. The *dot5*Δ mutant strain showed an increase in MDA levels from 94.0 ± 2.9 nmol MDA mg^{-1} protein at 0 µM Al to 146.5 ± 3.7 nmol MDA mg^{-1} protein at 250 µM Al. The MDA levels of *grx5*Δ at 0 µM Al were 102.6 ± 3.7 nmol MDA mg^{-1} protein and increased to 155.9 ± 3.8 nmol MDA mg^{-1} protein at 250 µM Al (Figure 2-9).

Malondialdehyde levels were also determined for the parental strain, BY4741, and single disruption mutants *trr1*Δ, *dot5*Δ and *grx5*Δ exposed to Cd. The MDA levels in the parental strain, BY4741, increased by 61 nmol MDA mg^{-1} protein above control in the presence of 10 µM Cd (Figure 2-10). In BY4741, the basal MDA levels were 85.69 ± 6.3 nmol MDA mg^{-1} protein at 0 µM and increased to 146.9 ± 3.7 nmol MDA mg^{-1} protein at 10 µM Cd. Levels of MDA were slightly induced in the *trr1*Δ mutant relative

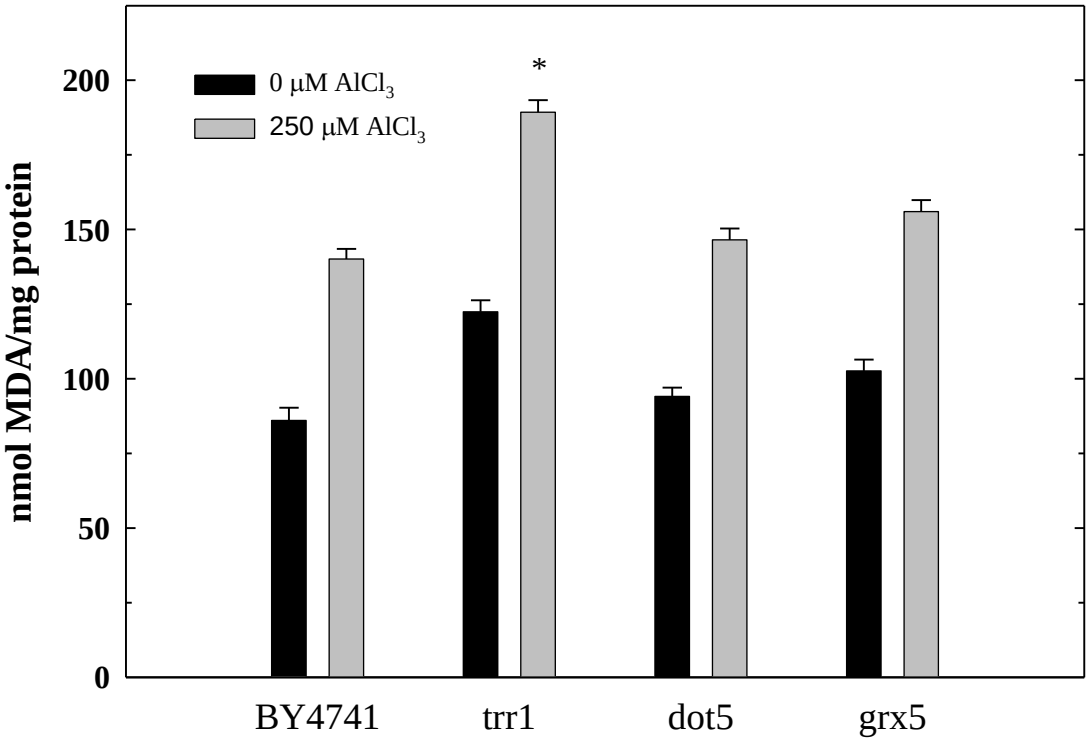

Figure 2-9. Levels of lipid peroxidation in parental strain, BY4741, and single disruption mutant strains *trr1*Δ, *dot5*Δ and *grx5*Δ. The parental strain and single disruption mutants were exposed to 250 μM $AlCl_3$ for 16 h. Malondialdehyde (MDA) values were normalized for protein concentration. * indicates a significant difference ($p<0.1$) between a particular mutant and WT (Student's t-test). Vertical bars represent standard error (n=3). Results shown are representative of three independent experiments.

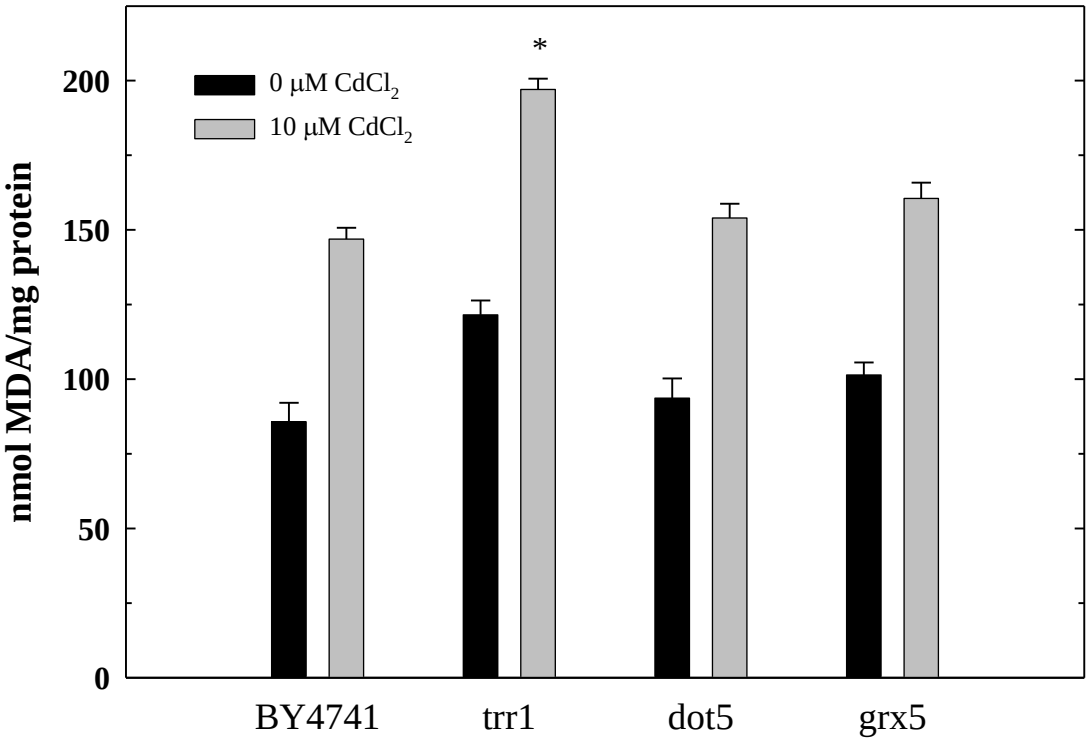

Figure 2-10. Levels of lipid peroxidation in the parental strain, BY4741, and single disruption mutant strains *trr1*Δ, *dot5*Δ and *grx5*Δ. The parental strain and single disruption mutants were exposed to 10 μM $CdCl_2$ for 16 h. Malondialdehyde (MDA) values were normalized for protein concentration. * indicates a significant difference ($p<0.1$) between a particular mutant and WT (Student's t-test). Vertical bars represent standard error (n=3). Results shown are representative of three independent experiments.

to the parental strain by 75 nmol MDA mg^{-1} protein at 10 µM Cd. Levels of MDA in the *trr1*Δ mutant increased from 121.5 ± 4.7 nmol MDA mg^{-1} protein at 0 µM to 197.05 ± 3.6 nmol MDA mg^{-1} protein at 10 µM Cd. (Figure 2-10). The MDA levels in the single disruption mutants *dot5*Δ and *grx5*Δ increased by 60 and 59 nmol MDA mg^{-1} protein above their controls, respectively, in the presence of 10 µM Cd (Figure 2-10).

2.3.9. Lipid peroxidation in multiple mutants under aluminum and cadmium stress

The levels of MDA were determined for multiple mutants hypersensitive to Al and Cd in order to investigate whether lipid peroxidation is induced in these mutants relative to their parental strain (CY4) in the presence of Al and Cd. The parental strain, CY4, showed an increase of MDA levels of 13 nmol MDA mg^{-1} protein upon Al exposure. The basal MDA levels in CY4 were 108.7 ± 3.9 nmol MDA mg^{-1} protein at 0 µM Al and increased to 122.07 ± 3.5 nmol MDA mg^{-1} protein at 250 µM Al (Figure 2-11). The greatest increase in MDA levels, however, was observed in the multiple mutant strain *trr1*Δ *trx1*Δ *trx2*Δ by 49 nmol MDA mg^{-1} protein above its control in the presence of Al (Figure 2-11). The MDA levels in the *trr1*Δ *trx1*Δ *trx2*Δ mutant strain increased from 146.2 ± 3.0 nmol MDA mg^{-1} protein at 0 µM Al to 195.7 ± 3.6 nmol MDA mg^{-1} protein at 250 µM Al (Figure 2-11). The MDA levels in the multiple mutant *grx1*Δ *grx2*Δ *trx2*Δ were also induced compared to the parental strain (CY4) and increased by 38 nmol MDA mg^{-1} protein above its control in the presence of Al (Figure 2-11). The MDA levels in the *grx1*Δ *grx2*Δ *trx2*Δ mutant increased from 169.1 ± 4.1 nmol MDA mg^{-1} protein at 0 µM Al to 207.3 ± 3.3 nmol MDA mg^{-1} protein at 250 µM Al (Figure 2-11).

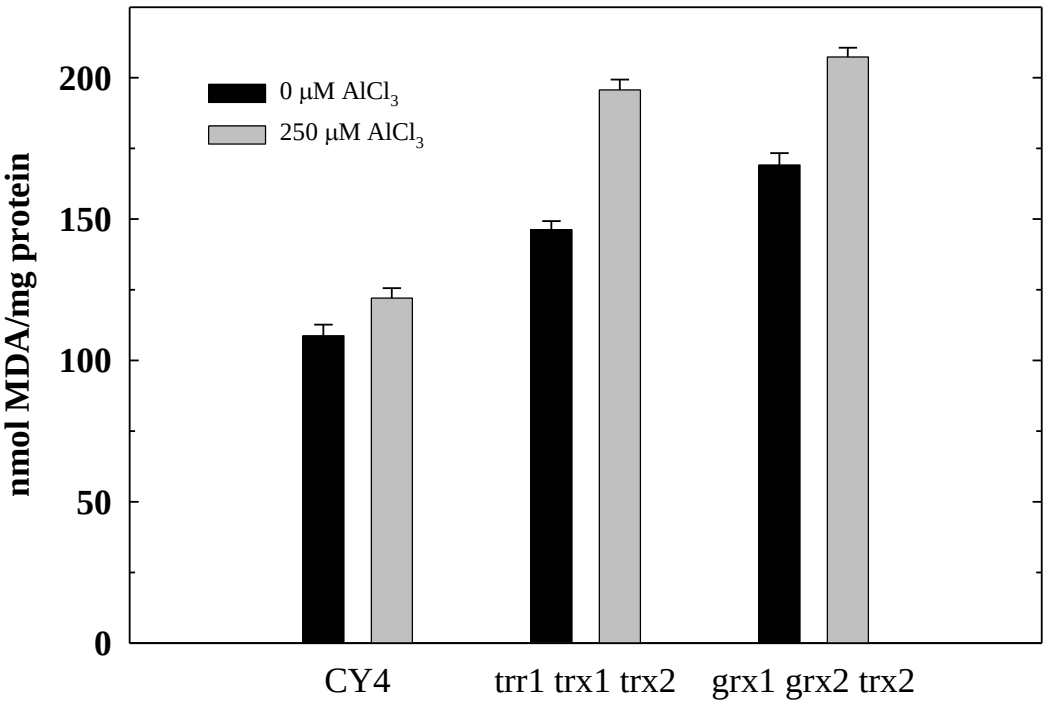

Figure 2-11. Levels of lipid peroxidation in parental strain, CY4, and multiple mutant strains defective in *TRR1*, *TRX1*, *TRX2*, *GRX1* and *GRX2*. The parental strain and multiple mutants were exposed to 250 μM $AlCl_3$ for 16 h. Malondialdehyde (MDA) values were normalized for protein concentration. Vertical bars represent standard error (n=3). Results shown are representative of three independent experiments.

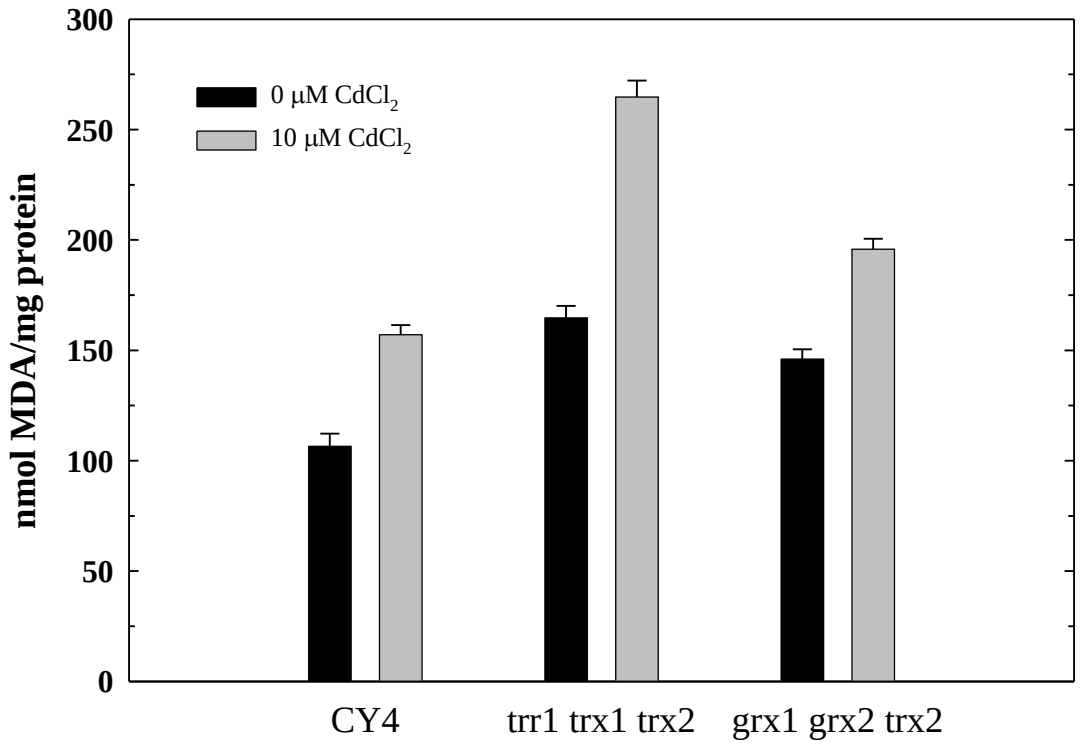

Figure 2-12. Levels of lipid peroxidation in parental strain, CY4, and multiple mutant strains defective in *TRR1*, *TRX1*, *TRX2*, *GRX1* and *GRX2*. The parental strain and multiple mutants were exposed to 10 μM $CdCl_2$ for 16 h. Malondialdehyde (MDA) values were normalized for protein concentration. Vertical bars represent standard error (n=3). Results shown are representative of three independent experiments.

The effect of Cd on MDA levels was also studied for the parental strain, CY4, and the hypersensitive multiple mutant strains *trr1*Δ *trx1*Δ *trx2*Δ and *grx1*Δ *grx2*Δ *trx2*Δ. The parental strain, CY4, showed an increase of 50 nmol MDA mg^{-1} protein in MDA levels in the presence of Cd (Figure 2-12). The basal MDA levels for CY4 were 106.5 ± 5.7 nmol MDA mg^{-1} protein and increased to 157 ± 4.3 nmol MDA mg^{-1} protein at 10 µM Cd (Figure 2-12). The greatest increase in MDA levels was observed for the mutant strain *trr1*Δ *trx1*Δ *trx2*Δ, with an increase of 100 nmol MDA mg^{-1} protein above its control in the presence of Cd. The MDA levels for *trr1*Δ *trx1*Δ *trx2*Δ increased from 164.6 ± 5.4 nmol MDA mg^{-1} protein at 0 µM Cd to 264.7 ± 7.4 nmol MDA mg^{-1} protein at 10 µM Cd (Figure 2-12). The MDA levels in the mutant strain *grx1*Δ *grx2*Δ *trx2*Δ increased by 49 nmol MDA mg^{-1} protein upon Cd exposure. The basal MDA levels for *grx1*Δ *grx2*Δ *trx2*Δ were 145.9 ± 4.5 nmol MDA mg^{-1} protein at 0 µM Cd and increased to 195.7 ± 4.7 nmol MDA mg^{-1} protein at 10 µM Cd (Figure 2-12).

2.4. Discussion

Metal-induced oxidative stress is a known mechanism of toxicity for redox active metal ions and some non-redox active metal ions, such as Al and Cd. Not surprisingly, a connection between isolated components of the antioxidant defense system and Al and Cd resistance has been observed in numerous studies (Cakmak and Horst, 1991; Chaoui *et al*., 1997; Richards *et al*., 1998; Ezaki *et al*., 2000; Boscolo *et al*., 2003; Basu *et al*., 2004; Smeets *et al*., 2008; Markovska *et al*., 2009). Antioxidant enzymes of the thioredoxin system, peroxiredoxins and glutaredoxins have been shown to play an

important role protecting yeast cells from oxidative stress (Rodriguez-Manzaneque *et al*., 1999; Carmel-Harel and Storz, 2000; Grant, 2001; Collinson *et al*., 2002; Wong *et al*, 2002), however, these enzymes have not been previously studied in the context of Al and Cd tolerance.

In this work, I used yeast (*Saccharomyces cerevisiae*) as a model system to identify putative genes encoding thioredoxins, thioredoxin reductases, peroxiredoxins and glutaredoxins that could be involved in Al and Cd tolerance in plants. This approach allowed me to dissect a less complex antioxidant system in a rapid manner with the purpose of identifying genes of interest to further study in plants. In this study, I used the readily available collection of single disruption mutants in *S. cerevisiae* to screen for hypersensitivity to Al and Cd and select genes of interest. Similarly, in a previous study, Basu *et al*., (2004) used yeast disruption mutants to study the genes involved in the glutathione metabolic pathway and demonstrated a role for the glutathione peroxidase gene, *PHGPX3*, in the response to Al stress in yeast.

In the present work, a total of 15 yeast single disruption strains defective in genes encoding all antioxidant enzymes of the thioredoxin system, peroxiredoxins and glutaredoxins were screened for hypersensitivity to Al and Cd. The yeast mutant strain defective in cytoplasmic thioredoxin reductase (*trr1*Δ) was particularly interesting, since it showed the most hypersensitive phenotype to both, Al and Cd. Upon Al and Cd exposure, the *trr1*Δ strain showed a 57% and 79% maximum growth inhibition respectively, whereas the WT showed a maximum growth inhibition of 27% and 42%, respectively. Strains harbouring single mutations that also showed strong hypersensitive

phenotypes to Al and Cd were those defective in peroxiredoxins *DOT5*, *TSA1* and *YBL064c*, and glutaredoxins *GRX3* and *GRX5*. Other yeast strains showed slightly higher sensitivity or did not show any higher sensitivity at all compared to the WT. This last observation might be explained by the overlapping function in redox regulation of many of these antioxidant enzymes. Thus, the loss of activity of one antioxidant enzyme in a single mutant strain could be compensated by another antioxidant enzyme. For instance, the yeast glutaredoxins act as antioxidants and have activity as glutathione peroxidases as well (Luikenhuis *et al*., 1997; Collinson *et al*., 2002). This overlapping function between glutaredoxins and glutathione peroxidases could explain the lack of phenotype observed by Basu *et al*., (2004) when yeast single mutants defective in glutathione peroxidases were exposed to Al.

In yeast, TRR1 is a key regulatory enzyme that determines the redox state of the cytoplasmic thioredoxin system (Trotter and Grant, 2003). Carmel-Harel *et al*., (2001) observed that yeast cells lacking cytoplasmic thioredoxin reductase are extremely sensitive to H_2O_2 and possess diminished capacity to detoxify oxidants and to repair oxidative stress-induced damage. The hypersensitivity of the *trr1*Δ strain to Al and Cd observed here suggests that cytoplasmic thioredoxin reductase is a key player of the defense mechanisms that yeast cells possess against Al- and Cd-induced stress. These results also suggest that oxidative damage is one of the mechanisms of Al and Cd toxicity in yeast and that TRR1 may protect yeast cells by detoxifying oxidants and repairing oxidative damage induced by these metal ions. Moreover, Trotter and Grant (2003) observed that loss of *TRR1* not only results in the oxidation of thioredoxins but also shifts the redox state of glutaredoxins to a more reduced form. Thus, the highly hypersensitive

phenotype to Al and Cd of the single mutant *trr1*Δ could be partly explained by the diminished antioxidant capacity of glutaredoxins. Similarly, Rodríguez-Manzaneque *et al*., (1999) reported that *GRX5* plays a particularly important role protecting yeast cells against oxidative stress and observed that mutants lacking *GRX5* are highly sensitive to oxidative damage caused by hydrogen peroxide and the superoxide anion. In this study, I observed that the mutant strain lacking *GRX5* (*grx5*Δ) had the most hypersensitive phenotype to Al and Cd among all glutaredoxins (Figures 2-2 and 2-3), which again suggests that Al and Cd cause oxidative stress and that *GRX5* plays an important role as a defense mechanism against this stress. Analysis of mutants lacking peroxiredoxin genes in yeast have shown that *TSA1* is more involved in hydrogen peroxide resistance than *AHP1*, which appears to be specific for organic peroxides (Lee *et al*., 1999). I observed that the yeast mutant lacking *TSA1* was one of the most hypersensitive strains to Al and Cd among the strains lacking peroxiredoxins. All together, these observations suggest that Al and Cd probably induce oxidative stress mainly by increasing the levels of hydrogen peroxide in yeast cells.

Since *TRR1* seemed to be a particularly interesting candidate to further study in plants, I decided to test whether the hypersensitive phenotype to Al and Cd observed in the *trr1*Δ mutant could be restored by complementation with the functional *TRR1* gene in yeast. The growth pattern in the presence of Al and Cd observed for the *trr1*Δ mutant strain transformed with a plasmid containing the *TRR1* gene (pRS426-*TRR1*) was similar to that of the parental strain, which provides further support for the role of *TRR1* in Al and Cd tolerance in yeast (Figure 2-8).

I also tested the sensitivity of all 9 viable yeast strains harbouring multiple mutations in genes of the thioredoxin system, peroxiredoxins and glutaredoxins to Al and Cd. All multiple mutants showed increased sensitivity to Al and Cd compared to the parental strain (CY4), and they also showed at least a 2-fold increase in hypersensitivity to Al and Cd compared to single mutants. Multiple mutant strains were extremely hypersensitive to the Cd concentrations used in the experiments with single mutants and lower concentrations of Cd were used to better observe growth differences. I observed that from all strains tested, the triple mutant *trr1*Δ *trx1*Δ *trx2*Δ was the most hypersensitive to Al and Cd, with a maximum growth reduction of 80% and 75% upon Al and Cd treatment, respectively (Figures 2-4 and 2-5). The highly hypersensitive phenotype to Al and Cd observed for this strain seems to correlate with its severely diminished antioxidant capacity. This strain, *trr1*Δ *trx1*Δ *trx2*Δ, lacks the entire cytoplasmic thioredoxin system, encoded by thioredoxin reductase *TRR1*, and thioredoxins *TRX1* and *TRX2*. Although the mitochondrial thioredoxin system is intact, it does not appear to compensate for the loss of function of the cytoplasmic thioredoxin system. The second most hypersensitive multiple mutant strain to Al (78% growth reduction at 300 µM) was that lacking the entire subfamily of dithiol glutaredoxins (*GRX1-2*) and also defective in cytoplasmic thioredoxin *TRX1* (*grx1*Δ *grx2*Δ *trx1*Δ). A multiple mutant strain with exactly the same mutations except that TRX2 was defective instead of TRX1 (*grx1*Δ *grx2*Δ *trx2*Δ) showed noticeably less sensitivity to Al and Cd than *grx1*Δ *grx2*Δ *trx1*Δ. This suggests that *TRX1* plays a unique role in Al- and Cd-induced oxidative stress. In the case of Cd, however, the second most hypersensitive strain was the double mutant *trx1*Δ *trx2*Δ, defective in both cytosolic thioredoxin

reductases, with a growth reduction of 72% by 15 µM Cd (Figure 2-5). It can be concluded from these results that a highly diminished antioxidant capacity caused by multiple mutations in genes of the thioredoxin system and glutaredoxins causes increased sensitivity to Al and Cd.

Aluminum and Cd enhanced the expression of various genes encoding antioxidant enzymes, such as genes encoding glutathione peroxidase, superoxide dismutase dehydroascorbate reductase, glutathione *S*-transferase and thioredoxin (Lemaire *et al*., 1999; Ezaki *et al*., 2001; Basu *et al*., 2004; Smeets *et al*., 2008). Based on my results from the sensitivity test, I decided to investigate whether Al and Cd enhance the expression of *TRR1*, *DOT5*, *TSA1*, *YBL064c*, *GRX3* and *GRX5*. However, given the high degree of homology between *TSA1* and *TSA2*, and between *GRX4* and *GRX5*, it was not possible to design a probe that would be specific enough for Northern analysis of *TSA1* and *GRX5* genes. Northern analysis of the WT strain (BY4741) showed that *TRR1* was the most induced gene upon Al and Cd treatment among the genes tested (Figures 2-6 and 2-7). However, this induction was stronger upon Al treatment (Figure 2-6). These results provide further support for the role of *TRR1* in the Al and Cd stress response. On the other hand, peroxiredoxins *DOT5* and YBL064c and glutaredoxin *GRX3* transcript levels increased slightly or not at all upon Al and Cd treatment (Figures 2-6 and 2-7).

To investigate whether yeast mutant strains that were hypersensitive to Al and Cd experience increased oxidative damage relative to the WT in the presence of Al and Cd, I quantified the levels of lipid peroxidation in selected yeast mutant strains upon Al and Cd exposure. Lipid molecules are sensitive to ROS generated under oxidative stress, and the

presence of lipid peroxides is considered a major indicator of oxidative stress (Becana *et al*., 1998). To quantify lipid peroxidation, I used the TBARS assay, which detects primarily products arising from the decomposition of lipid hydroperoxides such as malondialdehyde (MDA) (Aust, 1994). Among the single mutants studied (*trr1*Δ, *dot5*Δ, *grx5*Δ), *trr1*Δ was the only mutant strain that showed a significant Al- and Cd-induced increase of lipid peroxidation compared to the parental strain (BY4741) (Figures 2-9 and 2-10). These results support the notion that TRR1 plays a role protecting yeast cells from Al- and Cd- induced oxidative stress. Although the lack of peroxiredoxin *DOT5* and glutaredoxin *GRX5* in yeast cells results in hypersensitivity to Al and Cd, the levels of lipid peroxidation in the presence of Al and Cd remain similar to those observed for the WT (Figures 2-9 and 2-10). This observation suggests that other antioxidant enzymes are able to compensate for the loss of function of *DOT5* and *GRX5*, and thus protect yeast cells from Al- and Cd-induced oxidative damage. However, the hypersensitivity of *dot5*Δ and *grx5*Δ mutant strains remains unclear and suggests that *DOT5* and *GRX5* may have a different role in Al and Cd tolerance other than directly protecting yeast cells from Al- and Cd-induced oxidative damage. Among the multiple mutants studied, only the triple mutant *trr1*Δ *trx1*Δ *trx2*Δ showed a significant increase in lipid peroxidation in the presence of both Al and Cd compared to the parental strain (CY4). The triple mutant *grx1*Δ *grx2*Δ *trx2*Δ showed a significant increase in lipid peroxidation in the presence of Al but not in the presence of Cd (Figures 2-11 and 2-12). According to these results, it appears that the lack of *TRR1*, either as a single or multiple mutant strain, renders yeast cells more susceptible to Al- and Cd-induced oxidative stress. The lack of Al- and Cd-induced oxidative damage in some hypersensitive yeast strains studied here, also suggests

that some antioxidant enzymes may play a role in Al and Cd tolerance that is different from scavenging ROS or repairing oxidative damage directly.

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3. Investigating the role of peroxiredoxins and thioredoxin reductases in aluminum and cadmium tolerance in *Arabidopsis thaliana* using a reverse genetics approach

3.1. Introduction

In this study, a reverse genetics approach was used to investigate the role of peroxiredoxins and thioredoxin reductases in Al and Cd tolerance in the model plant *Arabidopsis thaliana*. Peroxiredoxins and thioredoxin reductases were selected for further study in plants, since yeast mutants defective in these antioxidant enzymes showed highest hypersensitivity to Al and Cd (Chapter 2). Selection of a smaller group of genes was important due to the fact that plants possess a complex thioredoxin and glutaredoxin system. In *Arabidopsis thaliana*, 40 genes coding for thioredoxin and thioredoxin-related proteins, 31 genes coding for glutaredoxins and 11 genes coding for peroxiredoxins have been identified in the whole sequenced genome (Meyer *et al*., 2002; Rouhier *et al*., 2004; Meyer *et al*., 2006). The reason for the abundance of Trx and Grx isoforms in plants remains unclear, although this complexity may explain how plants are able to maintain redox homeostasis under changing environmental conditions (Hisabori *et al*., 2007).

Peroxiredoxins (Prxs) are part of the major antioxidant enzymes in plants, which also include superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT) and glutathione peroxidase (GPX). However, knowledge of Prxs is limited, since they have only recently been identified as a new class of antioxidant enzymes in plants (Dietz 2003; Wood *et al*., 2003). Plant Prxs posses one or two characteristic cysteines in highly

conserved sequence motifs that participate in the reduction of hydrogen peroxide and various alkyl hydroperoxides. The cysteinyl residues in the catalytic center of plant Prxs are regenerated via intramolecular or intermolecular thiol-disulfide-reactions and finally by electron donors such as thioredoxins and glutaredoxins The *Arabidopsis thaliana* genome contains 10 Prxs genes and five of them are targeted to organelles, including one (Prx II F) targeted to the mitochondrion and four (Prx II E, 2-Cys Prx A, 2-Cys Prx B and Prx Q) targeted to the chloroplast (Dietz *et al*. 2002; Horling *et al*. 2002; Finkemeier *et al*. 2005). Various Prxs have been found to play a role in tolerance to salt, low-temperature and oxidative stress in plants (Horling *et al*., 2003; Jing *et al*., 2006).

Plants contain more types of thioredoxin systems than any other organism. The cytosolic thioredoxin system is composed of type *h* thioredoxins and NADPH-thioredoxin reductases. NADPH-dependent thioredoxin reductases (NTR) are homodimeric enzymes that reduce thioredoxins. NTRs belong to a superfamily of flavoprotein disulfide oxidoreductases that includes glutathione reductase, dihydrolipoamide reductase, mercuric reductase and alkylhydroperoxide reductase (Russel and Model, 1988). Plants contain also a chloroplastic and a mitochondrial thioredoxin system. The mitochondrial thioredoxin system in plants is composed of type *o* thioredoxins and NADPH-thioredoxin reductases, where as the chloroplastic thioredoxin system is composed of thioredoxins type *f, m,* and *x* which are reduced by ferredoxin in a reaction catalyzed by ferredoxin thioredoxin reductase (Schurmann and Jacquot, 2000). *Arabidopsis* contains two NTR genes, each expressing a short mRNA encoding a cytosolic NTR and a long mRNA encoding a mitochondrial NTR precursor (Laloi *et al*., 2001; Reichheld *et al*., 2005). These two genes encoding NADPH-

thioredoxin reductases in *Arabidopsis* (*AtNTRA* and *AtNTRB*) originated from a recent gene duplication event (~40 million years) and appear to be evolving to specialized cytosolic or mitochondrial functions (Blanc *et al*., 2003; Reichheld *et al*., 2005). Unlike yeast (*Saccharomyces cerevisiae*), where cytosolic and mitochondrial NTRs are encoded by *TRR1* and *TRR2*, respectively, *Arabidopsis* genes *AtNTRA* and *AtNTRB* encode both cytosolic and mitochondrial isoforms. However, it appears that *AtNTRA* expresses as the major cytosolic isoform and *AtNTRB* encodes the major mitochondrial isoform (Reichheld *et al*., 2005).

Although some peroxiredoxins such as *SsPrxQ* and the chloroplastic NADPH-thioredoxin reductase (*AtNTRC*) have been found to play a role in tolerance to oxidative stress, neither peroxiredoxins nor thioredoxin reductases have been functionally characterized as to their possible roles in Al or Cd tolerance (Serrato *et al*., 2004; Jing *et al*., 2006). In the present study, I used a reverse genetics approach to test the hypothesis that peroxiredoxins and/or thioredoxin reductases may play a role in Al and Cd tolerance in plants by ameliorating oxidative damage induced by Al and Cd toxicity. *Arabidopsis* T-DNA mutant lines defective in peroxiredoxins and NADPH-thioredoxin reductases were screened for hypersensitivity to Al and Cd, and sensitive knockout lines were further characterized in an attempt to elucidate the role of these antioxidant enzymes in Al and Cd tolerance.

3.2. Materials and Methods

3.2.1. Plant material and growth conditions

Salk T-DNA insertion lines (Alonso *et al*., 2003) and their parental wild-type (Columbia) used for this study were obtained from ABRC (Arabidopsis Biological Resource Center, The Ohio State University) (Table 3-1 and 3-2). Seeds were surface sterilized by vortexing for 10 min in a solution containing 20% bleach and 0.05% Tween-20. Seeds were pelleted and rinsed five times in sterile Milli-Q water and suspended in 0.1% agar. To break dormancy, seeds were kept in darkness for 2 d at 4^{o}C. Following stratification, seeds were sown in a soil mixture (Metromix 350, Scotts Company, Marysville OH) and transferred to a controlled environment chamber at 22^{o}C with 16 h light and 8 h dark cycle, 70% humidity and approximately 160 µmol m^{-2} s^{-1} photosynthetic photon flux density. Plants were fertilized weekly with Richards medium (Richards *et al*., 1998) containing 5mM KNO_3, 2.5 mM KH_2PO_4 2mM $MgSO_4$, 2 mM $Ca(NO_3)_2$, 12.5 µM FeEDTA, 7 µM H_3BO_3, 14 µM $MnCl_2$, 0.5 µM $CuSO_4$, 1 µM $ZnSO_4$, 10 µM NaCl, and 0.1 µM $CoCl_2$, pH 5.8.

3.2.2. Isolation of *Arabidopsis prx* and *ntr* mutants

The PCR screening protocol described on the Salk webpage (http://signal.salk.edu/tdnaprimers.2.html) was used to identify knockout lines homozygous for the T-DNA insertion. Briefly, genomic DNA was isolated from 3-week old seedlings using the Extract-N-Amp PCR kit (Sigma) and used as a template for PCR.

Table 3-1. *Arabidopsis* lines harbouring a T-DNA insert in genes encoding peroxiredoxins and thioredoxin reductases used in this study. Seeds were obtained from the Arabidopsis Biological Resource Center (ABRC).

Locus No.	Gene name	T-DNA line (s)	Insertion site
At2g17420	Thioredoxin reductase 2 (NTRA)	SALK_039152 SALK_014076	Exon Exon
At4g35460	Thioredoxin reductase (NTRB)	SALK_027935 SALK_045978	Exon Intron
At2g41680	Thioredoxin reductase (NTRC)	SALK_096776	Exon
At1g65990	Type II PRX A	SALK_075656	Exon
At1g65980	Type II PRX B	SALK_069621	300-UTR3
At1g65970	Type II PRX C	SALK_018070	Promoter
At3g52960	Type II PRX E	SALK_064512	Exon
At3g06050	Type II PRX F	SALK_043925	Intron
At1g48130	1-cys PRX	SALK_133714	Exon
At3g11630	2-cys PRX A	SALK_059673	Exon
At5g06290	2-cys PRX B	SALK_017213	Exon
At3g26060	PRX Q	SAIL_742_G10	Intron
At4g09143	Peroxiredoxin related	SALK_123174	Exon
At1g60740	Type II PRX	SALK_138035	Intron

Table 3-2. Salk lines isolated for *NTRA* and *NTRB* genes (after screening lines described above).

Gene	T-DNA line	Insert localization
AtNTRA	SALK_039152	exon
AtNTRA	SALK_014076	exon
AtNTRB	SALK_045978	intron
AtNTRB	SALK_027935	exon

Two reactions were set up for each T-DNA knockout line. The first PCR reaction was set up using gene specific primers designed using the T-DNA primer design tool (http://signal.salk.edu/tdnaprimers.2.html) (Tables 3-3 and 3-4). The second PCR reaction was set up using a primer specific for the left border sequence of T-DNA, LBb1 (5' - GCGTGGACCGCTTGCTGCAACT -3') for SALK lines, and LB3 (5' - TAGCATCTGAATTTCATAACCAATCTCGATACAC - 3') for SAIL lines, and gene specific right primers (Tables 3-3 and 3-4).

3.2.3. RT-PCR analysis

Total RNA was isolated from 3-week old seedlings using an RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's protocol. The isolated total RNA was treated with RNase-Free DNase (Qiagen) to eliminate DNA contamination and

Table 3-3. List of primers used for PCR to identify homozygous *Arabidopsis* lines containing a T-DNA insert in genes encoding peroxiredoxins.

Locus No.	T-DNA line	Primers (gene specific)
At3g26060	SAIL_742_G10	LP 5' CCACAAGCTTTTAAGGAGCTG 3' RP 5' GAGTTCATATGCCGTGTCAGC 3'
At1g65990	SALK_075656	LP 5' TCATTGAGAAAGCAGAGGAGC 3' RP 5' GTTCTTTAGCGCCCCAGTAAG 3'
At1g48130	SALK_133714	LP 5' GAGCGAGAAAGGAAACGAGAG 3' RP 5' ACCTTGCTTCCGTGCTATATC 3'
At1g65980	SALK_069621	LP 5' CTGATTTCTTCGTGAGCAAGC 3' RP 5' TAGCCAATGTCGAATCTGGTG 3'
At5g06290	SALK_017213	LP 5' CTAATTCCAGGGAGCTGTGAC 3' RP 5' GGCAGTCTGATAATCGAATGG 3'
At1g65970	SALK_018070	LP 5' GAGTGAAAGCACCAGGAACAC 3' RP 5' TGTTTTGTGCGGTATCTAATGC 3'
At4g09143	SALK_123174	LP 5' TCTAATACCGTGACCGTCGTC 3' RP 5' AAAAAGATTGTGTTTCGGGTG 3'
At1g60740	SALK_138035	LP 5' GGTGTGCGAGTTTGCAATAAG 3' RP 5' ATCAGCTTCAGCCGTCTCTG 3'
At3g06050	SALK_043925	LP 5' ACCAGGTAGCAGGAAAGAAG 3' RP 5' TGTAACTCTTGTTGACGTCCTG 3'
At3g52960	SALK_064512	LP 5' TCCGAAAACTAAACACAACGC 3' RP 5' AGGCCCAATAATGACATAGCC 3'
At3g11630	SALK_059673	LP 5' GGACTTTACTTTCGTCTGCCC 3' RP 5' TTTGGGGACAAAGTGAGAATC 3'

Table 3-4. List of primers used for PCR to identify homozygous *Arabidopsis* lines containing a T-DNA insert in genes encoding NADPH-thioredoxin reductases.

Locus	T-DNA line	Primers (gene specific)
At4g35460	SALK_027935	LP 5' GGAAGAATCATTCTGGTTTGG3' RP 5' TTTCAATCCTCCAAGCACATC3'
At2g17420	SALK_039152	LP 5'TTTGTTTAGGGTTAGGGATAGGG3' RP 5' CTTCACCAGATCCAGTGAAGC 3'
At2g41680	SALK_096776	LP 5' TCAGAAGTGCAACGATCACTG 3' RP 5' CAAGCATTTTCTCTGCCTCAC 3'

transcribed into cDNAs using RevertAid H Minus M-MuLV reverse transcriptase (Fermentas) and Oligo(dT)$_{18}$ primer (Fermentas) following manufacturer's instructions. The synthesized first strand cDNA was used as a template for PCR with the following primers:

NTRA (5' GCAAAATGTGTTGGATCTCAATGAG 3',
5' CATGGATCCTTCTCCTACAGCTTC 3'),

NTRB (5' CGAAAGCTTTGCACGGCTTGGTGGTG 3',
5' GATCAATCAACAATAACTCAATGACCT 3'),

β-tubulin (5' CGTGGATCACAGCAATACAGAGCC 3',
5' CCTCCTGCACTTCCACTTCGTCTTC 3').

3.2.4. Root elongation assay

Seeds of homozygous T-DNA lines and WT were surface sterilized as described in section 3.2.1. Seeds were then plated in 0.5 X MS medium containing 6.5 g phytagar and plates were kept in the dark at 4^{o}C for 2 d. Following cold stratification, plates were transferred to a controlled environment chamber (22^{o}C with 16 h light and 8 h dark cycle and 70% humidity) for 10 d before transferring seedlings to exposure plates containing Al or Cd. For Al treatment, plates were prepared using the Al exposure medium for *Arabidopsis* described by Snowden *et al*. (1995). This medium contains reduced phosphate levels to avoid precipitation of Al. Briefly, to prepare 1L of medium, 5 ml of four stock solutions (Table 3-5) were mixed in Milli-Q water and 0.0861 g $CaSO_4$ was added. The pH of the solution was adjusted to 4.3 using 0.1M HCl and 14 g phytagar were added prior to autoclaving. Media containing 0 µM, 100 µM, 125 µM and 150 µM $AlCl_3$ were prepared by adding 0 ml, 4 ml, 5ml, and 6 ml of a filter sterilized 25 mM $AlCl_3$ stock to a liter of the autoclaved solution and poured in 100x100x15 mm square plates with grid. Ten day-old seedlings were transferred to Al exposure plates in triplicate (8 plants per plate) ensuring that roots were adequately stuck to the agar and as straight as possible. The tip of each root was marked in the bottom of the plates with a permanent marker before transferring the plates to the growth chamber (Time 0), and this was done again after 24, 48 and 72 hours of Al exposure. For Cd treatment, solutions containing 0 µM, 25 µM, 75 µM, and 100 µM $CdCl_2$ were prepared by adding 0 ml, 2.5 ml, 7.5 ml, and 10 ml, respectively, of a filter sterilized 10 mM $CdCl_2$ solution to a liter of autoclaved 0.5 X MS medium containing 1.4% phytagar.

Table 3-5. List of components of the exposure medium used for Al treatment of *Arabidopsis* as described by Snowden *et al*. (1995).

Component	Final concentration	Component	Final concentration
Stock 1		**Stock 2**	
NH_4NO_3	0.15 mM	NaCl	0.2 mM
KNO_3	0.3 mM	$MgSO_4$	0.1 mM
		$(NH_4)H_2PO_4$	5 µM
Stock 3		**Stock 4**	
H_3BO_4	5 µM	$FeCl_3$	5 µM
$MnSO_4$	1 µM		
$CuSO_4$	0.2 µM		
$ZnSO_4$	1 µM		
$CoCl_2$	0.2 µM		

3.2.5. Hydroponics system for Al and Cd exposure

Seeds of *Arabidopsis* (Col-0) were surface sterilized as described in section 3.2.1 and sown in agar plugs containing 0.5 MS medium and 0.3 % phytagar within floating plastic rafts described by Kumari *et al*. (2008). These rafts or plastic disks (130 mm diameter, 3 mm thick) containing approximately 100 holes (3-4 mm diameter) were glued to a 0.5 mm thick nylon mesh using Dow sealant-732 (Dow Corning Corporation, Midland, MI) after both rafts and mesh had been pre-soaked in 95% ethanol overnight. Following cold stratification for 2 d at 4^{o} C, rafts were transferred to black polyethylene

tanks containing 1 L of sterile Richards medium, pH 5.8 (Richards *et al*., 1998). Plants were grown for 14 d in a controlled environment chamber (growth chamber conditions were the same as described in section 3.2.1), and the medium was replaced every 3 d to avoid algal growth and depletion of oxygen. For Al treatment, rafts were transferred to tanks containing 1 L of 200 µM $CaCl_2$ solution, pH 4.33 and either 0 µM, 25 µM, 50 µM or 75 µM $AlCl_3$ for 18 h. For Cd treatment, rafts were transferred to tanks containing 1 L of 200 µM $CaCl_2$ solution, pH 4.3 and either 0 µM, 2.5 µM, 5 µM or 7.5 µM $CdCl_2$ for 18 h. After Al and Cd treatment, roots were harvested using a sterile razor blade and immediately frozen in liquid nitrogen. Roots were then ground using a mortar and pestle and stored at -80^{o} C for RNA extractions and determination of lipid peroxidation.

3.2.6. Determination of lipid peroxidation

Levels of lipid peroxidation products, expressed as malondialdehyde (MDA), were estimated using the thiobarbituric acid (TBA)-reactive substances (TBARS) assay described by Heath and Packer (1968) for plant tissue. Fresh root tissue samples (500 mg) from plants treated with Al or Cd for 18 h using the hydroponic system and control samples were homogenized in a solution containing 0.25% thiobarbituric acid (TBA) in 10% trichloroacetic acid (TCA) using a mortar and pestle. The mixture was incubated at 95 °C for 25 min and then quickly cooled in an ice bath. Samples were centrifuged at 10 000 × *g* for 5 min and the absorbance of the supernatant was read at 532 and 600 nm. The value for non-specific absorption at 600 nm was subtracted from the value at 532 nm. The amount of lipid peroxidation products (MDA-TBA complex) was calculated using the extinction coefficient 155 mM^{-1} cm^{-1}.

3.2.7. Quantitative real time reverse transcription PCR (qRT-PCR) analysis

The effect of Al and Cd on transcript abundance of *AtNTRA* and *AtNTRB* in *Arabidopsis* plants was determined by qRT-PCR. Roots from three independently grown hydroponics tanks were pooled to make a single biological replicate. Total RNA from roots of hydroponically grown (as described in section 3.2.4) WT *Arabidopsis* plants treated with Al or Cd for 18 h and control plants was isolated using a silica membrane-based RNA isolation kit (RNeasy Plant Mini kit, Qiagen). All RNA samples were treated with RNase-Free DNase (Qiagen) to eliminate DNA contamination and precipitated according to Sanbrook and Russel (2001). Quality of RNA samples was tested on the Agilent 2100 Bioanalyzer (Agilent Technologies). Total RNA samples were transcribed into cDNAs using RevertAid H Minus M-MuLV reverse transcriptase (Fermentas) and Oligo$(dT)_{18}$ primer (Fermentas) following manufacturer's instructions. Primers for qRT-PCR analysis (Table 3-6) were designed using Primer Express 3.0 (Applied Biosystems) to amplify 125-150 bp sequences and the specificity of each primer was checked against the Arabidopsis genome using WU-BLAST 2.0 (http://www.Arabidopsis.org/wublast/index2.jsp). Ubiquitin-conjugating enzyme (*UBC*; At5g25760) has been used as a reference gene to quantify Al-induced transcript levels of various genes (Czechowski *et al*., 2005; Kumari *et al*., 2008). In the present study, *UBC* was also used as a reference gene to quantify AtNTRA and AtNTRB transcript levels induced by Al and Cd and validated for unchanged expression over treatments. For qRT-PCR, PCR amplification was performed using the qRT-PCR SYBR Green chemistry in a final volume of 10 µl with a master mix provided by the Molecular Biology Services Unit (MBSU, Department of Biological Sciences, University of Alberta). This master mix was

Table 3-6. List of primers used for qRT-PCR analysis. Pimers were designed using Primer Express 3.0 (Applied Biosystems).

Primer name	Primer sequence
AtNTRA-F	5'-TGGAAACTCACAAAACCAAGGTT-3'
AtNTRA-R	5'-TCCGCTCTCGATGCATAGATC-3'
AtNTRB-F	5'-CTCACAACACAAGGCTCTGTATCG-3'
AtNTRB-R	5'-AAGTTCAGCCCTAGCTGCGTAA-3'
Ubiquitin-F	5'-CTTAACTGCGACTCAGGGAAT-3'
Ubiquitin-R	5'-GGCGAGGCGTGTATACATTT-3'

optimized to work on the ABI 7500 Fast Real-Time PCR system (Applied Biosystems). Amplifications were performed in triplicate for each biological replicate in the ABI 7500 Fast Real-Time PCR system with the following conditions: Stage 1, 1 cycle at 95°C for 2 min; Stage 2, 40 cycles at 95°C for 0.15 min and 60°C for 1min; Stage 3: 95°C for 0.15 min; Stage 4: 60°C for 1 min; and Stage 5: 95°C for 0.15 min. Relative abundance of each transcript was estimated using the ΔΔ Ct method described by Livak and Schmittgen (2001). The ΔC_t (C_t target – C_t reference) was calculated using C_t values obtained for each primer set over a range of dilutions. ΔCt values obtained were plotted against log input amount to verify that the slope of the ΔCt vs. log input was < 0.1.

3.2.8. Enzyme activity assay

To confirm decreased levels of thioredoxin reductase activity in *ntr* mutants compared to WT (Col-0) *Arabidopsis*, plants were grown hydroponically in Richards medium as described in section 3.2.4 for 2 weeks. Thioredoxin reductase activity in WT *Arabidopsis* plants upon Al and Cd exposure was also determined and plants were treated with Al and Cd as described in section 3.2.4. Root tissue was harvested with a sterile razor blade and frozen in liquid nitrogen. Frozen root tissue (300 mg) was ground with a pinch of sand and a pinch of PVPP in an ice-cold mortar and pestle in 900 µl of extraction buffer (100 mM Tris-HCl pH 7.8, 5 mM EDTA, 1 mM DTT, 10 mM L-cysteine, 0.1 mM PMSF and 5 µl leupeptin). The slurry was then centrifuged for 15 min at 16,000 x g to remove cell debris. The supernatant was kept on ice for 15 min and used to determine protein concentration and to assay enzyme activity. Protein concentrations were quantified spectrophotometrically using the Bradford reagent (Sigma-Aldrich, Inc.) according to the manufacturer's recommendations. Thioredoxin reductase activity was assayed following the spectrophotometric method described by by Holmgren and Björnstedt (1995). In this assay, TNB (5´-thionitrobenzoic acid), which is a yellow compound with a maximum absorbance at 412 nm, is produced by the reduction of 5, 5´-dithiobis (2-nitrobenzoic acid) (DTNB) by thioredoxin reductase using NADPH. Briefly, a reaction mixture containing 50 µl of NADPH (40 mg ml^{-1}), 0.5 ml of 0.2 mM EDTA, 1 ml of 1 M potassium phosphate buffer, 0.8 ml of DTNB 25 mg ml^{-1} in 99.5% ethanol, 100 µl of BSA (20 mg ml^{-1}) was prepared in a final volume of 10 ml. Five-hundred µl of the reaction mixture were mixed with 100 µl of the protein sample for the sample cuvettes and with an equal volume of buffer for the reference cuvette. The reaction was

then followed at 412 nm. Thioredoxin reductase activity was calculated using the extinction coefficient 13,600 M^{-1} cm^{-1}.

3.2.9. Experimental design and statistical analyses

All experiments include three independent replicates and results are expressed as mean ± standard error (SE). Statistical analyses were performed using the program SigmaStat Version 3.5 (2006, Systat Software, Inc.). Student's t-test was used to determine significant differences between treatment and control. Experiments were repeated at least three times to ensure reproducibility of results.

3.3. Results

3.3.1. Isolation and characterization of *Arabidopsis prx* and *ntr* mutants

Salk T-DNA insertion lines (Alonso *et al*., 2003) were screened according to the PCR screening protocol (section 3.2.2) to isolate knockout mutants for *PRX* and *NTR* genes in *Arabidopsis* (Table 3-1). For WT plants, a PCR product of ~1 kb was generated with gene specific primers (LP + RP) and the second PCR reaction using T-DNA-specific and gene specific primers failed to generate a product, since WT plants do not harbor a T-DNA insert (Figure 3-1). Homozygous Salk T-DNA lines generated a PCR product from ~ 500-750 bp when T-DNA-specific and gene specific primer were used and no product was generated when gene specific primers were used (Figure 3-1). RT-PCR analysis

confirmed the absence of *AtNTRA* and *AtNTRB* transcript levels in *ntra* and *ntrb* mutants (Figure 3-2).

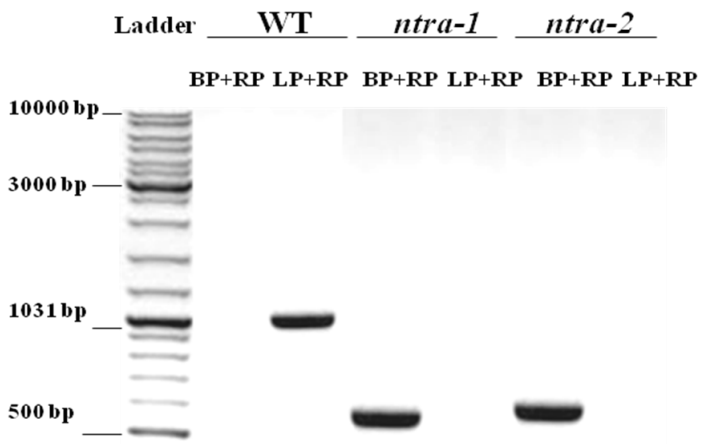


Figure 3-1. Agarose gel image showing the PCR products obtained in WT and *ntra* mutants. Genomic DNA from WT and mutant lines was extracted from 3-week old seedlings and used as a template for PCR. BP: Border primer (T-DNA-specific), RP: right primer (gene specific), LP: left primer (gene specific).

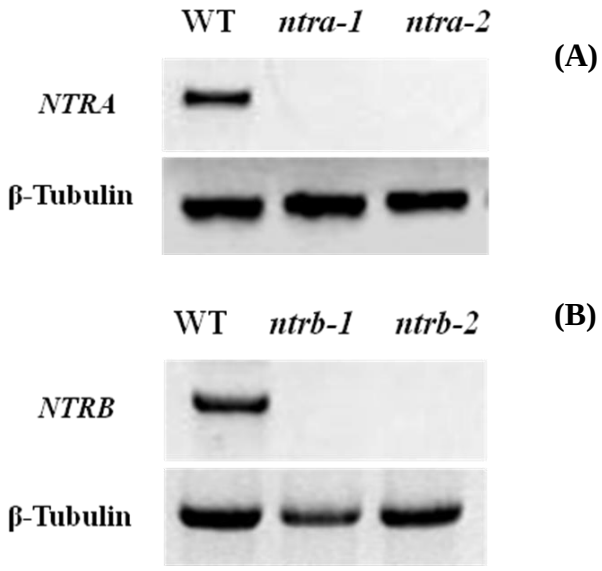


Figure 3-2. RT-PCR analysis showing the absence of normal *AtNTRA* **(A)** and *AtNTRB* **(B)** transcripts in *ntra* and *ntrb* mutants. Total RNA was isolated from 2-week old seedlings. PCR was performed with the synthesized first strand cDNA and with gene-specific primers.

Regarding the phenotypes of *prx* and *ntr* mutants, no abnormalities were observed in growth and development when grown in normal conditions. However, the seeds of various *prx* mutants showed a reduced germination rate after long-term (3-5 months) storage compared to the WT (Col-0) (data not shown).

3.3.2. Thioredoxin reductase enzyme activity in *ntr* mutants

To further confirm loss of gene function in *ntr* mutants, total thioredoxin reductase activity was determined in two-week old seedlings of *ntr* mutants and WT plants. All four *ntr* mutants showed a significant ($p<0.1$) decrease in thioredoxin reductase activity compared to the WT (Col-0). Thioredoxin reductase enzyme activity decreased by 15%, 19%, 20% and 17% compared to the WT in *ntra-1*, *ntra-2*, *ntrb-1* and *ntrb-2* mutants, respectively. The basal levels of thioredoxin reductase activity in two-week old WT seedlings were 4.7 ± 0.2 µmol min^{-1} mg $protein^{-1}$ and decreased to 3.9 ± 0.2, 3.8 ± 0.3, 3.7 ± 0.4, 3.9 ± 0.3 µmol min^{-1} mg $protein^{-1}$ in *ntra-1*, *ntra-2*, *ntrb-1* and *ntrb-2* mutants, respectively (Figure 3-3).

3.3.3. Effect of Al on root growth of *Arabidopsis prx* and *ntr* mutants

Root length was determined in ten-day-old seedlings of WT (Col-0) *Arabidopsis* and T-DNA lines defective in PRX and NTR genes (Table 3-1) treated with Al (150 µM $AlCl_3$) for 72 h as described above (section 3.2.3). *Arabidopsis* T-DNA lines defective in PRX genes showed similar root growth to WT (Col-0) *Arabidopsis* in the presence of Al (Figure 3-4). No significant differences in root growth were observed between WT (Col-0) *Arabidopsis* and *prx* mutants. Root growth of WT (Col-0) *Arabidopsis* was reduced to 59% of control in the presence of Al, whereas *prx* mutants root growth ranged from 57% to 63% of their control (0 µM $AlCl_3$). Only two T-DNA lines (Salk_027935 and Salk_039152) defective in NADPH-thioredoxin reductases (*AtNTRA* and *AtNTRB*)

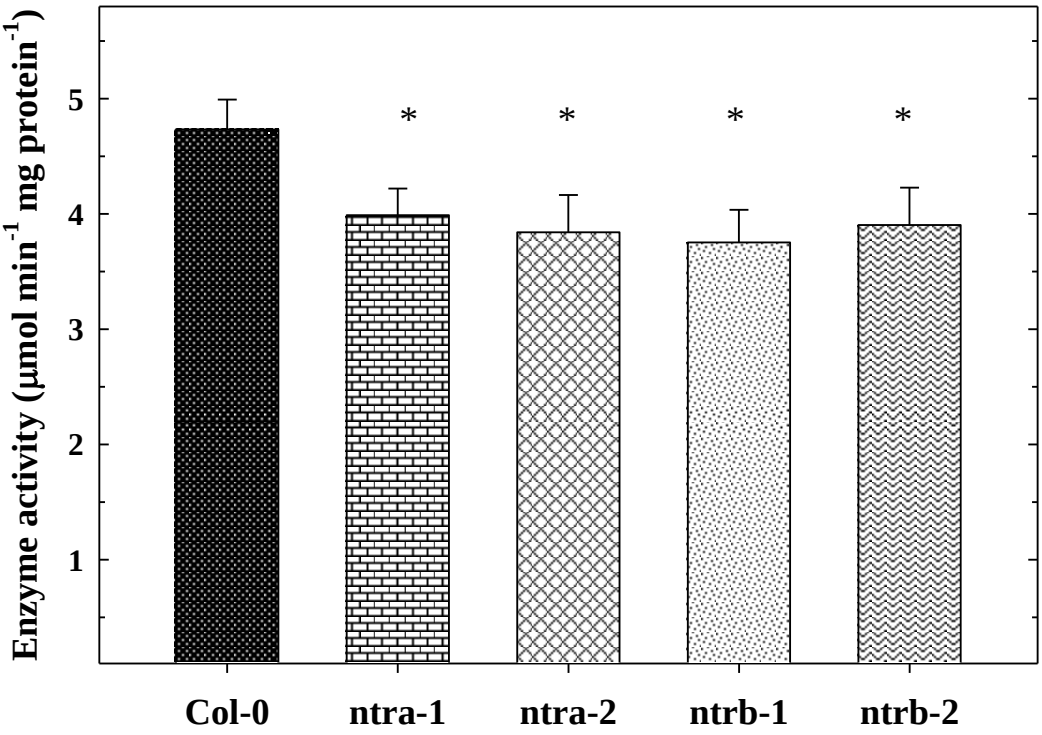
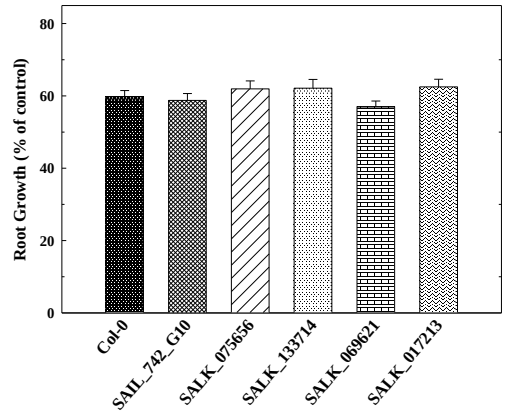
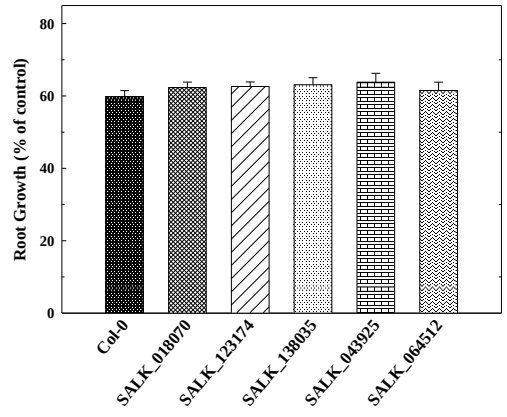


Figure 3-3. Thioredoxin reductase enzyme activity in WT (Col-0) and *ntr* mutants. Thioredoxin reductase acitivity was measured spectrophotometrically using enzyme extracts from roots and shoots of two-week-old seedlings. Vertical bars represent standard error (n=3). * indicates a significant difference ($p<0.1$) between a particular mutant and WT (Student's t-test). Results shown are representative of three independent experiments.

showed a significant inhibition of root growth (50% and 51% of their control, respectively) compared to the WT in the presence of Al ($p<0.01$) (Figure 3-4). To confirm these results, 2 different *ntr* T-DNA insertion alleles were obtained from the Salk collection. The homozygous mutants of these alleles were designated *ntra-1*, *ntra-2* for *AtNTRA* and *ntrb-1* and *ntrb-2* for *AtNTRB*. In the presence of Al, *ntra-1* and *ntra-2* mutants showed a significant reduction in root growth (53% and 50% of their control, respectively) compared to the WT ($p<0.01$) (Figure 3-5). Similarly, root growth in *ntrb-1* and *ntrb-2* mutants was significantly affected by Al ($p<0.01$). Root growth for both, *ntrb-1* and *ntrb-2*, was reduced to 51% of control (0 µM $AlCl_3$) (Figure 3-5).





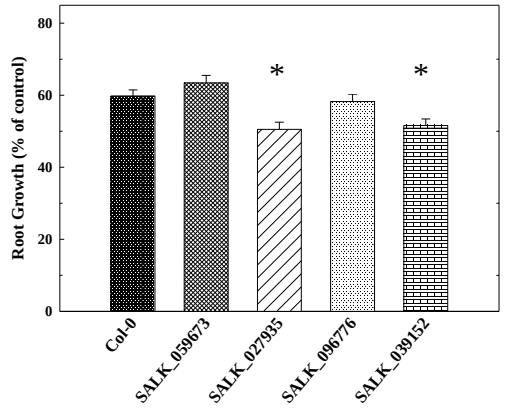


Figure 3-4. The effect of Al on root growth (% of control) of WT (Col-0), *prx* mutants and *ntr* mutants. Seedlings were treated with 150 µM $AlCl_3$ for 72 h. Vertical bars represent standard error (n=10). * indicates $p<0.01$ (Student's t-test). Results shown are representative of three independent experiments.

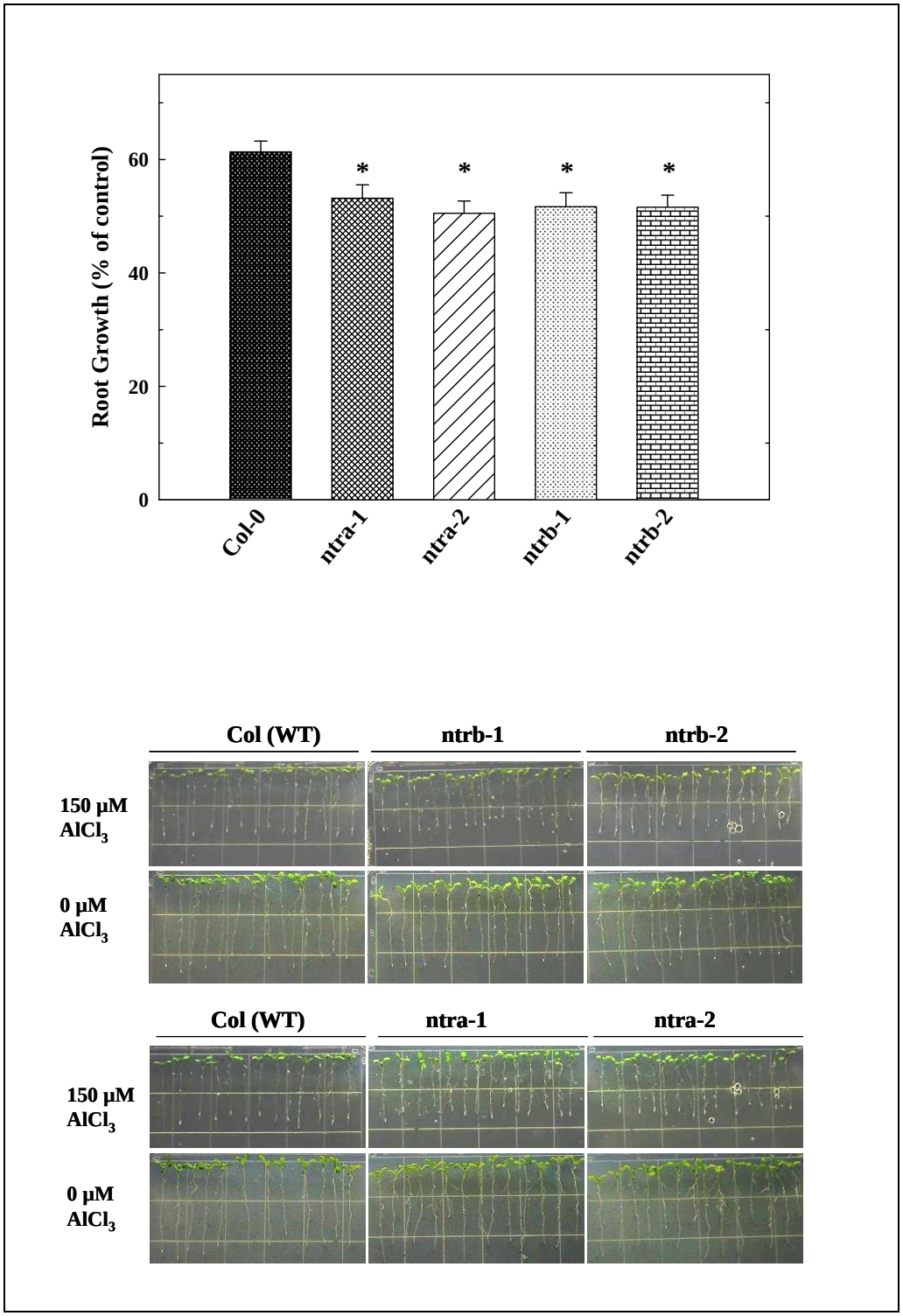


Figure 3-5. The effect of Al on root growth (% of control) of WT (Col-0) and *ntr* mutants. Seedlings were treated with 150 µM $AlCl_3$ for 72 h. Vertical bars represent standard error (n=10). * indicates $p<0.01$ (Student's t-test). Results shown are representative of three independent experiments.

3.3.4. Effect of Cd on root growth of *Arabidopsis prx* and *ntr* mutants

The effect of Cd in root growth of *prx* and *ntr* mutants was also investigated as described in section 3.2.3. *Arabidopsis* T-DNA lines defective in PRX genes showed similar root growth to WT (Col-0) *Arabidopsis* in the presence of Cd (Figure 3-6). No significant differences in root growth were observed between WT (Col-0) *Arabidopsis* and *prx* mutants. In the presence of Cd (75 µM $CdCl_2$), root growth of WT (Col-0) *Arabidopsis* was reduced to 53% of control (0 µM $CdCl_2$), whereas root growth of *prx* mutants ranged from 51% to 56% of control (Figure 3-6). Similar to what was found for Al, the only mutants sensitive to Cd were the *ntr* mutants defective in mitochondrial and cytosolic NADPH-thioredoxin reductases. Root growth for both of these mutants was significantly reduced to 44% of control ($p<0.01$) (Figure 3-6).

The different T-DNA insertion alleles obtained for these two genes, *ntra-1* and *ntra-2* for *AtNTRA* and *ntrb-1* and *ntrb-2* for *AtNTRB* were also tested to confirm these results. In the presence of Cd, *ntra-1* and *ntra-2* mutants showed a significant reduction in root growth (43% and 42% of their control, respectively) compared to the WT ($p<0.01$) (Figure 3-7). Similarly, root growth in *ntrb-1* and *ntrb-2* mutants was significantly affected by Cd ($p<0.01$). Root growth for *ntrb-1* and *ntrb-2* was reduced to 43% and 44% of control (0 µM $CdCl_2$), respectively (Figure 3-7).

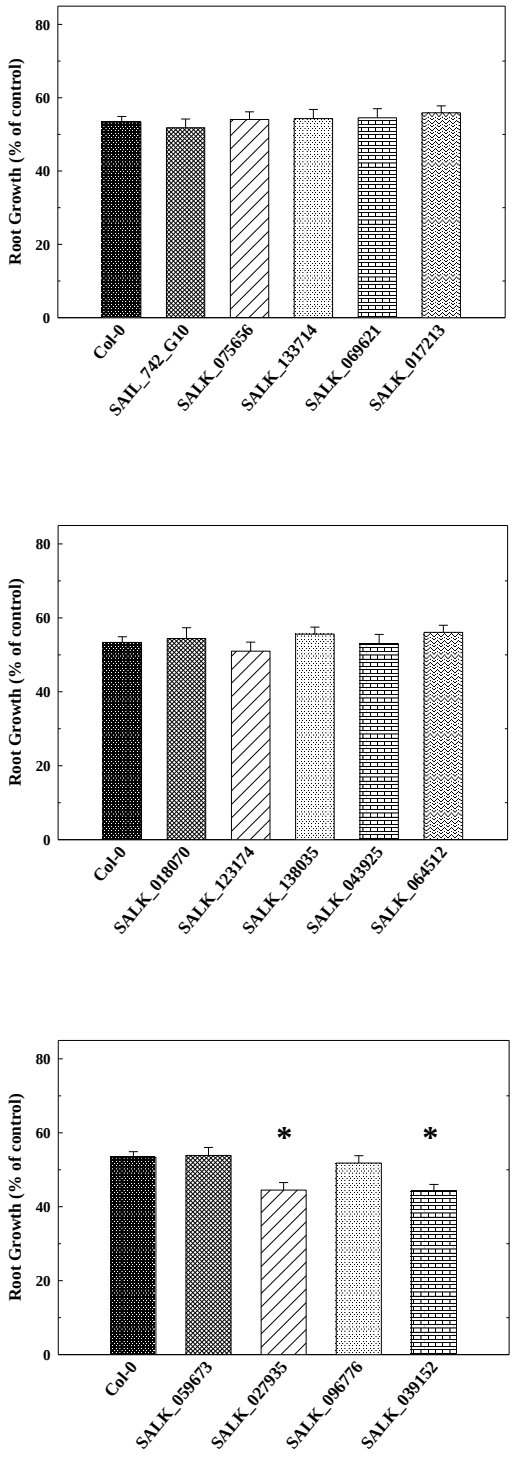


Figure 3-6. The effect of Cd on root growth (% of control) of WT (Col-0), *prx* mutants and *ntr* mutants. Seedlings were treated with 75 µM $CdCl_2$ for 72 h. Vertical bars represent standard error (n=10). * indicates $p<0.01$ (Student's t-test). Results shown are representative of three independent experiments.

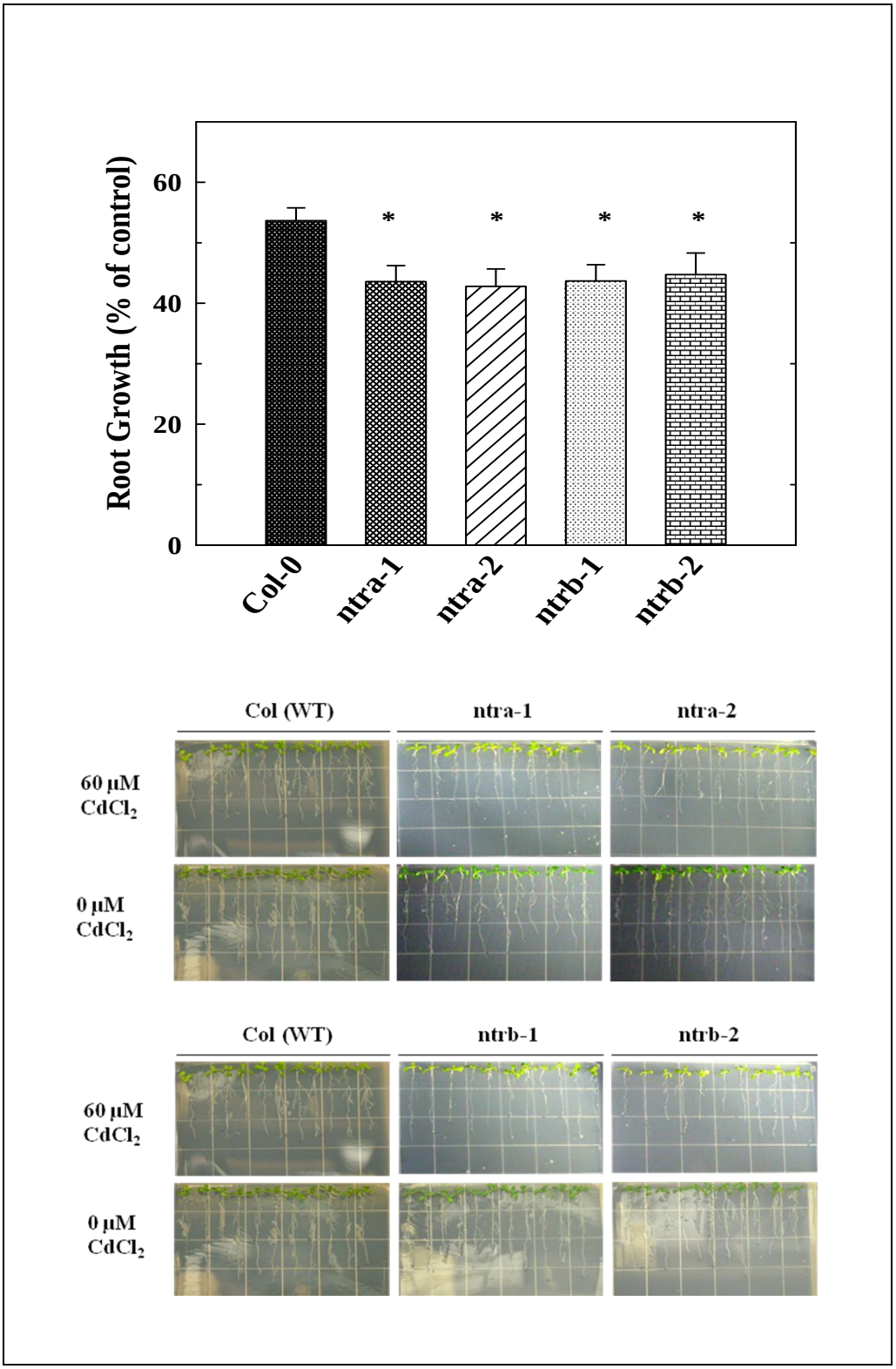


Figure 3-7. The effect of Cd on root growth (% of control) of WT (Col-0) and *ntr* mutants. Seedlings were treated with 75 µM $CdCl_2$ for 72 h. Vertical bars represent standard error (n=10). * indicates $p<0.01$ (Student's t-test). Results shown are representative of three independent experiments.

3.3.5. Effect of Al on lipid peroxidation levels in *Arabidopsis* WT (Col-0) and *ntr* mutants

To determine whether lipid peroxidation was induced in the Al-sensitive mutants relative to the WT (Col-0), the amounts of MDA produced in roots of *Arabidopsis* plants grown in the presence of 0 and 75 µM Al were estimated. Malondialdehyde levels in WT roots increased by 2.8 µmol g^{-1} FW above their control in the presence of 75 µM Al. The basal levels of MDA in WT *Arabidopsis* plants were 1.7 ± 0.2 µmol g^{-1} FW at 0 µM Al and increased to 4.6 ± 0.7 µmol g^{-1} FW at 75 µM Al (Figure 3-8). Compared to the WT (Col-0), a significant increase in MDA levels was observed in *ntr* mutants when exposed to 75 µM Al ($p<0.01$). Malondialdehyde levels in *ntr* mutants increased by 5.5, 5.8, 5.6 and 5.6 µmol g^{-1} FW in *ntra-1*, *ntra-2*, *ntrb-1* and *ntrb-2*, respectively, above their control in the presence of 75 µM Al. Basal levels of MDA were 2.0 ± 0.5, 2.0 ± 0.6, 2.2 ± 0.5 and 2.2 ± 0.4 µmol g^{-1} FW for *ntra-1*, *ntra-2*, *ntrb-1* and *ntrb-2*, respectively, and increased to 7.5 ± 0.8, 7.8 ± 0.3, 7.9 ± 0.2 and 7.9 ± 0.4 µmol g^{-1} FW for *ntra-1*, *ntra-2*, *ntrb-1* and *ntrb-2*, respectively, in the presence of 75 µM Al (Figure 3-8).

3.3.6. Effect of Cd on lipid peroxidation levels in *Arabidopsis* WT (Col-0) and *ntr* mutants

Malondialdehyde levels were also quantified in *ntr* mutants and WT treated with Cd. In WT roots, MDA levels increased by 3.1 µmol g^{-1} FW above their control in the presence of 7.5 µM Cd.

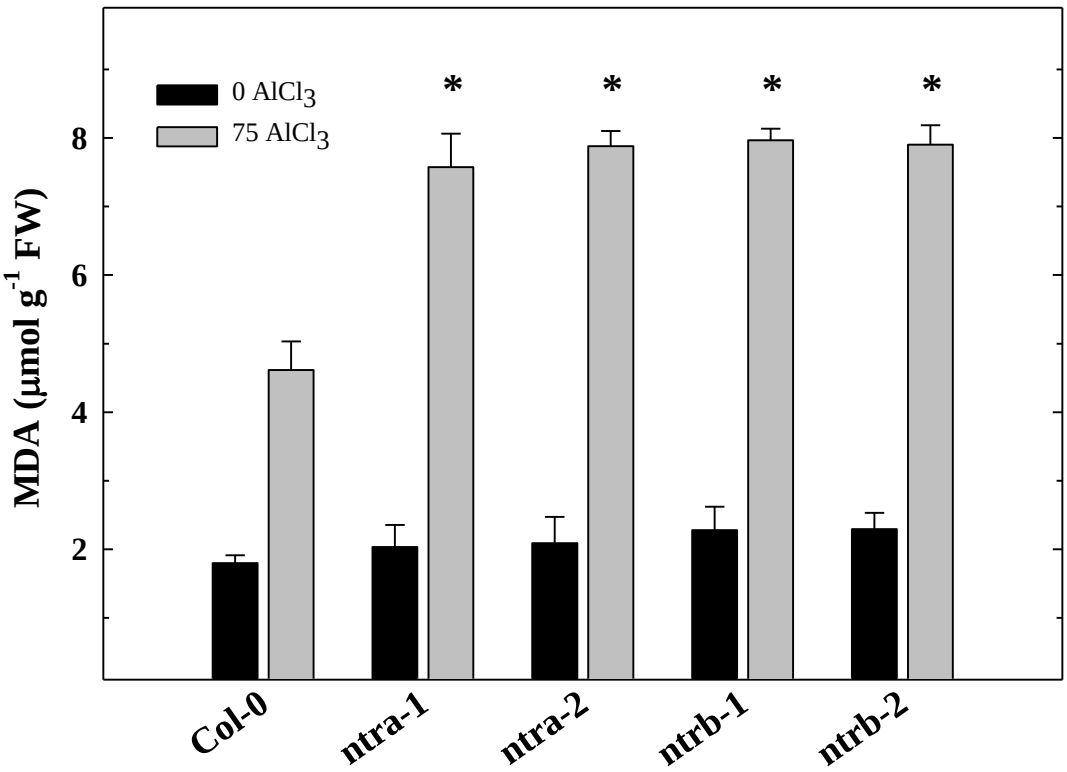


Figure 3-8. The effect of Al on lipid peroxidation levels of WT (Col-0) and *ntr* mutants. Seedlings were treated with 75 µM $AlCl_3$ for 18 h in a hydroponic system. Vertical bars represent standard error (n=3). * indicates $p<0.01$ (Student's t-test). Results shown are representative of three independent experiments.

The basal levels of MDA in WT *Arabidopsis* plants were 1.8 ± 0.4 µmol g^{-1} FW at 0 µM Cd and increased to 4.9 ± 0.7 µmol g^{-1} FW at 7.5 µM Cd (Figure 3-9). Compared to the WT (Col-0), a significant increase in MDA levels was observed in *ntr* mutants when exposed to 7.5 µM Cd ($p<0.01$). Malondialdehyde levels in *ntr* mutants increased by 6.1, 6.1, 6.3 and 6.3 µmol g^{-1} FW in *ntra-1*, *ntra-2*, *ntrb-1* and *ntrb-2*, respectively, above their control in the presence of 7.5 µM Cd. Basal levels of MDA were 2.0 ± 0.5, 2.1 ± 0.6, 2.1 ± 0.6 and 2.2 ± 0.3 µmol g^{-1} FW for *ntra-1*, *ntra-2*, *ntrb-1* and *ntrb-2*, respectively, and increased to 8.1 ± 0.4, 8.2 ± 0.5, 8.4 ± 0.4 and 8.5 ± 0.6 µmol g^{-1} FW for *ntra-1*, *ntra-2*, *ntrb-1* and *ntrb-2*, respectively, in the presence of 7.5 µM Cd (Figure 3-9).

3.3.7. Effect of Al on *AtNTRA* and *AtNTRB* transcript abundance in WT (Col-0) *Arabidopsis*

The effect of Al on transcript abundance of *AtNTRA* and *AtNTRB* in *Arabidopsis* roots was determined by qRT-PCR analysis in three biologically independent replicates treated with 0, 25, 50 and 75 µM Al for 18 h grown hydroponically as described in section 3.2.4. Transcript levels for both genes increased by less than twofold in the presence of Al relative to control samples. Transcript levels of *AtNTRA* increased by 1.2-fold, 1.1-fold and 1.2-fold in the presence of 25, 50 and 75 µM Al, respectively (Figure 3-10). In the case of *AtNTRB*, transcript levels increased by 1.5-fold and 1.4-fold in the presence of 50 and 75 µM Al, respectively, whereas transcript levels in roots treated with 25 µM Al remained unchanged (Figure 3-11). Overall, transcript levels for both genes did not increase in a dose response manner in the presence of Al.

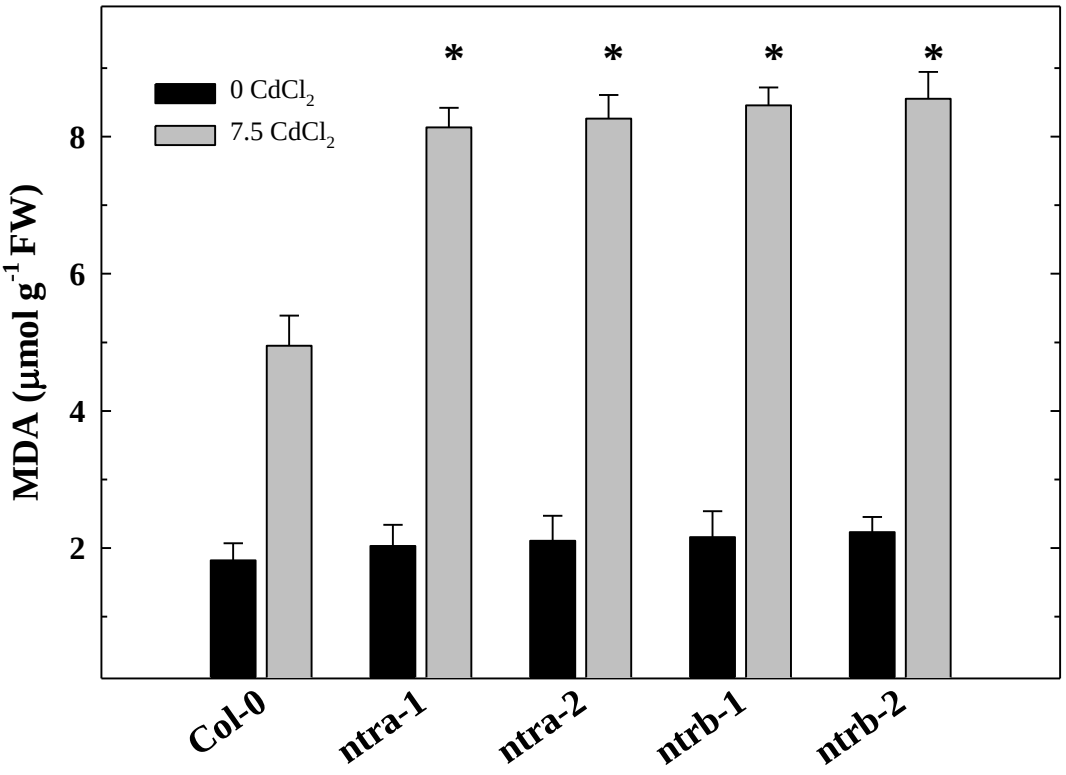


Figure 3-9. The effect of Cd on lipid peroxidation levels of WT (Col-0) and *ntr* mutants. Seedlings were treated with 7.5 µM $CdCl_2$ for 18 h in a hydroponic system. Vertical bars represent standard error (n=3). * indicates $p<0.01$ (Student's t-test). Results shown are representative of three independent experiments.

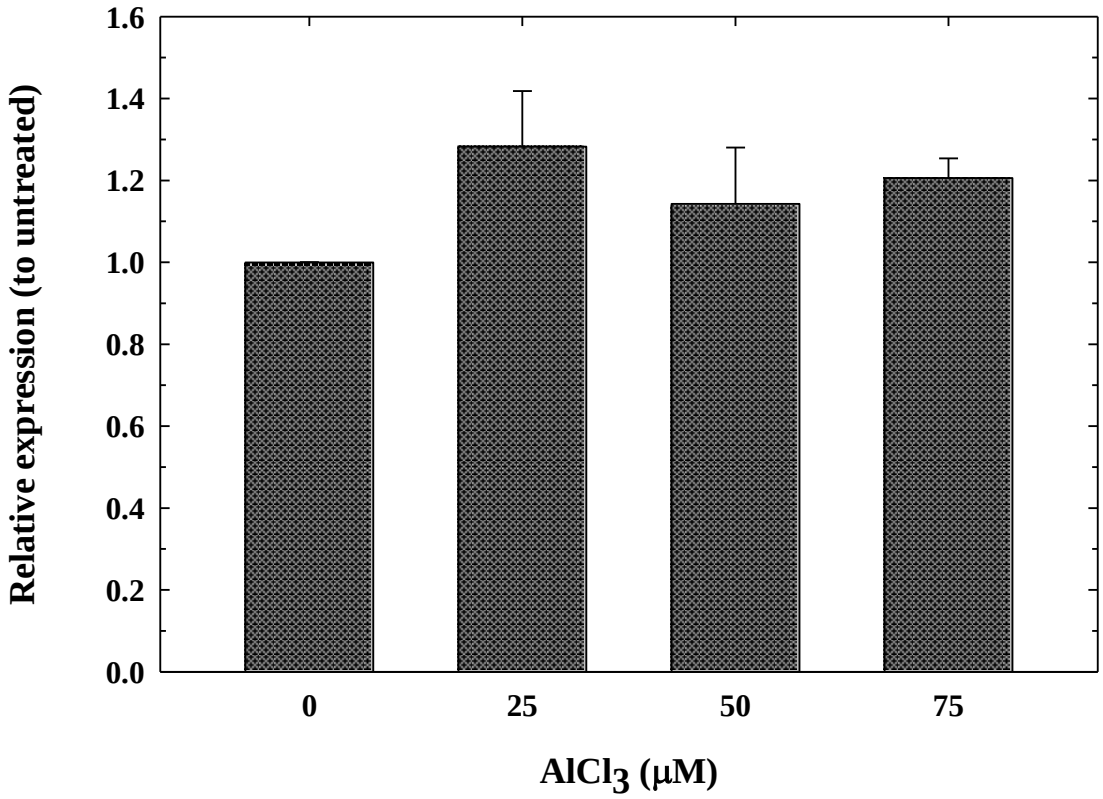


Figure 3-10. Relative expression of *AtNTRA* in *Arabidopsis* plants treated with 0 µM, 25 µM, 50 µM and 75 µM $AlCl_3$. Expression was calculated using Ubiquitin-conjugating enzyme (*UBC*; At5g25760) as an endogenous control and expressed relative to untreated samples (-ΔΔCT). No significant differences were found between treatments and control. Bars represent the standard deviation.

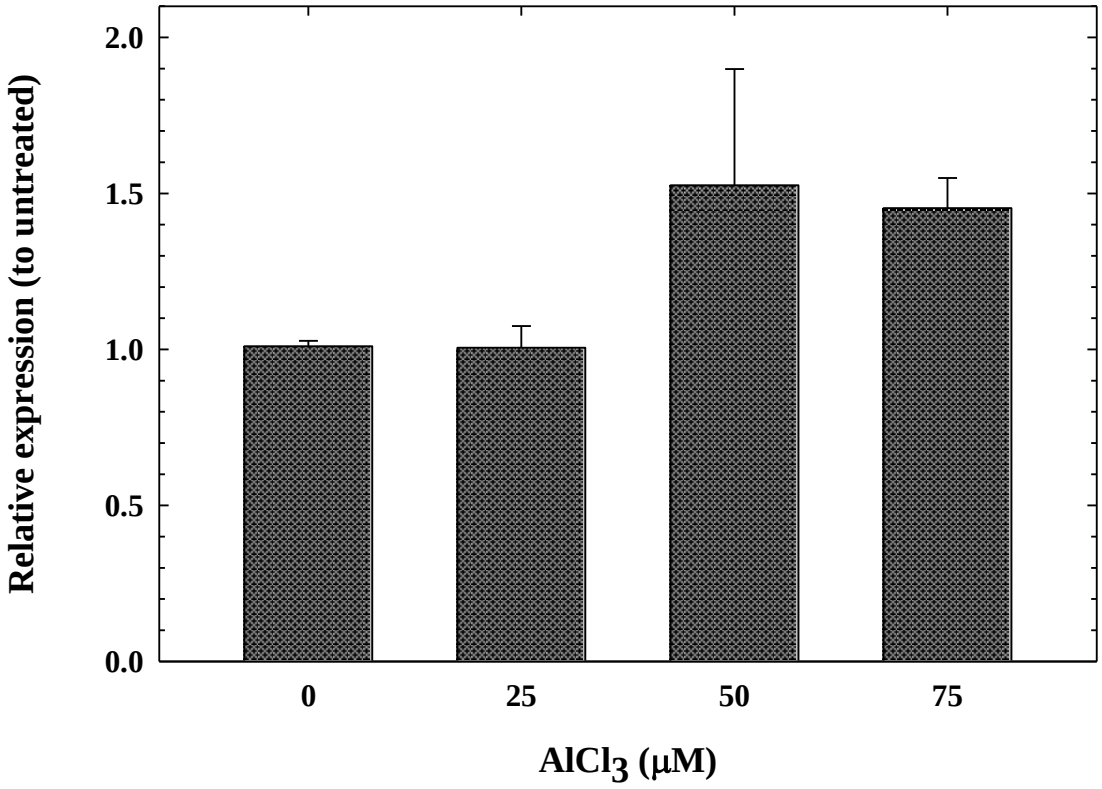


Figure 3-11. Relative expression of *AtNTRB* in *Arabidopsis* plants treated with 0 µM, 25 µM, 50 µM and 75 µM $AlCl_3$. Expression was calculated using Ubiquitin-conjugating enzyme (*UBC*; At5g25760) as an endogenous control and expressed relative to untreated samples (-ΔΔCT). No significant differences were found between treatments and control. Bars represent the standard deviation.

3.3.8. Effect of Cd on *NTRA* and *NTRB* transcript abundance in WT (Col-0) *Arabidopsis*

Transcript abundance of *AtNTRA* and *AtNTRB* in *Arabidopsis* roots treated with 0, 2.5, 5.0 and 7.5 µM Cd was also determined by qRT-PCR analysis. Similarly to the treatment with Al, transcript levels for both genes increased by less than twofold in the presence of Cd relative to control samples. Transcript levels of *AtNTRA* increased by 1.4-fold, 1.3-fold and 1.2-fold in the presence of 2.5, 5.0 and 7.5 µM Cd, respectively (Figure 3-12). A similar pattern was observed in the case of *AtNTRB* where transcript levels increased by 1.6-fold, 1.5-fold and 1.3-fold in the presence of 2.5, 5.0 and 7.5 µM Cd, respectively (Figure 3-13). Transcript levels for both genes appeared to be induced by the lowest concentration of Cd and then decreased with higher Cd concentrations.

3.3.9. Effect of Al on thioredoxin reductase enzyme activity in WT (Col-0) *Arabidopsis*

To further investigate the role of thioredoxin reductases in Al tolerance, thioredoxin reductase enzyme activity was determined in roots of WT (Col-0) *Arabidopsis* plants treated with 0, 25, 50 and 75 µM $AlCl_3$ hydroponically. A significant increase in thioredoxin reductase enzyme activity ($p<0.01$) was observed when *Arabidopsis* plants were treated with 25 µM Al. In this treatment, thioredoxin reductase

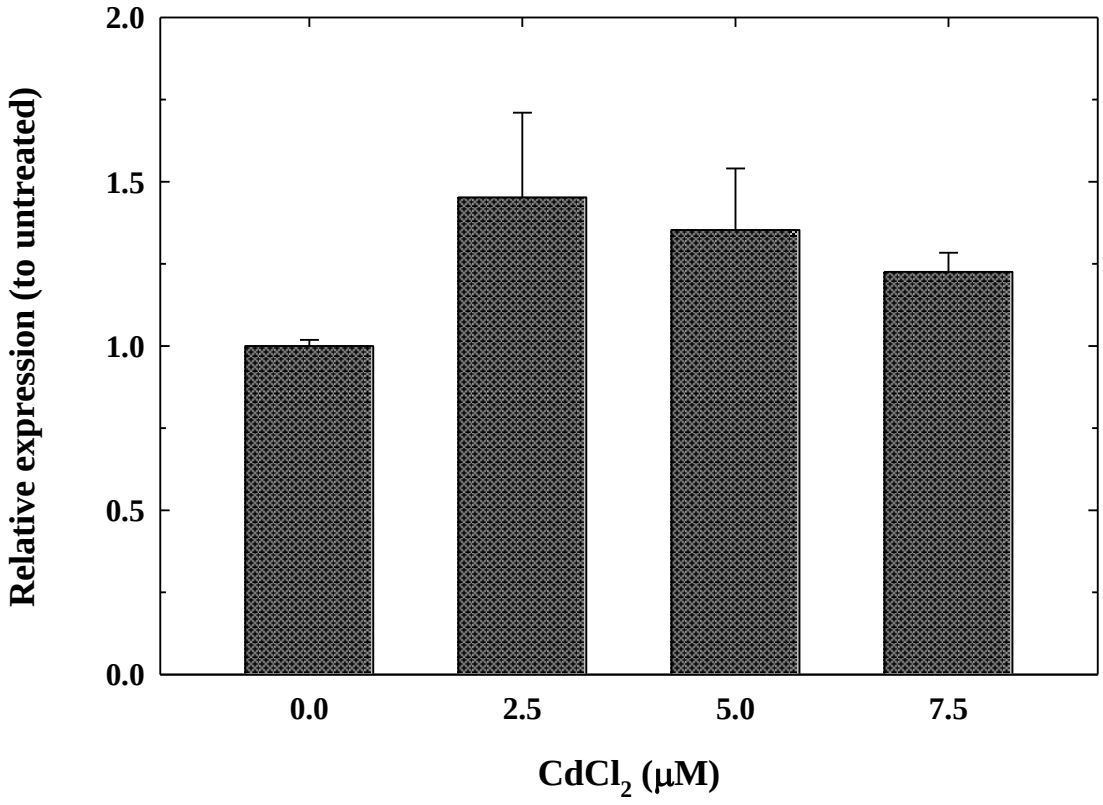


Figure 3-12. Relative expression of *AtNTRA* in *Arabidopsis* plants treated with 0 µM, 2.5 µM, 5.0 µM and 7.5 µM $CdCl_2$. Expression was calculated using Ubiquitin-conjugating enzyme (*UBC*; At5g25760) as an endogenous control and expressed relative to untreated samples (-ΔΔCT). No significant differences were found between treatments and control. Bars represent the standard deviation.

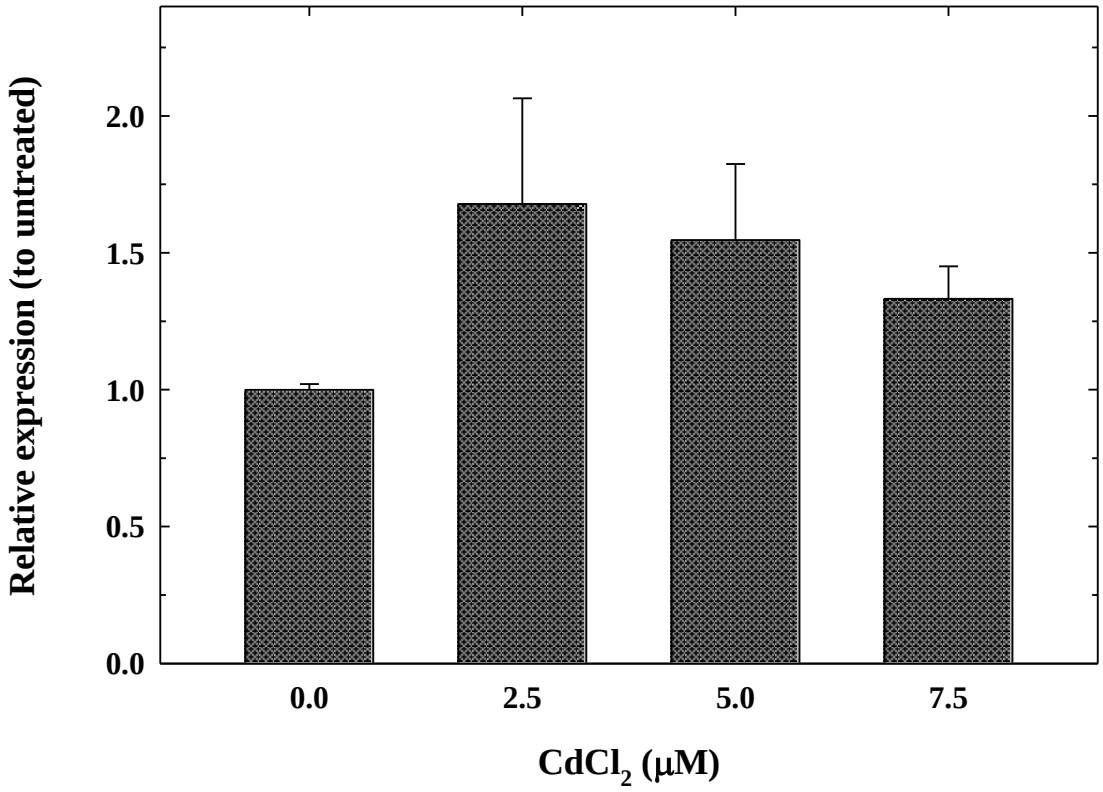


Figure 3-13. Relative expression of *AtNTRB* in *Arabidopsis* plants treated with 0 µM, 2.5 µM, 5.0 µM and 7.5 µM $CdCl_2$. Expression was calculated using Ubiquitin-conjugating enzyme (*UBC*; At5g25760) as an endogenous control and expressed relative to untreated samples (-ΔΔCT). No significant differences were found between treatments and control. Bars represent the standard deviation.

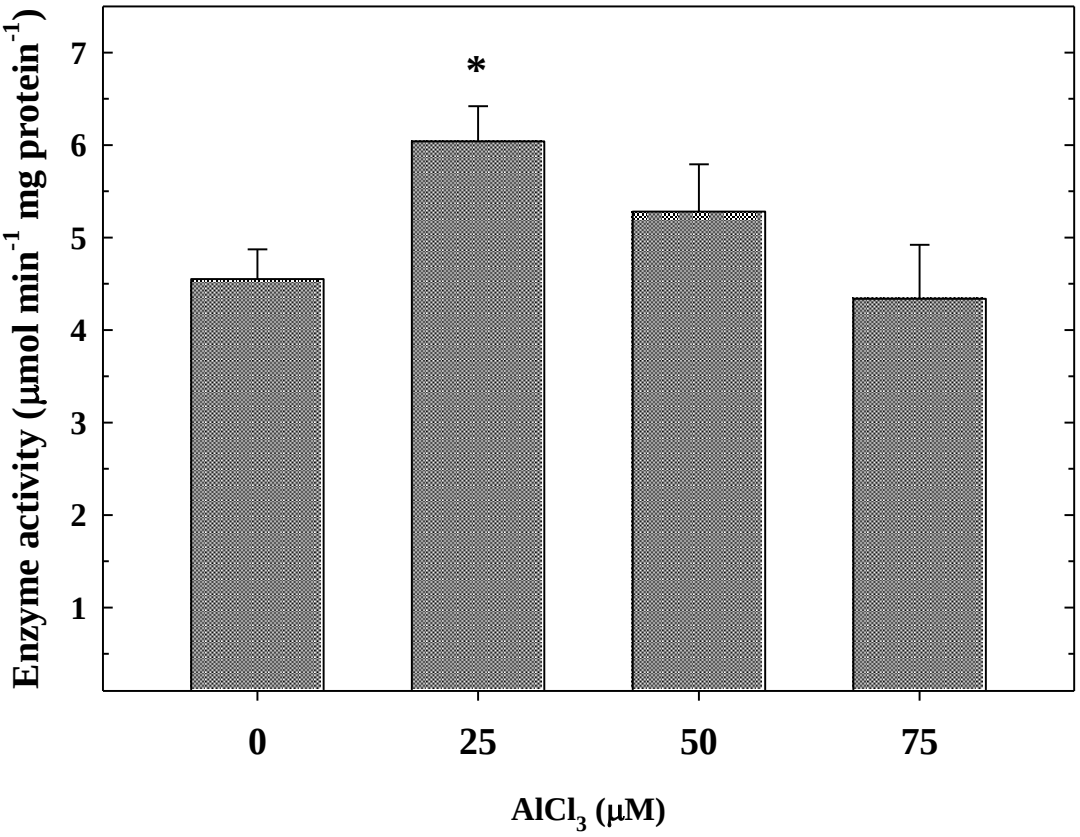


Figure 3-14. The effect of Al on thioredoxin reductase activity in WT (Col-0) *Arabidopsis*. Thioredoxin reductase acitivity was measured spectrophotometrically using enzyme extracts from roots and shoots of two-week-old seedlings treated with 0 µM, 25 µM, 50 µM and 75 µM $AlCl_3$ for 18 h in a hydroponic system. Vertical bars represent standard error (n=3). * indicates $p<0.01$ (Student's t-test). Results shown are representative of three independent experiments.

activity increased 1.3-fold relative to control. Basal levels of thioredoxin reductase activity in untreated samples were 4.5 ± 0.3 µmol min^{-1} mg $protein^{-1}$ and increased to 6.0 ± 0.3 µmol min^{-1} mg $protein^{-1}$ in roots treated with 25 µM Al (Figure 3-14). In plants treated with 50 µM Al, no significant increase in thioredoxin reductase enzyme activity was detected. Thioredoxin reductase enzyme activity detected for this treatment was 1.1-fold of control (5.2 ± 0.5 µmol min^{-1} mg $protein^{-1}$) (Figure 3-14). A further decrease in thioredoxin reductase enzyme activity was detected at 75 µM Al (4.3 ± 0.5 µmol min^{-1} mg $protein^{-1}$) relative to untreated samples (Figure 3-14).

3.3.10. Effect of Cd on thioredoxin reductase enzyme activity in WT (Col-0) *Arabidopsis*

Thioredoxin reductase enzyme activity was also determined in roots of WT (Col-0) *Arabidopsis* plants treated with 0, 2.5, 5.0 and 7.5 µM $CdCl_2$ hydroponically. Similarly to Al, a significant increase in thioredoxin reductase enzyme activity ($p<0.01$) was detected when *Arabidopsis* plants were treated with 2.5 µM Cd. In this treatment, thioredoxin reductase activity increased 1.4-fold relative to control. Basal levels of thioredoxin reductase activity in untreated samples were 4.3 ± 0.2 µmol min^{-1} mg $protein^{-1}$ and increased to 6.2 ± 0.4 µmol min^{-1} mg $protein^{-1}$ in roots treated with 2.5 µM Cd (Figure 3-15). In plants treated with 5.0 and 7.5 µM Cd, no significant increase in thioredoxin reductase enzyme activity was detected. Thioredoxin reductase enzyme activity detected in roots treated with 5.0 µM Cd was 1.2-fold of control (5.5 ± 0.4 µmol min^{-1} mg $protein^{-1}$) (Figure 3-15). Thioredoxin reductase enzyme activity decreased

further when plants were treated with 7.5 µM Cd (4.4 ± 0.5 µmol min^{-1} mg $protein^{-1}$) relative to untreated samples (Figure 3-15).

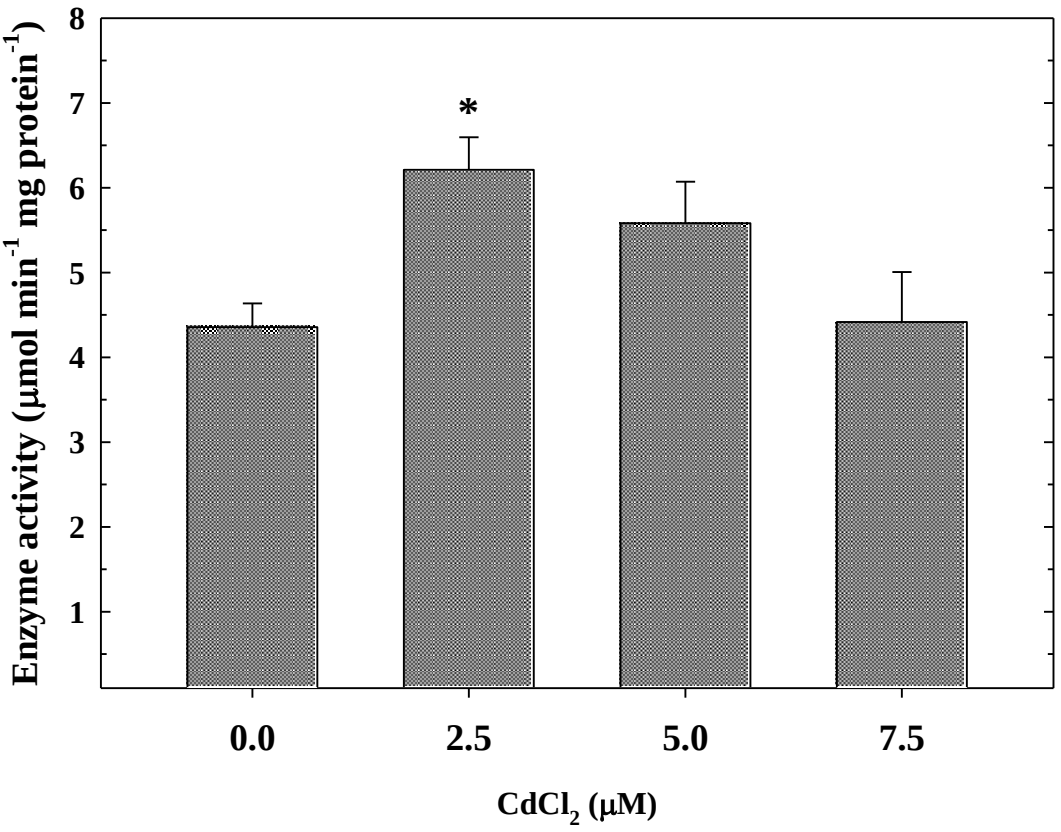


Figure 3-15. The effect of Cd on thioredoxin reductase activity in WT (Col-0) *Arabidopsis*. Thioredoxin reductase acitivity was measured spectrophotometrically using enzyme extracts from roots and shoots of two-week-old seedlings treated with 0 µM, 2.5 µM, 5.0 µM and 7.5 µM $CdCl_2$ for 18 h in a hydroponic system. Vertical bars represent standard error (n=3). * indicates $p<0.01$ (Student's t-test), respectively. Results shown are representative of three independent experiments.

3.4. Discussion

Based on the results from my previous study with yeast (*S. cerevisiae*), I decided to focus only on peroxiredoxins and thioredoxin reductases in *Arabidopsis*. These components of the antioxidant system in plants have not been previously characterized in the context of Al and Cd tolerance. To investigate the role of peroxiredoxins and NADPH-thioredoxin reductases in Al and Cd tolerance in *Arabidopsis*, I isolated 11 T-DNA knockout lines defective in PRXs and 3 T-DNA knockout lines defective in NTRs. Since inhibition of root growth caused by Al is a widely used indicator of Al toxicity in plants, I quantified root growth in T-DNA lines and WT upon Al and Cd in order to identify relevant PRXs or NTRs.

Arabidopsis knockout lines defective in PRXs did not show any significant reduction in root growth upon Al and Cd exposure compared to WT (Col-0). Enhanced Al and Cd sensitivity was observed in *Arabidopsis* T-DNA mutant lines lacking *AtNTRA* (the major cytosolic NADPH-thioredoxin reductase isoform) and *AtNTRB* (the major mitochondrial NADPH-thioredoxin reductase isoform). Root growth upon Al and Cd exposure of WT (Col-0) plants was 59% and 53% of controls, respectively. Compared to WT plants, a significant reduction in root growth was observed upon Al and Cd exposure in T-DNA knockout lines defective in *AtNTRA* and *AtNTRB*. Root growth in the T-DNA knockout line defective in *AtNTRA* was 50% and 44% of controls in Al- and Cd- treated plants, respectively. Similarly, root growth in the T-DNA knockout line defective in *AtNTRB* was 51% and 44% of controls in Al- and Cd- treated plants, respectively.

In order to confirm that mutants defective in *AtNTRA* and *AtNTRB* are more sensitive to Al and Cd stress than WT plants, I isolated different T-DNA insertion mutant alleles in each of the *NTRA* and *NTRB* genes. Root growth in these different alleles was also significantly reduced compared to WT plants upon Al and Cd exposure. The enhanced sensitivity to Al and Cd in *ntra* and *ntrb* mutants suggests that *AtNTRA* and *AtNTRB* may play a role in tolerance to these metal ions. Based on our current knowledge on the functions of these enzymes, and the fact that Al and Cd induce oxidative stress, I hypothesized that NTRs in *Arabidopsis* may play a role in Al and Cd tolerance by ameliorating oxidative stress. The exact functions of NTRs in plants, however, are still not fully understood. Reichheld *et al*. (2005) observed that *ntra* and *ntrb* mutant plants failed to reveal any phenotype, which suggests that NTRA and NTRB may share redundant functions. However, recent studies with a double mutant (*ntra ntrb*) in *Arabidopsis*, suggest that *AtNTRA* and *AtNTRB* may play important roles in several plant development programs, including pollen fitness, seed development and cell proliferation (Reichheld *et al*., 2007). Although single mutations of *NTRA* and *NTRB* do not influence root growth, Reichheld *et al*. (2007) observed that NTRs play a role in meristematic activities. Shoot growth in *rml1* (a mutant defective in GSH synthesis) is dependent on a functional NTR, as shown by the complete block of the apical meristem in the triple mutant *ntra ntrb rml1* (Reichheld *et al*., 2007; Reichheld *et al*., 2010).

In the present study, a significant increase in lipid peroxidation was observed in *ntra* and *ntrb* mutants compared to WT plants upon Al and Cd exposure. These results suggest that *ntra* and *ntrb* mutants are more prone to oxidative damage induced by Al and Cd. Thus, we could hypothesize that a double mutant defective in cytosolic and

mitochondrial thioredoxin reductase would be sensitive to conditions that generate oxidative stress. However, Reichheld *et al*. (2007) recently observed that *ntra ntrb* mutant plants do not show modified sensitivity to several abiotic stress conditions that generate cellular oxidative stress (Reichheld *et al*., 2007). These results are somewhat conflicting with what was found in the present study, since we would expect that the double mutant would be more sensitive to oxidative damage induced by Al or Cd than single mutations of *NTRA* and *NTRB*. Reichheld *et al*. (2007) showed that the double mutant, *ntra ntrb*, was extremely sensitive when grown in the presence of an inhibitor of glutathione biosynthesis, which suggests that glutathione is able to complement the absence of NTRs in the *ntra ntrb* mutant plants.

In order to better understand the role of NTRs in Al and Cd tolerance in plants, I quantified *AtNTRA* and *AtNTRB* transcripts to determine whether NTRs are induced upon Al and Cd stress. This approach has been widely used to further our understanding of how plants respond to Al and several genes whose expression is affected by Al toxicity have been identified (Snowden and Gardner, 1993; Snowden *et al*., 1995; Richards *et al*., 1998). In the present study, I found that levels of *AtNTRA* and *AtNTRB* transcripts increased by less than two-fold relative to control samples in *Arabidopsis* WT plants exposed to Al or Cd for 18 h. Transcript levels of both genes did not increase in a dose response manner. The highest increase in transcript levels of *AtNTRA* in WT plants treated with Al or Cd was observed at the lowest concentrations (25 µM Al and 2.5 µM Cd). At higher Al or Cd concentrations, *AtNTRA* transcript levels decreased progressively. The same pattern for *AtNTRA* transcript levels in plants treated with Al was observed by Northern analysis (data not shown). In contrast to the results for

AtNTRA transcripts, the highest increase of *AtNTRB* transcript levels in plants treated with Al, was not observed at the lowest Al concentration (25 µM), but rather at an intermediate concentration (50 µM). The response of *AtNTRB* transcript levels to Cd was similar to the response of *AtNTRA* transcript levels to Al and Cd, with the highest increase observed at the lowest concentration of Cd (2.5 µM). In my previous study, transcript levels of cystosolic thioredoxin reductase in yeast (*TRR1*) were significantly induced by Al and Cd stress, but transcript levels increased in a dose dependent manner. In contrast, NTRs in *Arabidopsis*, appear to be induced slightly only at low Al or Cd concentrations.

To gain further information about the role of NTRs in Al and Cd tolerance, I also quantified total NADPH-thioredoxin reductase enzyme activity in WT plants treated with various concentrations of Al or Cd. In a pattern similar to much of the transcript level data, total NTR enzyme activity increased at lower concentrations of Al (25 µM) and decreased with increasing concentrations of Al. Plants treated with the lowest concentration of Al (25 µM) showed a significant increase ($p<0.01$) in NTR enzyme activity compared to WT plants. A similar pattern was observed in WT plants treated with various concentrations of Cd. A significant increase ($p<0.01$) in NTR enzyme activity was observed in WT plants treated with 2.5 µM $CdCl_2$. However, total NTR enzyme activity decreased progressively as plants where exposed to increasing concentrations of Cd.

We can conclude from these results that NTR enzyme activity is not induced by high concentrations of Al and Cd, but is enhanced by low concentrations of Al and Cd.

Similar results have been observed for glutathione reductase (GR) activity, which is another thiol-disulfide oxidoreductase. Glutathione reductase activity decreased progressively with increasing Cu concentrations in copper-treated *Scenedesmus bijugatus* cells, and it also decreased progressively with increased concentrations of nickel (Ni) and Cd in hyperaccumulator plants of the genus *Alyssum* (Shickler and Caspi, 1999; Nagalakshmi and Prasad, 2001). Given the affinity that Cd has for sulfydryl groups, we could hypothesize that the decrease in GR and NTR activity at high concentrations of Cd could be the direct interaction of Cd with sulfhydryl groups present in this type of enzymes. However, since Al doesn't have such a high affinity for sulhydryl groups, this would not explain the decrease in NTR activity caused by higher concentrations of Al observed in the current study.

It can be concluded from the present study that NTRs (NTRA and NTRB) may play a role in Al and Cd tolerance in *Arabidopsis*. However, T-DNA lines defective in PRXs did not show any phenotype, which could be explained by the overlapping roles of PRXs. Unlike the previous dose-response pattern observed in yeast (*S. cerevisiae*) in my previous study, it appears that NTRs are only induced by low concentrations of Al and Cd in *Arabidopsis*. Interestingly, my work with yeast (Chapter 2) showed that the mutant defective in cytosolic thioredoxin reductase (*TRR1*) was hypersensitive to Al and Cd, but the mutant defective in mitochondrial thioredoxin reductase (*TRR2*) was not. In *Arabidopsis*, however, both mutants defective in *NTR*A and *NTRB* showed similar hypersensitivity to Al and Cd. This difference between the results from yeast and *Arabidopsis* could be explained by the fact that both *Arabidopsis* NTRs are capable of synthesizing cytosolic and mitochondrial isoforms, whereas in yeast NTR genes have

specialized to produce either the cytosolic or the mitochondrial isoform. Overall, the differences observed between my results from yeast and *Arabidopsis* could be explained by the fact that the antioxidant system in *Arabidopsis* is more complex than the antioxidant system in yeast. Thus, it would be possible that these differences can be accounted for by the higher number of thiol-disulfide oxidoreductases, and the overlapping roles they might play, in *Arabidopsis*. To further investigate the role of NTRs in Al and Cd tolerance in *Arabidopsis*, in the following chapter I tested the hypothesis that increased expression of NTRs in *Arabidopsis* would lead to increased tolerance to Al and Cd by developing *Arabidopsis* transgenic lines overexpressing *AtNTRA* and *AtNTRB*.

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4. Investigating the potential role of NADP(H)-thioredoxin reductases in aluminum and cadmium tolerance in transgenic *Arabidopsis* overexpressing *AtNTRA* and *AtNTRB*

4.1 Introduction

Based on the findings from my work with *Aradidopsis* T-DNA knockout lines (Chapter 3), I decided to focus the present study on the major cytosolic (*AtNTRA*) and the major mitochondrial (*AtNTRB*) NADPH-thioredoxin reductases. In my previous work, *Arabidopsis* mutants defective in *NTRA* and *NTRB* showed increased sensitivity to Al and Cd compared to the WT. A T-DNA knockout line defective in chloroplastic NADPH-thioredoxin reductase (NTRC) was also found to be sensitive to Al and Cd. However, *AtNTRC* was not included in this study since the chloroplastic thioredoxin system is not expected to have a role in Al tolerance, which is primarily a root-related phenomenon.

The *Arabidopsis thaliana* genome contains two genes that encode typical NTRs (*NTRA* and *NTRB*). Both NTRs in *Arabidopsis* produce two different mRNAs: one shorter (~35 kD), encoding a cytosolic protein, and one longer (~38 kD), featuring a signal peptide that targets the protein to the mitochondria. Although both NTR genes encode cytosolic and mitochondrial isoforms, it has been shown that *NTRB* expresses the major mitochondrial isoform, while *NTRA* expresses as the major cytosolic isoform (Reichheld *et al*., 2005). The two NTR genes in *Arabidopsis* originated from a recent duplication event, and it has been suggested that their gene products are evolving to

support specialized cytosolic or mitochondrial functions (Blanc *et al*., 2000; Reichheld *et al*., 2005). A third NTR gene is present in *Arabidopsis*, *NTRC*, which in addition to the thioredoxin reductase domain also contains a chloroplastic transit peptide. It has been shown that *NTRC* in *Arabidopsis* functions as an electron donor for plastidial peroxiredoxins and represents the NADPH-dependent thioredoxin system in chloroplasts (Schürmann and Jacquot, 2000; Bréhélin *et al*., 2004; Moon *et al*., 2006).

Our current knowledge on the functions of NTRs in plants is limited. Reichheld *et al*. (2007) has shown that in contrast with mammals, neither cytosolic nor mitochondrial NTRs are essential in plants. Reichheld *et al*. (2007) developed a double mutant lacking both mitochondrial and cytosolic NTRs in *Arabidopsis* (*ntra ntrb*) and observed that this double mutant is viable, but the pollen in these plants has reduced fitness. Lack of NTRs also leads to reduced plant growth, modified seed shape, and high accumulation of anthocyanins (Reichheld *et al*., 2007). The loss of NTRs, however, was compensated by GSH, which suggests that GSH and NTRs may have overlapping roles in *Arabidopsis*. Interestingly, the *ntra ntrb* double mutant was not found to be hypersensitive to oxidant stresses (Reichheld *et al*., 2007). It has been found that NTRs also constitute a functional backup for cytosolic glutathione reductase in *Arabidopsis* (Marty *et al*., 2009).

The purpose of the current study is to investigate whether overexpression of *AtNTRA* and *AtNTRB* confers increased tolerance to Al and Cd in *Arabidopsis*. Overexpression is a common approach used to further understand the role of relevant genes in metal tolerance. This approach has been widely used in the past 15 years to test whether increased expression of a particular gene can confer increased resistance to Al.

One of the main goals of many of these studies has been to engineer crops with increased Al resistance that could be cultivated in acid soils. This strategy has resulted in small to moderate gains in resistance compared to controls for most genes overexpressed. Currently, the largest increases in Al resistance have been achieved by overexpressing transport proteins that facilitate export of organic anions to the rhizosphere (Delhaize *et al*., 2004; Pereira *et al*., 2010).

Attempts to increase Al tolerance have also been made by overexpressing genes associated with oxidative stress. These manipulations have resulted in modest gains in Al tolerance. For instance, Ezaki *et al*. (2000, 2005) found that overexpressing genes encoding glutathione *S*-transferase, peroxidase, GDP dissociation inhibitor, and a blue copper protein in *Arabidopsis* increased relative root growth by 1.5 to 2.5-fold compared with the controls. In another study, Basu *et al*. (2001) found that overexpressing a wheat manganese superoxide dismutase gene in *Brassica napus* increased relative root growth by 2.5-fold compared with the controls. Similarly, overexpression of glutathione synthetase in Indian mustard has been shown to improve Cd tolerance (Zhu *et al*., 1999).

Although NTRs are part of the antioxidant system in plants, these enzymes have not been previously linked to Al or Cd tolerance. The findings in my previous study (Chapter 3) suggest that NTRs may play a role in Al and Cd tolerance in *Arabidopsis*. In this chapter, I describe experiments carried out to test the hypothesis that enhanced ability to synthesize NADPH-dependent thioredoxin reductases in *Arabidopsis* seedlings would lead to increased antioxidant capacity, which ultimately would allow for improved root growth when grown in the presence of Al and Cd compared to WT seedlings.

4.2. Materials and Methods

4.1.1 Plant materials and growth conditions

Arabidopsis ecotype Columbia seeds were used to create lines overexpressing *AtNTRA* and *AtNTRB* and as control plants. Seeds were surface sterilized by vortexing for 10 min in a solution containing 20% bleach and 0.05% Tween-20. Seeds were pelleted and rinsed five times in sterile Milli-Q water and suspended in 0.1% agar. To break dormancy, seeds were kept in darkness for 2 d at 4^{o}C. Following stratification, seeds were sown in a soil mixture (Metromix 350, Scotts Company, Marysville OH) and transferred to a controlled environment chamber at 22^{o}C with 16 h light and 8 h dark cycle, 70% humidity and approximately 160 µmol m^{-2} s^{-1} photosynthetic photon flux density. Plants were fertilized weekly with Richard's medium (Richards *et al*., 1998) containing 5mM KNO_3, 2.5 mM KH_2PO_4 2 mM $MgSO_4$, 2 mM $Ca(NO_3)_2$, 12.5 µM FeEDTA, 7 µM H_3BO_3, 14 µM $MnCl_2$, 0.5 µM $CuSO_4$, 1 µM $ZnSO_4$, 10 µM NaCl, and 0.1 µM $CoCl_2$, pH 5.8.

4.2.2. Development of transgenic lines overexpressing *AtNTRA* and *AtNTRB*

4.2.2.1. Preparation of overexpression constructs

To generate overexpression constructs of the *AtNTRA* and *AtNTRB* coding regions, the full-length coding sequences of NADPH- thioredoxin reductases (*AtNTRA* and *AtNTRB*) were amplified by RT-PCR with primers designed to create an *XbaI* site at

the 5' end and a *KpnI* site at the 3' end (Table 4-1). Total RNA was isolated from *A. thaliana* wild-type Col-0 two-week-old seedlings using RNeasy kit (Qiagen) following the manufacturer's recommendations. First strand cDNA synthesis was performed using 4 µg of total RNA in a 20 µl reaction with 200 U of Superscript^{TM} II Reverse Transcriptase (Invitrogen). The cDNA obtained was treated with RNAse H (Invitrogen) for 20 min at 37^{o}C. PCR was performed using iProof^{TM} High-Fidelity DNA polymerase (Bio-Rad) according to manufacturer's instructions and with the following cycling conditions: initial denaturation 98^{o} C for 30 s; 30 cycles of: 98^{o} C for 10 s, 64^{o} C for 30 s, 72^{o} C for 45 s, and a final extension of 72^{o} C for 10 min. The amplified fragments were gel-purified (Qiagen) and cloned into pGEM-T (Promega) vector and sequenced before subcloning into the overexpression binary vector. The binary vector pPZPY112 (Hajdukiewicz *et al*., 1994) was used for constitutive overexpression of *AtNTRA* and *AtNTRB*, and it was obtained from ABRC (Arabidopsis Biological Resource Center, stock donated by Yamamoto *et al*., 1998). The *XbaI*/*KpnI* fragments from pGEM-T/*AtNTRA* and pGEM-T/*AtNTRB* were ligated into *XbaI*/*KpnI* digested pPZPY112 binary vector resulting in the overexpression cassettes used for transformation of *Arabidopsis* (Figure 4-1).

Table 4-1. List of primers containing added restriction sites used for amplification and cloning of *Arabidopsis* NADPH-thioredoxin reductases (*AtNTRA* and *AtNTRB*).

Primer name	Primer sequence
NTRA-XbaI	5’ TCTAGAGGATCTCAATGAGCCAGTCAAGATTC 3’
NTRA-KpnI	5’ GGTACCCTCCTACAGCTTCTTCAATCACTC 3’
NTRB-XbaI	5’ TCTAGACGTTCCTAATGAACTGTGTGAGTCG 3’
NTRB-KpnI	5’ GGTACCTCCTCAATCACTCTTACCTTGCTG 3’

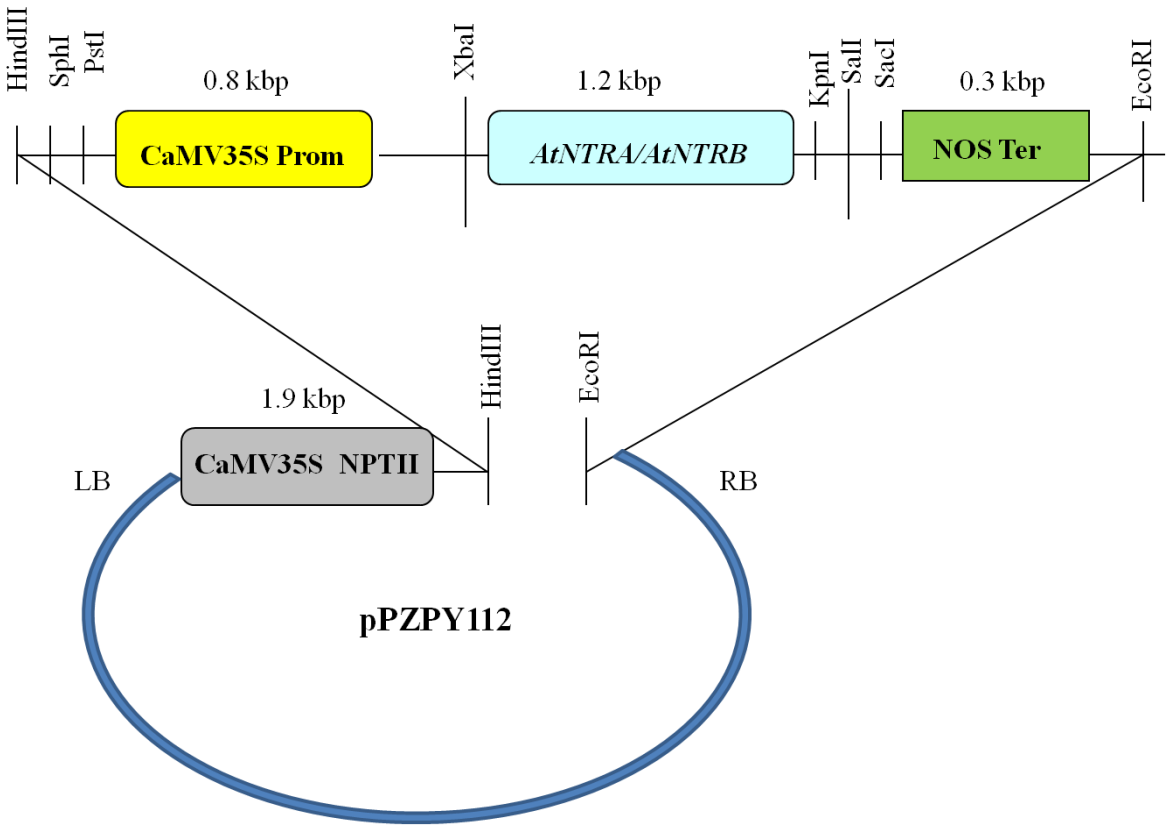


Figure 4-1. Schematic representation of the constructs carrying *AtNTRA* or *AtNTRB* for overexpression in *Arabidopsis thaliana*. A fragment (~1.2 kb) containing either *AtNTRA* or *AtNTRB* coding regions was cloned into the *XbaI* and *KpnI* restriction sites of the overexpression vector pPZPY112. LB, left border. RB, right border. CaMV35S, constitutive cauliflower mosaic virus 35S promoter. *NPTII*, neomycin phosphotransferase II. NOS ter, nopaline synthase terminator.

4.2.2.2. *Arabidopsis* transformation

Transformation of *Agrobacterium tumefaciens* (strain GV3101) with the overexpression binary vectors, pPZPY112/*AtNTRA* and pPZPY112/*AtNTRB*, was not performed successfully using the direct transformation method or "freeze-thaw" method described by Höfgen and Willmitzer (1988). Instead, transformation of *Agrobacterium* was obtained using the triparental mating method. Briefly, overnight liquid cultures were prepared for the *Agrobacterium* strain (GV3101), *E. coli* RK2013 helper strain, and *E.coli* DH5α carrying the pPZPY112/*AtNTRA* or pPZPY112/*AtNTRB* plasmid. Cells for each culture (500 µl aliquots) were washed using 500 µl 10 mM $MgSO_4$ and resuspended in the same volume of 10 mM $MgSO_4$. The 3 cultures were combined and grown on plates of 2xYT medium (1.6% bactotryptone, 1% yeast extract and 0.5% NaCl) overnight at 28^{o}C. Cells from overnight cultures were harvested and diluted using 10 mM $MgSO_4$, and plated on AB minimal medium (Lichtenstein and Draper, 1986) containing appropriate antibiotics (rifampicin 10 mg L^{-1}, gentamycin 25 mg L^{-1} and kanamycin 50 mg L^{-1}). Well isolated colonies were streaked again for single cells on fresh plates of the same medium. In order to confirm positive clones, cells from isolated colonies were grown in liquid 2xYT medium with antibiotics.

Transformation of *Arabidopsis* plants was performed using the floral dip method (Clough and Bent, 1998; Weigel and Glazebrook, 2002). Briefly, both *Agrobacterium* strains carrying *AtNTRA* and *AtNTRB* on the binary vector pPZPY112 were grown in liquid LB containing antibiotics for 2 d. *Agrobacterium* cells were resuspended to OD_{600} = 0.8 in 5% sucrose solution and Silwet L-77 was added to a concentration of 0.05% just

before dipping plants. The first bolts of *Arabidopsis* plants were clipped and secondary bolts were used for transformation. The solution was applied by pipet to unopened flowers and reapplied every 4 d for 16 d.

4.2.2.3. Screening transgenic lines

Transformed seedlings were identified following the method described by Harrison *et al*. (2006). Briefly, the harvested seeds were surface sterilized and plated on ½ MS media containing 50 µg ml^{-1} and stratified in the dark for 2 d at 4^{o} C. Following stratification, seeds were incubated in continuous white light (120 µmol m^{-2} s^{-1}) for 6 h at 22^{o} C in order to stimulate germination. Plates were covered in aluminum foil and incubated for 2 d at 22^{o} C. Finally, plates were incubated for 48 h in a 16 h light/8 h dark regime without the foil and transformed seedlings (kanamycin-resistant seedlings) were identified from non-transformed (non-resistant seedlings) (Figure 4-2). Seedlings that exhibited resistance to kanamycin were transferred to soil and selfed to obtain T_2 seeds.

The copy number of T-DNA inserts was determined by calculating the ratio of kanamycin-resistant to kanamycin-sensitive seedlings in the T_2 generation. Seeds from 8 lines (T_1 plants) for each transgene and WT were plated on ½ MS containing 6.5 g L^{-1} phytagar (Sigma-Aldrich) and grown for 5 d in a growth chamber at 22^{o} C with 16 h light/8 h dark. Twenty-four seedlings for each line and WT were plated in triplicate in ½ MS medium containing 50 µg ml^{-1} kanamycin and scored for resistant and non-resistant seedlings.

Figure 4-2. Screening for putative *Arabidopsis* transformants using kanamycin. Seedlings of putative *Arabidopsis* transformants and WT were grown in ½ MS medium containing 50 µg ml^{-1} kanamycin under the conditions described above. Kanamycin-resistant seedlings showed green cotyledons, whereas cotyledons in kanamycin-sensitive seedlings appeared pale (bleached).

To confirm the presence of a transgene within putative transformants, genomic DNA was isolated according to the method described by Edwards *et al*. (1991) for PCR analysis. Briefly, leaf tissue of four-week old plants was collected using the lid of an

Eppendorf tube, and the tissue was macerated at room temperature for 15 s. Extraction buffer (200 mM Tris-HCl pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5 % SDS) was added to the samples, vortexed, centrifuged, and the DNA was precipitated using isopropanol. PCR was performed with the following vector-specific and gene-specific primers:

35S-L (5'- CGCAATGATGGCATTTGTAG -3'),

OxNTRA-R (5'- CTAGAGACGGCGGATTTGAG -3'),

OxNTRB-R (5'- TTTCGAGACCATTCATGACG -3'),

and the following conditions: initial denaturation 94^{o} C for 3 min; 30 cycles of: 94^{o} C for 1 min, 58^{o} C for 1 min, 72^{o} C for 1 min, and a final extension of 72^{o} C for 10 min. PCR products were visualized on a 1% agarose gel and compared to expected fragment sizes.

4.2.3. Northern analysis

Tissue of one-week old *Arabidopsis* seedlings overexpressing *AtNTRA* and *AtNTRB* was harvested and immediately frozen using liquid nitrogen. The tissue was then ground to a fine powder using a morter and pestle. Total RNA (8 µg) was isolated using the QIAGEN RNeasy mini kit (QIAGEN Inc., Canada), separated by electrophoresis on agarose formaldehyde denaturing gels and transferred to nitrocellulose membranes following the manufacturer's recommendations (Genescreen, NEN Research Products). Briefly, a capillary blot was set up using 10X SSPE buffer (1.5 M NaCl, 100 mM NaH_2PO_4, Na_2EDTA) and blotted overnight. The membrane was then rinsed in 2X SSPE and baked at 80^{o} C for 2 h. Probes used for hybridization were amplified by PCR from *S. cerevisiae* genomic DNA using gene specific primers (Table 2-4) and confirmed by sequencing. Probes used for hybridization were amplified by RT-PCR from *Arabidopsis*

genomic DNA and radioactively labeled with [^{32}P] dCTP using using the Ready·To·Go DNA Labeling Beads (Amersham Biosciences-GE Healthcare, Ontario, Canada). Approximately 150-200 ng of denatured DNA was added to the labeling reaction with 5 µl of [^{32}P] dCTP in a total volume of 50 µl, and incubated at 37 °C for 30 min. Radioactive-labeled probes were separated from the unincorporated ^{32}P labeled nucleotides using the Sephadex G-50TM DNA grade NICK columns (Amersham Biosciences-GE Healthcare). Membranes were prehybridized, hybridized and washed under standard stringent conditions recommended by Genescreen. Briefly, membranes were prehybridized with 1 µg denatured salmon sperm DNA at 42^{o} C in 20 ml of prehybridization solution overnight (For 100 ml prehybridization/hybridization solution: 10 g dextran sulfate, 15 ml water, 25 ml 20X SSPE, 5 ml 20% SDS, 5 ml Denhardt's solution, 50 ml deionized formamide). Membranes were then hybridized in 20 ml of new hybridization solution at 42^{o} C for 20 h with 200 µl of denatured radioactively-labeled probe. After hybridization, membranes were washed three times: first in 2X SSPE at room temperature for 15 min, a second wash in 2X SSPE and 2% SDS at 65^{o} C for 15 min and a third wash in 0.1X SSPE at room temperature for 15 min. Membranes were then wrapped in plastic film (Saran wrap) and exposed to KODAK BioMax MR autoradiography films (Marketlink Scientific, Burlington, Ontario) for 6 h and 24 h at -80^{o} C. All the x-ray films were developed by the Kodak X-OMAT 2000 processor in a dark room.

4.2.4. Western analysis

Two-week old seedlings of *Arabidopsis* overexpression lines and WT plants were used for protein extraction. Harvested tissue (300 mg) was ground with a pinch of sand

and a pinch of PVPP in an ice-cold mortar and pestle in 900 µl of extraction buffer (100 mM Tris-HCl pH 7.8, 5 mM EDTA, 1 mM DTT, 10 mM L-cysteine, 0.1 mM PMSF and 5 µl leupeptin). The slurry was then centrifuged for 15 min at 16,000 x g to remove cell debris. The supernatant was kept on ice for 15 min and used to determine protein concentration. Protein concentrations were quantified using the Bradford reagent (Sigma-Aldrich, Inc.) according to the manufacturer's recommendations. Proteins (10 µg) were separated on a SDS polyacrylamide gel (10% resolving gel, 4% stacking gel) using a Mini-PROTEAN II Electrophoresis Cell (Bio-Rad) and transferred to a nitrocellulose membrane using a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad) according to the manufacturer's specifications. Rabbit polyclonal antibodies against AtNTRB diluted to 1:10,000 (Reichheld *et al*., 2005) were used as the primary antibody. This antibody also recognizes AtNTRA (Reichheld *et al*., 2005). Anti-rabbit antibodies conjugated to horseradish peroxidase (Amersham Biosciences) were used as secondary antibodies and revealed with an ECL Western detection kit (Amersham Biosciences) following the manufacturer's protocol.

4.2.5. Enzyme activity assay

Seedlings of 2-week old *Arabidopsis* lines overexpressing *AtNTRA* and *AtNTRB* and WT plants grown hydroponically were used to determine if increased levels of thioredoxin reductase enzyme activity occur in overexpression lines. To determine thioredoxin reductase enzyme activity in plants exposed to Al and Cd, control and treatment samples were grown hydroponically as described in Chapter 3 and exposed to Al (25 µM) or Cd (2.5 µM) for 18 h. Root tissue was harvested with a sterile razor blade

and frozen in liquid nitrogen following Al and Cd exposure. Frozen tissue (300 mg) was ground with a pinch of sand and a pinch of PVPP in an ice-cold mortar and pestle in 900 µl of extraction buffer (100 mM Tris-HCl pH 7.8, 5 mM EDTA, 1 mM DTT, 10 mM L-cysteine, 0.1 mM PMSF and 5 µl leupeptin). The slurry was then centrifuged for 15 min at 16,000 x g to remove cell debris. The supernatant was kept on ice for 15 min and used to determine protein concentration and to assay enzyme activity. Protein concentrations were quantified using the Bradford reagent (Sigma-Aldrich, Inc.) according to the manufacturer's recommendations. Thioredoxin reductase activity was assayed following the spectrophotometric method described by by Holmgren and Björnstedt (1995). In this assay, TNB (5´-thionitrobenzoic acid), which is a yellow compound with a maximum absorbance at 412 nm, is produced by the reduction of 5, 5´- dithiobis (2-nitrobenzoic acid) (DTNB) by thioredoxin reductase using NADPH. Briefly, a reaction mixture containing 50 µl of NADPH (40 mg ml^{-1}), 0.5 ml of 0.2 mM EDTA, 1 ml of 1 M potassium phosphate buffer, 0.8 ml of DTNB 25 mg ml^{-1} in 99.5% ethanol, 100 µl of BSA (20 mg ml^{-1}) was prepared in a final volume of 10 ml. Five-hundred µl of the reaction mixture were mixed with 100 µl of the protein sample for the sample cuvettes and with an equal volume of buffer for the reference cuvette. The reaction was then followed at 412 nm. Thioredoxin reductase activity was calculated using the extinction coefficient 13,600 M^{-1} cm^{-1}.

4.2.6. Lipid peroxidation assay

The level of lipid peroxidation products, expressed as malondialdehyde (MDA), was estimated using the thiobarbituric acid (TBA)-reactive substances (TBARS) assay

described by Heath and Packer (1968) for plant tissue. Fresh root tissue (500 mg) from plants treated with Al or Cd for 18 h using the hydroponics system and control samples were homogenized in a solution containing 0.25% thiobarbituric acid (TBA) in 10% trichloroacetic acid (TCA) using a mortar and pestle. The mixture was incubated at 95 °C for 25 min and then quickly cooled in an ice bath. Samples were centrifuged at 10,000 x g for 5 min and the absorbance of the supernatant was read at 532 and 600 nm. The value for non-specific absorption at 600 nm was subtracted from the value at 532 nm. The amount of lipid peroxidation products (MDA-TBA complex) was calculated using the extinction coefficient 155 mM^{-1} cm^{-1}.

4.2.7. Root elongation assay

Seeds of *Arabidopsis AtNTRA*- and *AtNTRB*- overexpressing lines and WT were surface sterilized as described in Chapter 3. Seeds were then plated in 0.5 X MS medium containing 6.5 g phytagar and plates were kept in the dark at 4^{o} C for 2 d. Following cold stratification, plates were transferred to a controlled environment chamber (22^{o} C with 16 h light and 8 h dark cycle and 70% humidity) for 10 d before transferring seedlings to exposure plates containing Al or Cd. For Al treatment, plates were prepared using the Al exposure medium for *Arabidopsis* described by Snowden *et al* (1995). This medium contains reduced phosphate levels to avoid precipitation of Al. Briefly, to prepare 1L of medium, 5 ml of four stock solutions (Chapter 3) were mixed in Milli-Q water and 0.0861 g $CaSO_4$ was added. The pH of the solution was adjusted to 4.3 using 0.1M HCl and 14 g phytagar were added prior to autoclaving. Media containing 0 µM and 150 µM $AlCl_3$ were prepared by adding 0 ml and 6 ml of a filter sterilized 25 mM $AlCl_3$ stock to a

liter of the autoclaved solution and poured in 100x100x15 mm square plates with grid. Ten day-old seedlings were transferred to Al exposure plates in triplicate (8 plants per plate) ensuring that roots were adequately stuck to the agar and as straight as possible. The tip of each root was marked in the bottom of the plates with a permanent marker before transferring the plates to the growth chamber (Time 0), and this was done again after 24, 48 and 72 hours of Al exposure. For Cd treatment, solutions containing 0 µM and 75 µM $CdCl_2$ were prepared by adding 0 ml and 7.5 ml, respectively, of a filter sterilized 10 mM $CdCl_2$ solution to a liter of autoclaved 0.5 X MS medium containing 1.4 % phytagar.

4.2.8. Experimental design and statistical analyses

All experiments include three independent replicates and results are expressed as mean ± standard error (SE). Statistical analyses were performed using the program SigmaStat Version 3.5 (2006, Systat Software, Inc.). Student's t-test was used to determine significant differences between treatment and control. Experiments were repeated at least three times to ensure reproducibility of results.

4.2 Results

4.3.1. Isolation and characterization of *Arabidopsis* lines overexpressing *AtNTRA* (*OxNTRA* lines) and *AtNTRB* (*OxNTRA* lines)

4.3.1.1. Determination of copy number of T-DNA inserts

The ratio of kanamycin-resistant to kanamycin-sensitive individuals was determined in 8 overexpression lines for each transgene. A total of four overexpression lines for each transgene showed a 3:1 ratio, which suggests that those transgenic lines possess only one T-DNA insert (Table 4-2 and Table 4-3). These transgenic lines were selected and self-fertilized to the T_4 generation to be used for subsequent experimental work.

4.3.1.2 Confirmation of increased expression of transgenes

Northern analysis was performed on total RNA isolated from transgenic lines that appeared to contain a single T-DNA insert and WT plants to determine if NTRA and NTRB transcript levels were enhanced when overexpressed under control of the 35S promoter. Transgenic lines OxNTRA-9, OxNTRA-12 and OxNTRA-15 showed enhanced NTRA transcript levels compared to the WT, whereas OxNTRA-2 showed WT-like NTRA transcript levels (Figure 4-3). In contrast, none of the *Arabidopsis* lines transformed with the pPZPY112/AtNTRB overexpression binary vector showed increased NTRB transcript levels compared to the WT (Figure 4-3).

Table 4-2. Determination of number of T-DNA inserts in putative *Arabidopsis thaliana* plants overexpressing *AtNTRA* (Ox-NTRA lines). Copy number of T-DNA inserts was measured by segregation of kanamycin resistance in T_2 progeny. Seedlings were grown in ½ MS medium containing 50 µg ml^{-1} kanamycin and scored as kanamycin-resistant (green cotyledons) or kanamycin-sensitive (pale cotyledons).

Arabidopsis line	Kanamycin-resistant T_2 progeny	Kanamycin-sensitive T_2 progeny	Ratio	Copy Number
Col-0 (parent)	0	24	0:24	0
Ox-NTRA-1	24	0	24:0	multiple
Ox-NTRA-2	**17**	**7**	**3:1**	**1**
Ox-NTRA-6	12	12	1:1	silenced
Ox-NTRA-9	**19**	**5**	**3:1**	**1**
Ox-NTRA-10	24	0	24:0	multiple
Ox-NTRA-11	24	0	24:0	multiple
Ox-NTRA-12	**18**	**6**	**3:1**	**1**
Ox-NTRA-15	**17**	**7**	**3:1**	**1**

Table 4-3. Determination of number of T-DNA inserts in putative *Arabidopsis thaliana* plants overexpressing *AtNTRB* (Ox-NTRB lines). Copy number of T-DNA inserts was measured by segregation of kanamycin resistance in T_2 progeny. Seedlings were grown in ½ MS medium containing 50 µg ml^{-1} kanamycin and scored as kanamycin-resistant (green cotyledons) or kanamycin-sensitive (pale cotyledons).

Arabidopsis line	Kanamycin-resistant T_2 progeny	Kanamycin-sensitive T_2 progeny	Ratio	Copy Number
Col-0 (parent)	0	24	0:24	0
Ox-NTRB-1	0	24	0:24	0
Ox-NTRB-3	**19**	**5**	**3:1**	**1**
Ox-NTRB-6	14	10	1:1	silenced
OxNTRB-8	13	11	1:1	silenced
Ox-NTRB-10	**19**	**5**	**3:1**	**1**
Ox-NTRB-13	23	1	23:1	multiple
Ox-NTRB-15	**17**	**7**	**3:1**	**1**
Ox-NTRB-16	**18**	**6**	**3:1**	**1**

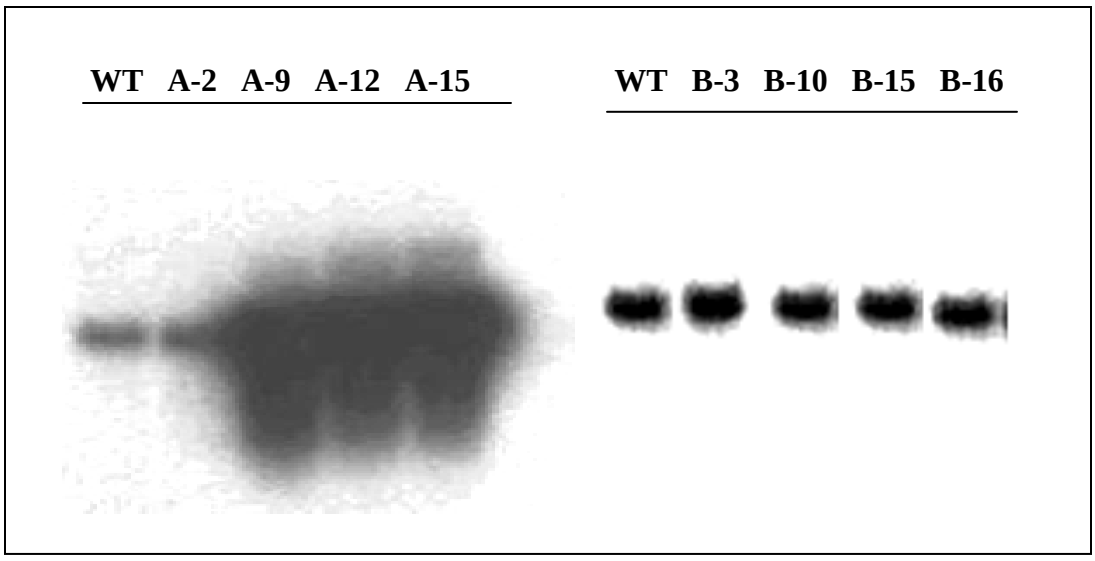


Figure 4-3. Northern analysis on WT (Col-0) and transgenic lines overexpressing *AtNTRA* (A-2, A-9, A-12 and A-15) and *AtNTRB* (B-3, B-10, B-15 and B-16). Total RNA (8µg) isolated from 1-week old seedlings was fractionated by gel electrophoresis, blotted onto membranes, and hybridized with ^{32}P-labelled cDNA probes. The experiment was repeated two times and pictures of representative blots are shown.

4.3.1.3. Determination of NTR protein levels in overexpression lines

Western analysis was performed on transgenic lines and WT in order to determine whether enhanced NTR mRNA had translated into enhanced NTR protein in *Arabidopsis* transgenic lines. NTR protein levels were enhanced in transgenic lines OxNTRA-9, OxNTRA-12 and OxNTRA-15, whereas NTR protein levels in OxNTRA-2 remained unchanged compared to the WT (Figure 4-4) Transgenic lines OxNTRA-9, OxNTRA-12 and OxNTRA-15 showed two bands in the film, thus it appears that protein levels in these lines increased for the major mitochondrial isoform (38 kD). NTR protein levels in transgenic lines overexpressing NTRB remained unchanged compared to the WT (Figure 4-4).

4.3.1.4. Thioredoxin reductase activity in overexpression lines

In order to determine if NTR activity was enhanced in *Arabidopsis* lines overexpressing *AtNTRA* and *AtNTRB* compared to the WT, NTR activity was measured in transgenic lines and WT. Thioredoxin reductase activity was significantly enhanced ($p<0.01$) in transgenic lines OxNTRA-9, OxNTRA-12 and OxNTRA-15 compared to the WT (Figure 4-5). NTR activity increased up to 1.6-fold in transgenic line OxNTRA-12 (Figure 4-5). Thioredoxin reductase activity remained unchanged compared to the WT in OxNTRA-2 transgenic line (Figure 4-5). Transgenic lines overexpressing *AtNTRB* did not show increased NTR activity compared to the WT (Figure 4-6).

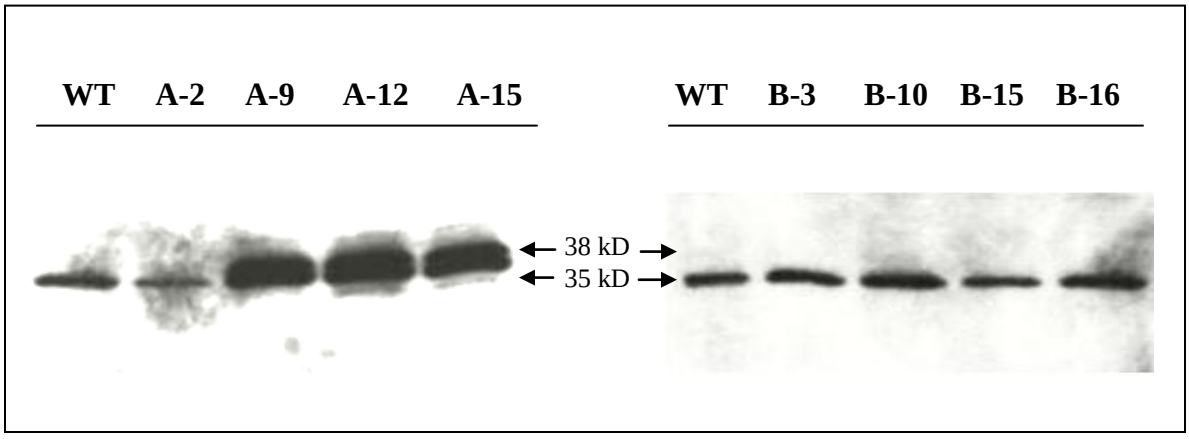


Figure 4-4. Western analysis in WT (Col-0), transgenic lines overexpressing *AtNTRA*: Ox*AtNTRA* (A-2, A-9, A-12, A-15) and transgenic lines overexpressing *AtNTRB*: Ox*AtNTRB* (B-3, B-10, B-15, B-16). Proteins (10 μg) extracted from 2-week old seedlings were separated by SDS-PAGE, immunoblotted and probed with an anti-AtNTRB antibody that recognizes both NTRA and NTRB. Antibody labeling was detected using horseradish peroxidase-conjugated secondary antibodies. Increased levels of the major mitochondrial isoform (38 kD) were observed in transgenic lines A-9, A-12 and A-15. The experiment was repeated three times and pictures of representative blots are shown.

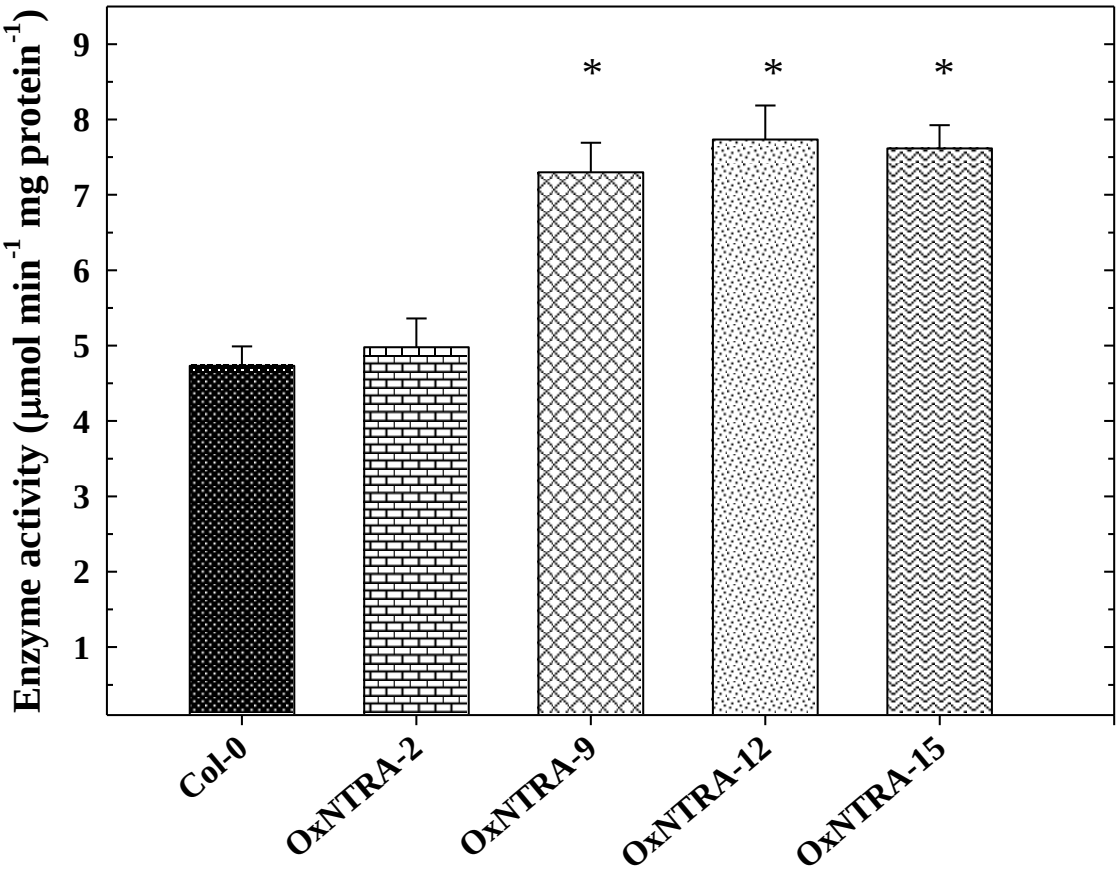


Figure 4-5. Thioredoxin reductase activity in WT (Col-0) and *AtNTRA*- overexpressing lines. Thioredoxin reductase activity was measured spectrophotometrically using enzyme extracts from roots and shoots of two-week-old seedlings. Vertical bars represent standard error (n=3). * indicates $p<0.01$ (Student's t-test). Results shown are representative of three independent experiments.

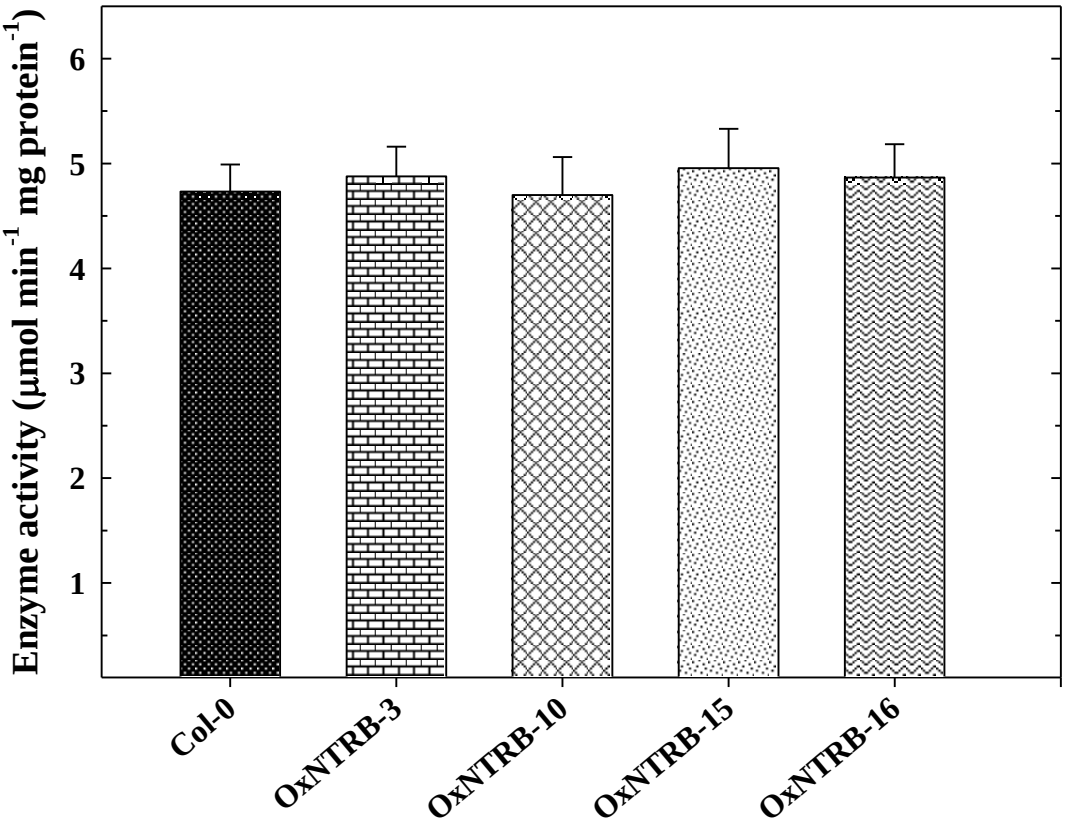


Figure 4-6. Thioredoxin reductase activity in WT (Col-0) and *AtNTRB*- overexpressing lines. Thioredoxin reductase activity was measured spectrophotometrically using enzyme extracts from roots and shoots of two-week-old seedlings. Vertical bars represent standard error (n=3). Results shown are representative of three independent experiments.

4.3.2. Effect of Al on root growth of transgenic lines overexpressing *AtNTRA* and *AtNTRB*

In order to test whether overexpression of NTRs in *Arabidopsis* confer increased tolerance to Al, root growth of transgenic lines and WT in the presence of Al was measured. In the presence of Al (150 µM), relative root growth among WT and transgenic lines overexpressing *AtNTRA* varied between 63% and 65% of control (Figure 4-7A). However, the variation was not statistically significant (p=0.6). Relative root growth in the presence of Al for transgenic lines OxNTRA-2, Ox NTRA-9 OxNTRA-12 and OxNTRA-15 was 65%, 61% 65% and 62% of their control, respectively.

Similarly, in the presence of Al, *Arabidopsis* transgenic lines overexpressing *AtNTRB* did not show a statistically significant difference in relative root growth from WT. *Arabidopsis* lines overexpressing *AtNTRB*, OxNTRB-3, OxNTRB-10, OxNTRB-15 and OxNTRB-16 showed a relative root growth of 59%, 60%, 65% and 64% of their control, respectively, whereas relative root growth in the WT was 63% of control (Figure 4-8).

4.3.3. Effect of Cd on root growth of transgenic lines overexpressing *AtNTRA* and *AtNTRB*

Relative root growth in the presence of Cd was also measured in transgenic lines overexpressing *Arabidopsis* NTRs and WT in order to determine whether transgenic lines are more tolerant to Cd than WT *Arabidopsis* plants. In the presence of 75 µM Cd, WT

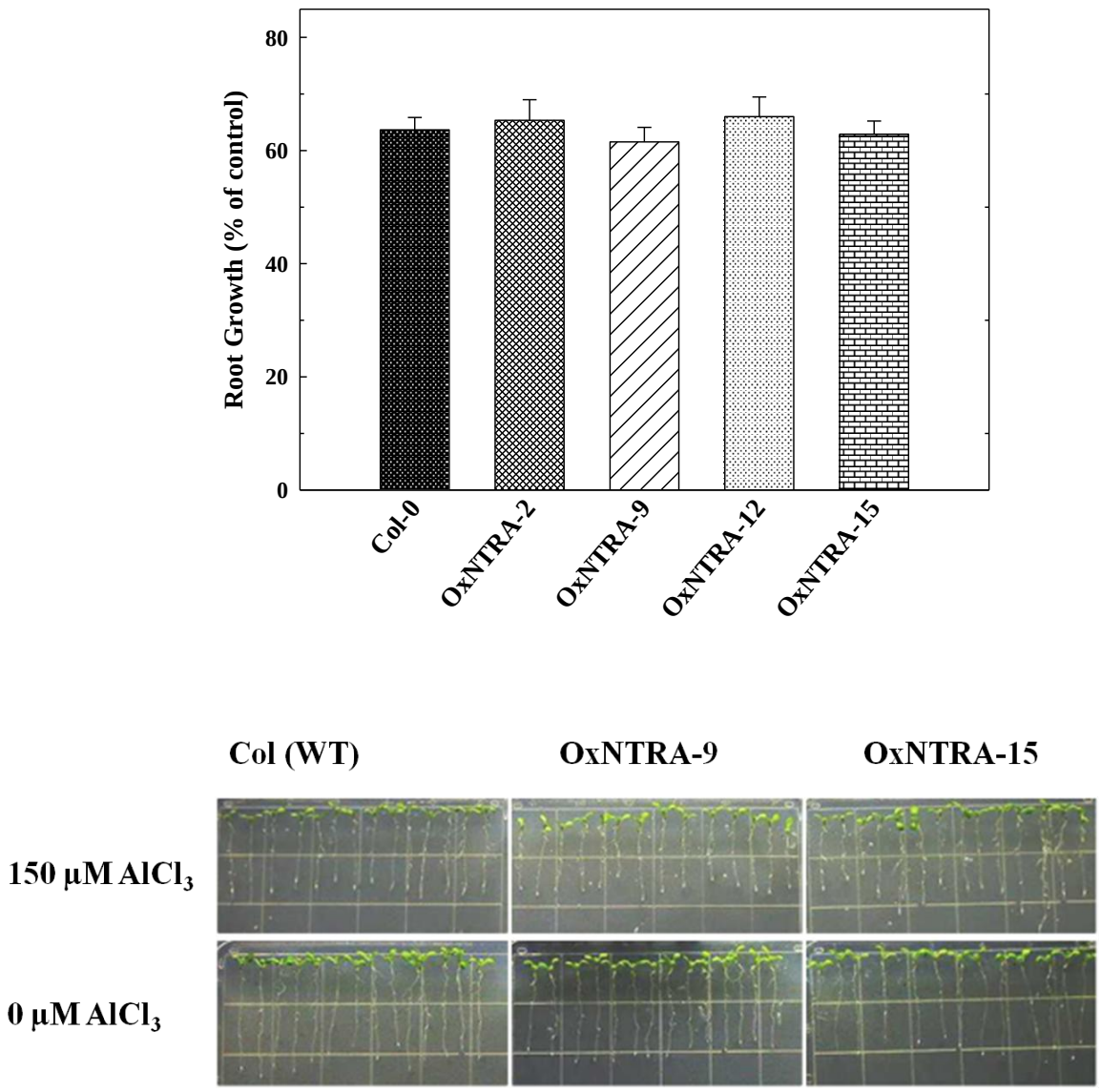
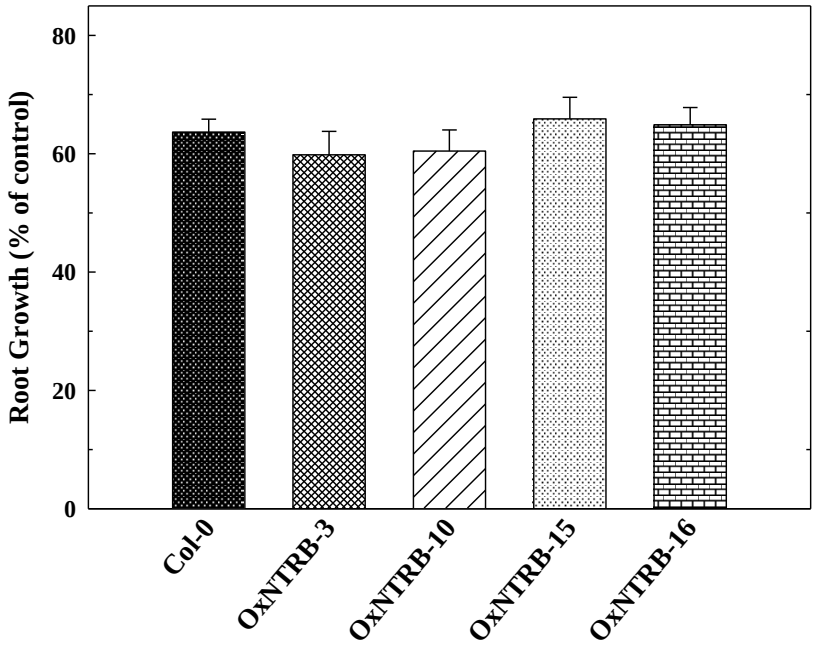


Figure 4-7. The effect of Al on root growth (% of control) of WT (Col-0) and *AtNTRA*-overexpressing lines. Seedlings were treated with 150 µM $AlCl_3$ for 72 h. Vertical bars represent standard error (n=10). Results shown are representative of three independent experiments.



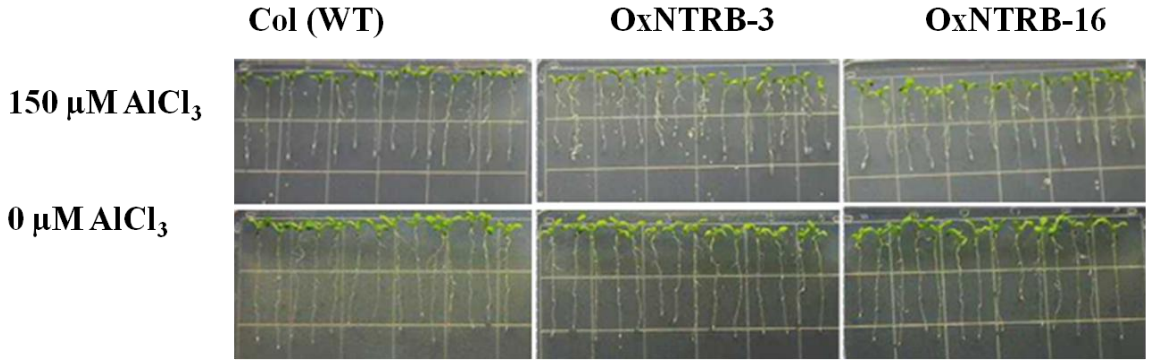


Figure 4-8. The effect of Al on root growth (% of control) of WT (Col-0) and *AtNTRB*-overexpressing lines. Seedlings were treated with 150 µM $AlCl_3$ for 72 h. Vertical bars represent standard error (n=10). Results shown are representative of three independent experiments.

relative root growth was 52% of control, whereas transgenic lines overexpressing *AtNTRA*, OxNTRA-2, OxNTRA-9, OxNTRA-12 and OxNTRA-15 showed a relative root growth of 54%, 51%, 55% and 51% of their control, respectively (Figure 4-9). The differences in relative root growth between WT and *AtNTRA*-overexpressing lines were not statistically significant (p=0.5).

Similarly, the differences in relative root growth between transgenic lines overexpressing *AtNTRB* and WT in the presence of Cd were not statistically significant. In the presence of Cd, relative root growth of transgenic lines OxNTRB-3, OxNTRB-10, OxNTRB-15 and OxNTRB-16 was 51%, 52%, 56% and 50% of their control, respectively, whereas relative root growth in WT was 53% of control (Figure 4-10).

4.3.4. Effect of Al on lipid peroxidation in transgenic lines overexpressing *AtNTRA* and *AtNTRB*

To determine whether lipid peroxidation levels decreased in *Arabidopsis* transgenic lines overexpressing NTRs compared to the WT (Col-0), the amounts of MDA produced in roots of *Arabidopsis* plants grown in the presence of 0 and 75 µM Al were estimated. Malondialdehyde levels in WT roots increased by 2.6 µmol g^{-1} FW above their control in the presence of 75 µM Al. The basal levels of MDA in WT *Arabidopsis* plants were 2.1 ± 0.2 µmol g^{-1} FW at 0 µM Al and increased to 4.8 ± 0.3 µmol g^{-1} FW at 75 µM

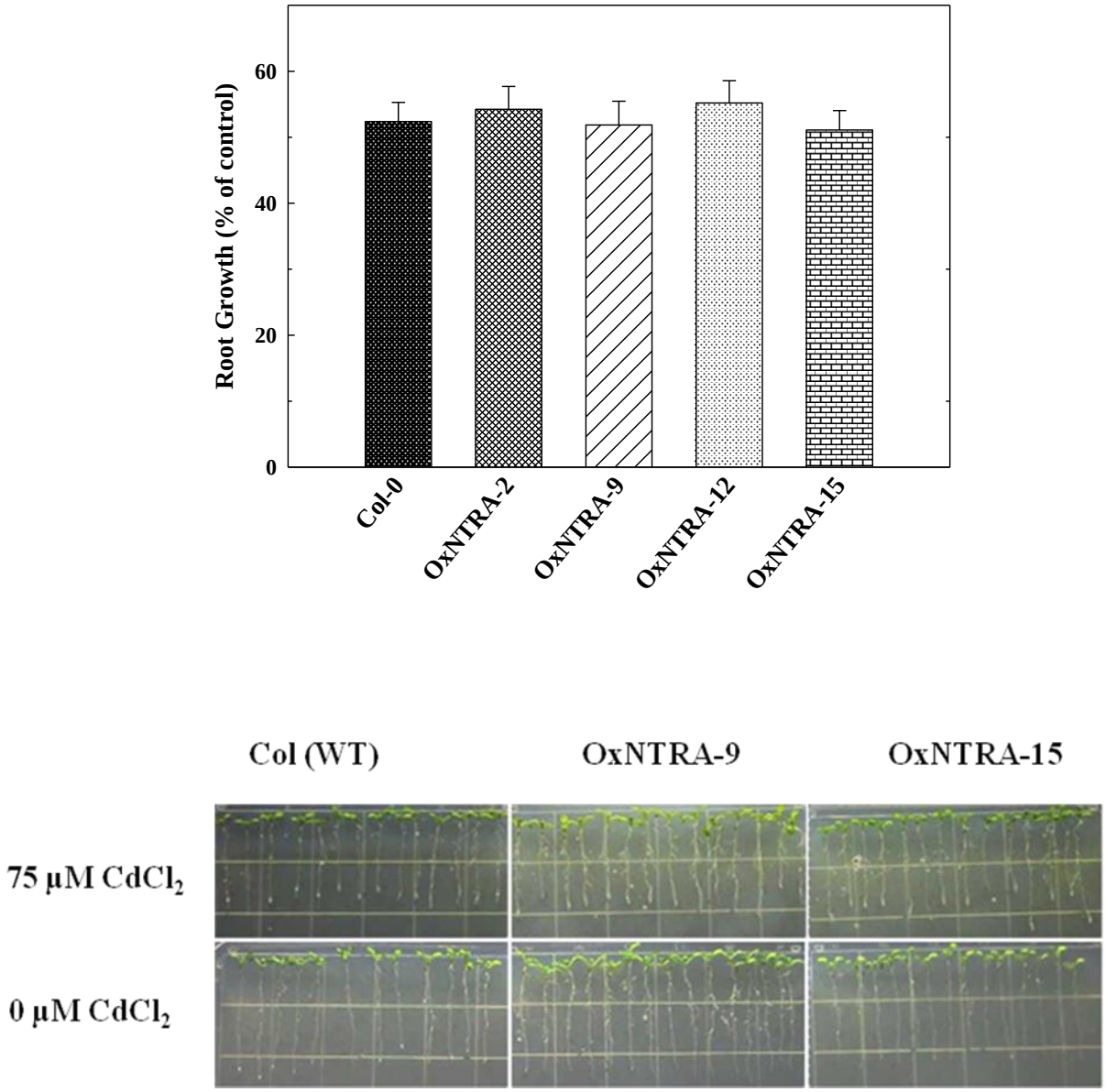


Figure 4-9. The effect of Cd on root growth (% of control) of WT (Col-0) and *AtNTRA*-overexpressing lines. Seedlings were treated with 75 µM $CdCl_2$ for 72 h. Vertical bars represent standard error (n=10). Results shown are representative of three independent experiments.

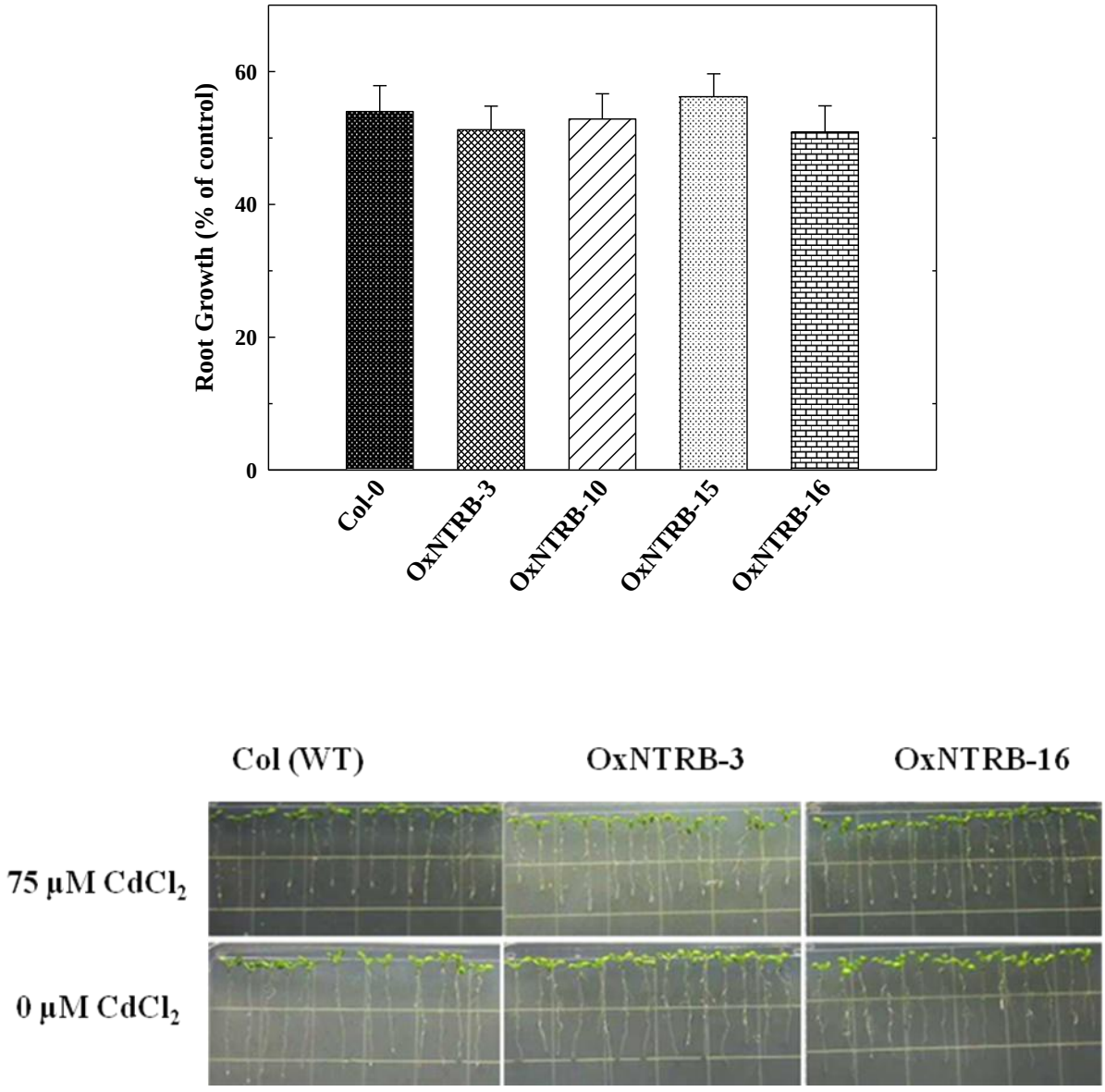


Figure 4-10. The effect of Cd on root growth (% of control) of WT (Col-0) and *AtNTRB*-overexpressing lines. Seedlings were treated with 75 µM $CdCl_2$ for 72 h. Vertical bars represent standard error (n=10). Results shown are representative of three independent experiments.

Al (Figure 4-11). Malondialdehyde levels in transgenic lines overexpressing *AtNTRA* increased by 2.0, 1.9, 2.2 and 2.0 µmol g^{-1} FW in OxNTRA-2, OxNTRA-9, OxNTRA-12 and OxNTRA-15, respectively, above their control in the presence of 75 µM Al. Basal levels of MDA in transgenic lines OxNTRA-2, OxNTRA-9, OxNTRA-12 and OxNTRA-15 were 2.1 ± 0.3, 2.3 ± 0.3, 2.2 ± 0.4 and 2.1 ± 0.3 µmol g^{-1} FW, respectively, and increased to 4.1 ± 0.4, 4.3 ± 0.3, 4.5 ± 0.2 and 4.2 ± 0.4 µmol g^{-1} FW in the presence of 75 µM Al (Figure 4-11). However, the differences in levels of lipid peroxidation between transgenic plants and control were not statistically significant (p=0.3).

Malondialdehyde levels in transgenic lines overexpressing *AtNTRB* increased by 2.3, 2.2, 2.4 and 2.2 µmol g^{-1} FW in OxNTRB-3, OxNTRB-10, OxNTRB-15 and OxNTRB-16, respectively, above their control in the presence of 75 µM Al. Basal levels of MDA in transgenic lines OxNTRB-3, OxNTRB-10, OxNTRB-15 and OxNTRB-16 were 2.3 ± 0.4, 2.7 ± 0.3, 2.3 ± 0.3 and 2.2 ± 0.3 µmol g^{-1} FW, respectively, and increased to 4.6 ± 0.3, 4.9 ± 0.3, 4.7 ± 0.2 and 4.4 ± 0.4 µmol g^{-1} FW in the presence of 75 µM Al (Figure 4-12). The differences in MDA levels between transgenic lines overexpressing *AtNTRB* and WT *Arabidopsis* plants treated with Al were not statistically significant.

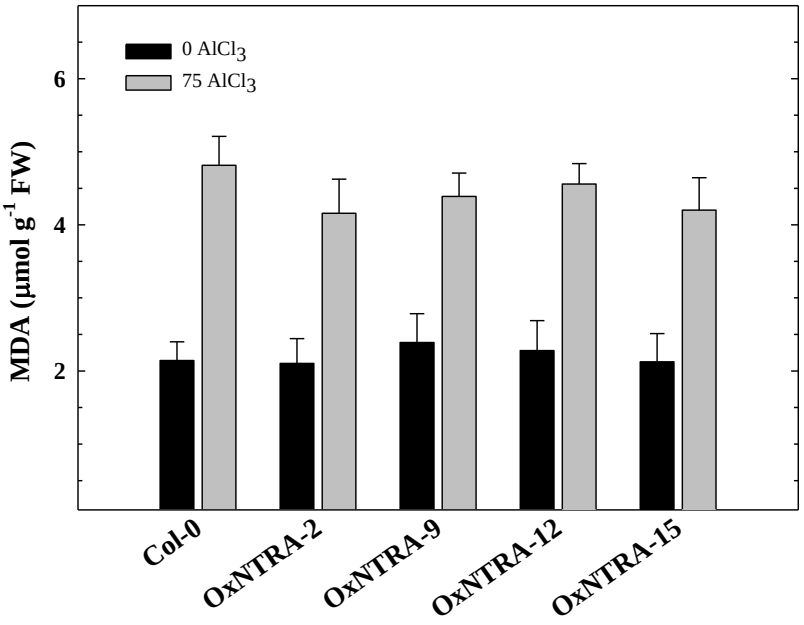


Figure 4-11. The effect of Al on lipid peroxidation levels of WT (Col-0) and *AtNTRA*-overexpressing lines. Seedlings were treated with 75 µM $AlCl_3$ for 18 h in a hydroponic system. Vertical bars represent standard error (n=3). Results shown are representative of three independent experiments.

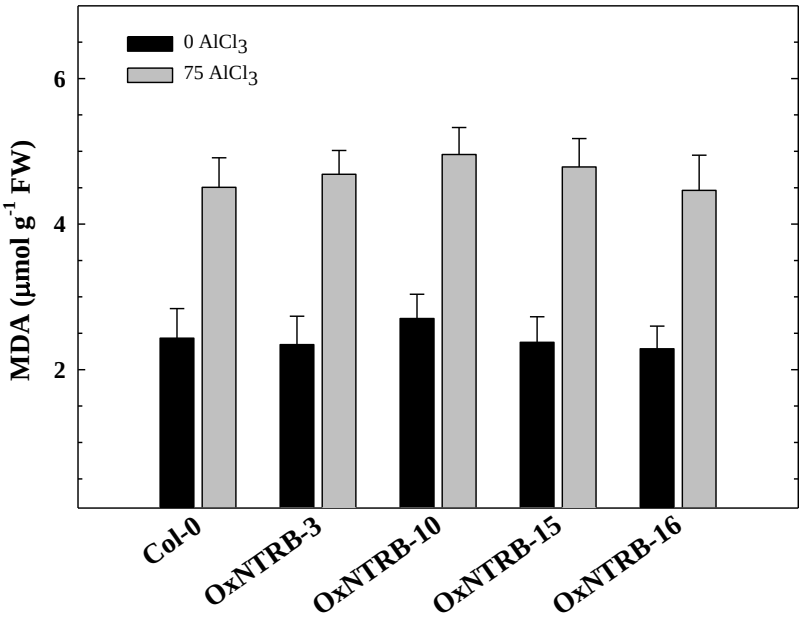


Figure 4-12. The effect of Al on lipid peroxidation levels of WT (Col-0) and *AtNTRB*-overexpressing lines. Seedlings were treated with 75 µM $AlCl_3$ for 18 h in a hydroponic system. Vertical bars represent standard error (n=3). Results shown are representative of three independent experiments.

4.3.5. Effect of Cd on lipid peroxidation in transgenic lines overexpressing *AtNTRA* and *AtNTRB*

Lipid peroxidation levels were also determined in *Arabidopsis* transgenic lines overexpressing NTRs and WT (Col-0) *Arabidopsis* plants grown in the presence of 0 and 7.5 µM Cd. Malondialdehyde levels in WT roots increased by 4.1 µmol g^{-1} FW above their control in the presence of 7.5 µM Cd. The basal levels of MDA in WT *Arabidopsis* plants were 2.3 ± 0.3 µmol g^{-1} FW at 0 µM Cd and increased to 6.4 ± 0.4 µmol g^{-1} FW at 7.5 µM Cd (Figure 4-13). Malondialdehyde levels in transgenic lines overexpressing *AtNTRA* increased by 4.0, 3.8, 4.0 and 4.1 µmol g^{-1} FW in OxNTRA-2, OxNTRA-9, OxNTRA-12 and OxNTRA-15, respectively, above their control in the presence of 7.5 µM Cd. Basal levels of MDA in transgenic lines OxNTRA-2, OxNTRA-9, OxNTRA-12 and OxNTRA-15 were 2.8 ± 0.3, 2.6 ± 0.4, 2.8 ± 0.3 and 2.5 ± 0.3 µmol g^{-1} FW, respectively, and increased to 6.9 ± 0.4, 6.5 ± 0.3, 6.8 ± 0.4 and 6.7 ± 0.3 µmol g^{-1} FW in the presence of 7.5 µM Cd (Figure 4-13). In the presence of Cd, the difference in levels of MDA between transgenic lines overexpressing *AtNTRA* and WT *Arabidopsis* plants was not statistically significant.

Malondialdehyde levels in transgenic lines overexpressing *AtNTRB* increased by 3.7, 3.8, 4.5 and 3.9 µmol g^{-1} FW in OxNTRB-3, OxNTRB-10, OxNTRB-15 and OxNTRB-16, respectively, above their control in the presence of 7.5 µM Cd. Basal levels of MDA in transgenic lines OxNTRB-3, OxNTRB-10, OxNTRB-15 and OxNTRB-16 were 2.6 ± 0.4, 2.4 ± 0.3, 2.1 ± 0.3 and 2.3 ± 0.2 µmol g^{-1} FW, respectively, and increased to 6.3 ± 0.4, 6.3 ± 0.3, 6.6 ± 0.3 and 6.2 ± 0.3 µmol g^{-1} FW in the presence of

7.5 µM Cd (Figure 4-14). The differences in MDA levels between transgenic lines overexpressing *AtNTRB* and WT *Arabidopsis* plants treated with Cd were not statistically significant.

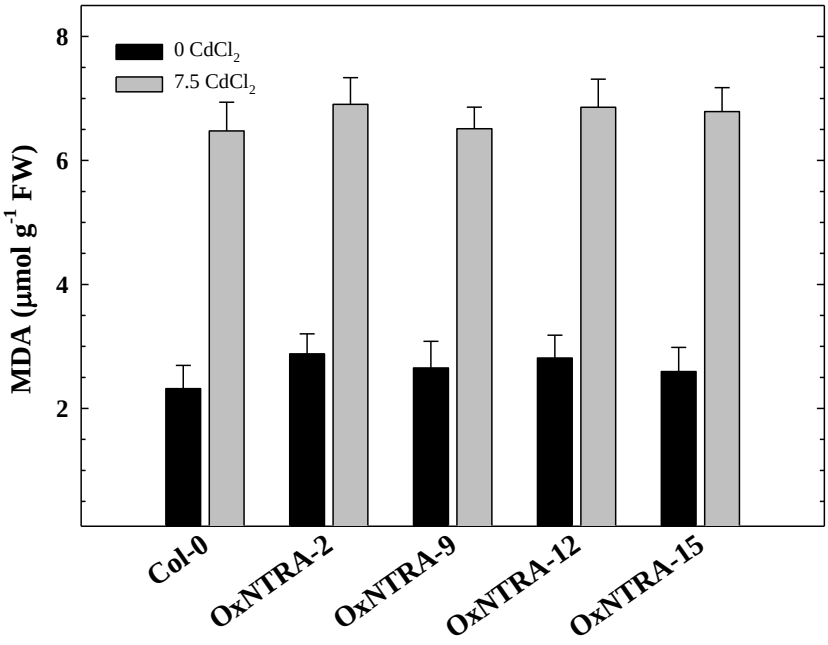


Figure 4-13. The effect of Cd on lipid peroxidation levels of WT (Col-0) and *AtNTRA*-overexpressing lines. Seedlings were treated with 7.5 µM $CdCl_2$ for 18 h in a hydroponic system. Vertical bars represent standard error (n=3). Results shown are representative of three independent experiments.

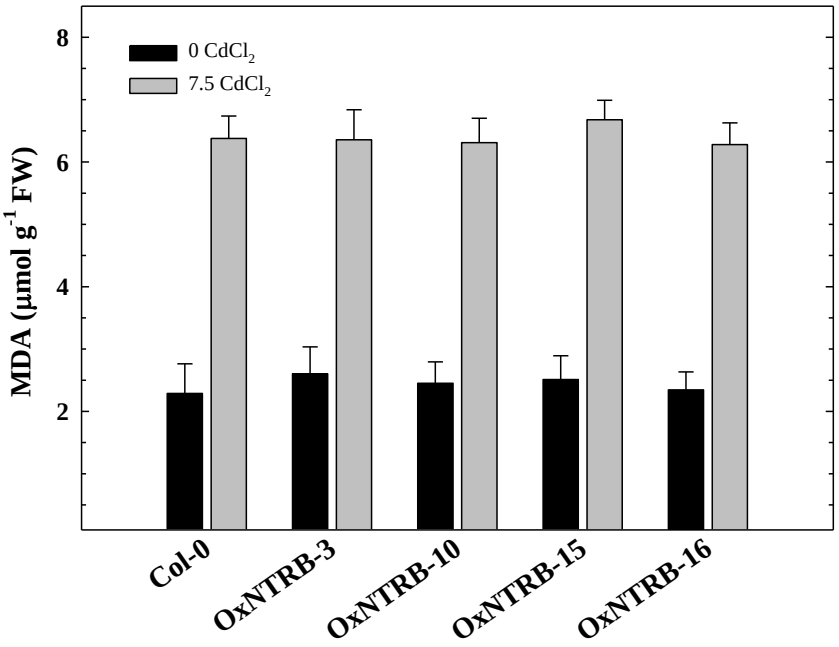


Figure 4-14. The effect of Cd on lipid peroxidation levels of WT (Col-0) and *AtNTRB*-overexpressing lines. Seedlings were treated with 7.5 µM $CdCl_2$ for 18 h in a hydroponic system. Vertical bars represent standard error (n=3). Results shown are representative of three independent experiments.

4.3.6. Effect of Al on thioredoxin reductase activity in transgenic lines overexpressing *AtNTRA* and *AtNTRB*

Thioredoxin reductase activity was determined in roots of WT (Col-0) *Arabidopsis* plants and transgenic plants overexpressing NTRs treated with 0 and 25 µM $AlCl_3$ hydroponically. Thioredoxin reductase activity increased by 1.2-fold in WT plants treated with 25 µM Al relative to control. Basal levels of thioredoxin reductase activity in WT plants were 4.5 ± 0.2 µmol min^{-1} mg $protein^{-1}$and increased to 5.3 ± 0.3 µmol min^{-1} mg $protein^{-1}$ in roots treated with 25 µM Al (Figure 4-15). In the presence of Al, no significant differences were observed in the increment of thioredoxin reductase activity between WT and transgenic lines overexpressing *AtNTRA.* Thioredoxin reductase activity in transgenic lines OxNTRA-2, OxNTRA-9, OxNTRA-12 and OxNTRA-15 increased by 1.3, 1.2, 1.2 and 1.1-fold, respectively, in the presence of Al. Basal levels of thioredoxin reductase activity in transgenic lines overexpressing *AtNTRA* were 4.1 ± 0.3, 6.7 ± 0.3, 6.5 ± 0.4 and 6.6 ± 0.3 µmol min^{-1} mg $protein^{-1}$for OxNTRA-2, OxNTRA-9, OxNTRA-12 and OxNTRA-15, respectively (Figure 4-15). In the presence of Al, thioredoxin reductase activity in transgenic lines overexpressing *AtNTRA*, OxNTRA-2, OxNTRA-9, OxNTRA-12 and OxNTRA-15 increased to 5.1 ± 0.4, 7.8 ± 0.3, 7.5 ± 0.2 and 7.3 ± 0.4 µmol min^{-1} mg $protein^{-1}$, respectively (Figure 4-15).

Thioredoxin reductase activity in transgenic lines OxNTRB-3, OxNTRB-10, OxNTRB-15 and OxNTRB-16 increased by 1.2, 1.2, 1.2 and 1.1-fold, respectively, in the presence of Al. However, in the presence of Al, no significant differences were observed

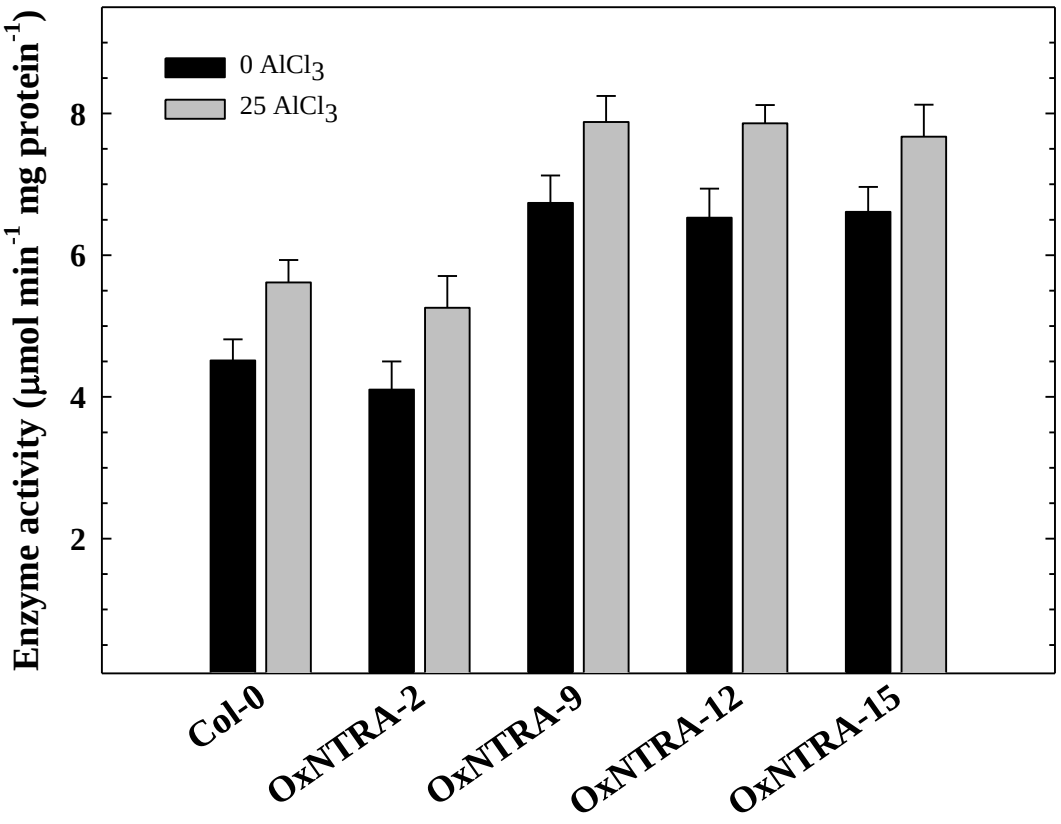


Figure 4-15. The effect of Al on thioredoxin reductase activity in WT (Col-0) and *AtNTRA*- overexpressing lines. Thioredoxin reductase activity was measured spectrophotometrically using enzyme extracts from roots and shoots of two-week-old seedlings treated with 25 µM for 18 h in a hydroponic system. Vertical bars represent standard error (n=3). Results shown are representative of three independent experiments.

in the increment of thioredoxin reductase activity between *AtNTRB*-overexpressing lines and WT (1.2-fold). Basal levels of thioredoxin reductase activity in transgenic lines overexpressing *AtNTRB* were 4.6 ± 0.3, 4.7 ± 0.3, 4.6 ± 0.4 and 4.5 ± 0.3 μmol min^{-1} mg $protein^{-1}$ for OxNTRB-3, OxNTRB-10, OxNTRB-15 and OxNTRB-16, respectively (Figure 4-16). In the presence of Al, thioredoxin reductase activity in transgenic lines overexpressing *AtNTRB*, OxNTRB-3, OxNTRB-10, OxNTRB-15 and OxNTRB-16 increased to 5.4 ± 0.4, 5.5 ± 0.3, 5.7 ± 0.3 and 5.2 ± 0.4 μmol min^{-1} mg $protein^{-1}$, respectively (Figure 4-16).

4.3.7. Effect of Cd on thioredoxin reductase activity in transgenic lines overexpressing *AtNTRA* and *AtNTRB*

Thioredoxin reductase activity was also determined in roots of WT (Col-0) *Arabidopsis* plants and transgenic plants overexpressing NTRs treated with 0 and 2.5 μM $CdCl_2$ hydroponically. Thioredoxin reductase activity increased by 1.3-fold in WT plants treated with 2.5 μM Cd relative to control. Basal levels of thioredoxin reductase activity in WT plants were 4.4 ± 0.3 μmol min^{-1} mg $protein^{-1}$ and increased to 5.6 ± 0.3 μmol min^{1} mg $protein^{-1}$ in roots treated with 2.5 μM Cd (Figure 4-17). In the presence of Cd, no significant differences were observed in the increment of thioredoxin reductase activity between WT and transgenic lines overexpressing *AtNTRA*. Thioredoxin reductase activity in transgenic lines OxNTRA-2, OxNTRA-9, OxNTRA-12 and OxNTRA-15 increased by 1.2, 1.2, 1.2 and 1.3-fold, respectively, in the presence of Cd. Basal levels of thioredoxin reductase activity in transgenic lines overexpressing *AtNTRA* were 4.7 ± 0.4,

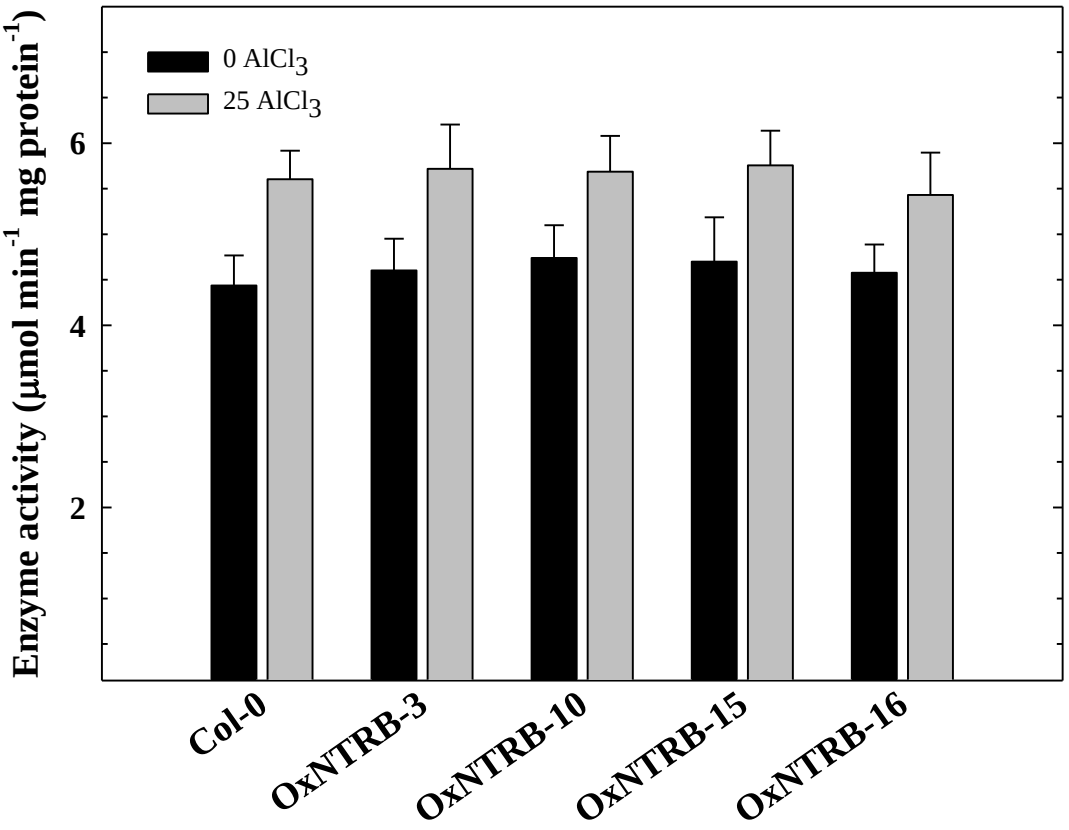


Figure 4-16. The effect of Al on thioredoxin reductase activity in WT (Col-0) and *AtNTRB*- overexpressing lines. Thioredoxin reductase activity was measured spectrophotometrically using enzyme extracts from roots and shoots of two-week-old seedlings treated with 25 µM for 18 h in a hydroponic system. Vertical bars represent standard error (n=3). Results shown are representative of three independent experiments.

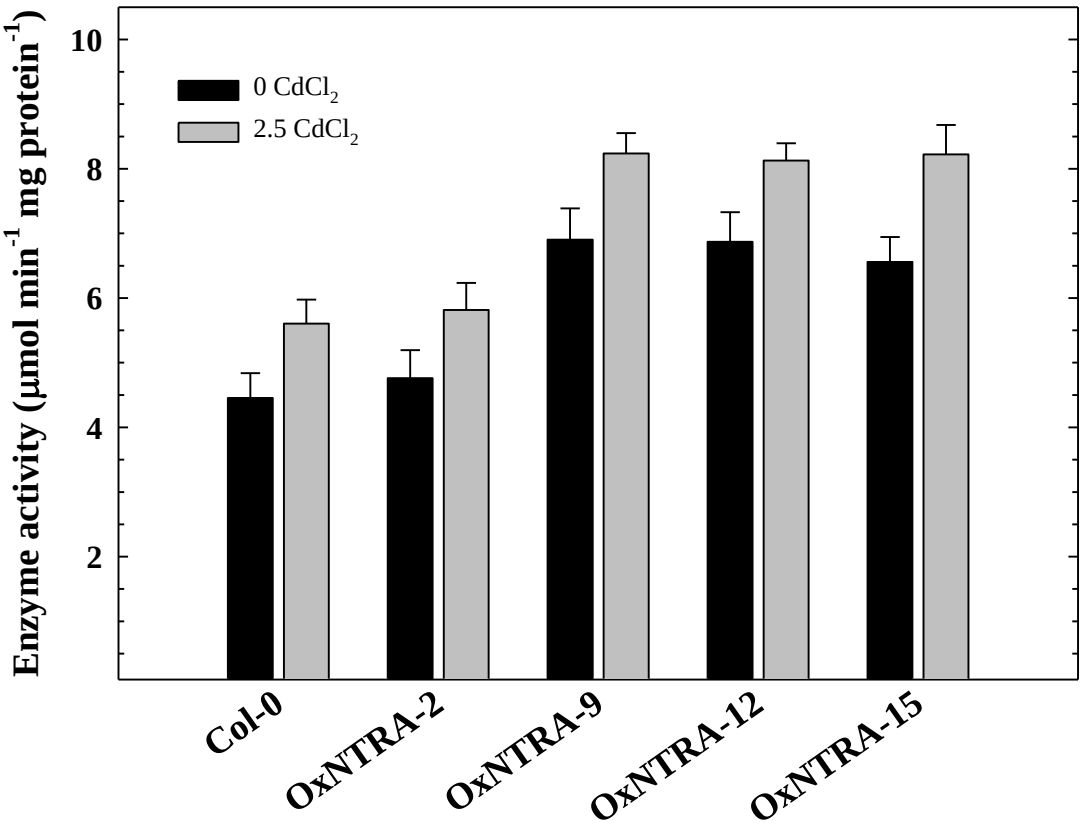


Figure 4-17. The effect of Cd on thioredoxin reductase activity in WT (Col-0) and *AtNTRA*- overexpressing lines. Thioredoxin reductase activity was measured spectrophotometrically using enzyme extracts from roots and shoots of two-week-old seedlings treated with 2.5 µM $CdCl_2$ for 18 h in a hydroponic system. Vertical bars represent standard error (n=3). Results shown are representative of three independent experiments.

6.9 ± 0.4, 6.8 ± 0.4 and 6.5 ± 0.3 µmol min^{-1} mg $protein^{-1}$ for OxNTRA-2, OxNTRA-9, OxNTRA-12 and OxNTRA-15, respectively (Figure 4-17). In the presence of Cd, thioredoxin reductase activity in transgenic lines overexpressing *AtNTRA*, OxNTRA-2, OxNTRA-9, OxNTRA-12 and OxNTRA-15 increased to 5.8 ± 0.4, 8.2 ± 0.3, 8.1 ± 0.2 and 8.2 ± 0.3 µmol min^{-1} mg $protein^{-1}$, respectively (Figure 4-17).

Thioredoxin reductase activity in transgenic lines OxNTRB-3, OxNTRB-10, OxNTRB-15 and OxNTRB-16 increased by 1.4, 1.5, 1.5 and 1.4-fold, respectively, in the presence of Cd. No significant differences were observed in the increment of thioredoxin reductase activity between WT and *AtNTRB*-overexpressing lines treated with Cd. Basal levels of thioredoxin reductase activity in transgenic lines overexpressing *AtNTRB* were 4.5 ± 0.3 , 4.3 ± 0.4, 4.4 ± 0.3 and 4.5 ± 0.3 µmol mg^{-1} protein for OxNTRB-3, OxNTRB-10, OxNTRB-15 and OxNTRB-16, respectively (Figure 4-18). In the presence of Cd, thioredoxin reductase activity in transgenic lines overexpressing *AtNTRB*, OxNTRB-3, OxNTRB-10, OxNTRB-15 and OxNTRB-16 increased to 6.4 ± 0.4, 6.4 ± 0.4, 6.7 ± 0.4 and 6.2 ± 0.2 µmol mg^{-1} protein, respectively (Figure 4-18).

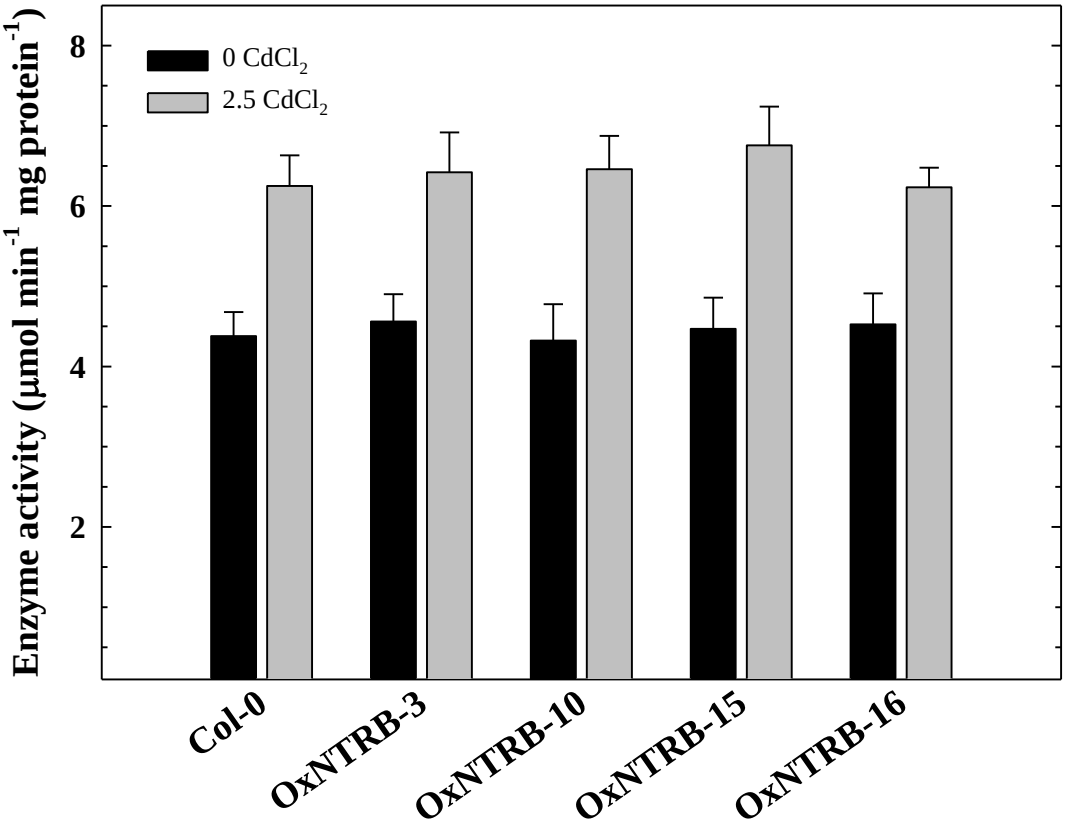


Figure 4-18. The effect of Cd on thioredoxin reductase activity in WT (Col-0) and *AtNTRB*- overexpressing lines. Thioredoxin reductase activity was measured spectrophotometrically using enzyme extracts from roots and shoots of two-week-old seedlings treated with 2.5 µM $CdCl_2$ for 18 h in a hydroponic system. Vertical bars represent standard error (n=3). Results shown are representative of three independent experiments.

4.4. Discussion

Results from my previous work with *Arabidopsis* T-DNA mutant lines (Chapter 3) suggest that NTRs may play a role in Al and Cd tolerance in *Arabidopsis*. In my previous work, T-DNA lines defective in NTRs showed increased sensitivity to Al and Cd compared to the WT. To increase our understanding of the role of NADPH-thioredoxin reductases in Al and Cd tolerance in plants, I developed *Arabidopsis* transgenic lines overexpressing *AtNTRA* and *AtNTRB* under the control of a constitutive promoter (CaMV). Homozygous (T_4 generation) *Arabidopsis* transgenic lines overexpressing *AtNTRA* and *AtNTRB* carrying a single T-DNA insertion were used for all the experiments, and transgenic lines did not show any difference in growth and development compared to WT. Four single-insert transgenic lines for each gene were selected for further characterization. Northern analysis revealed increased transcript levels compared to WT in 3 transgenic lines overexpressing *AtNTRA* (OxNTRA-9, OxNTRA-12 and OxNTRA-15), but OxNTRA-2 transgenic line showed no increase in *AtNTRA* transcript levels. Transcript levels in all transgenic lines overexpressing *AtNTRB* (OxNTRB-3, OxNTRB-10, OxNTRB-15 and OxNTRB-16) remained unchanged compared to WT. These results suggest that only 3 transgenic lines (OxNTRA-9, OxNTRA-12 and OxNTRA-15) are overexpressing NADPH-thioredoxin reductase (*NTRA*) and that all transgenic lines transformed with the *AtNTRB* construct failed to overexpress the *NTRB* gene.

Western analysis was performed in transgenic lines to determine whether enhanced NTR mRNA had translated into enhanced NTR protein in *Arabidopsis*

transgenic lines that showed increased *AtNTRA* transcript levels (OxNTRA-9, OxNTRA-12 and OxNTRA-15) , and also to confirm that none of the transgenic lines transformed with the *AtNTRB* construct were overexpressing the *AtNTRB* gene. NTR protein levels were enhanced in transgenic lines OxNTRA-9, OxNTRA-12 and OxNTRA-15, whereas NTR protein levels in OxNTRA-2 remained unchanged compared to WT. NTR protein levels in transgenic lines overexpressing *NTRB* also remained unchanged compared to WT. These results confirm that only transgenic lines OxNTRA-9, OxNTRA-12 and OxNTRA-15 are indeed overexpressing *AtNTRA* and that none of the transgenic lines transformed with the *AtNTRB* construct are actually overexpressing the *AtNTRB* gene. Since the antibody used for Western analysis is able to recognize both isoforms, it was also revealed that transgenic lines transformed with the *AtNTRA* overexpression construct were overexpressing the mitochondrial isoform. Two bands of similar intensity but slightly different molecular weight were observed in the films. One of the bands observed for transgenic lines overexpressing *AtNTRA* is of approximately 35 kD and corresponds to the major cytosolic isoform according to Reichheld *et al*. (2005), and the second band appears at 38 kD, which corresponds to the major mitochondrial isoform. Only one band of approximately 35 kD is observed for WT and transgenic lines transformed with the *AtNTRB* construct. This observation also suggests that only transgenic lines OxNTRA-9, OxNTRA-12 and OxNTRA-15 are overexpressing NTR, since OxNTRB transgenic lines show only one band just like the WT.

Further characterization of transgenic lines was done by measuring thioredoxin reductase activity. Transgenic lines overexpressing *AtNTRA* (OxNTRA-9, OxNTRA-12 and OxNTRA-15) showed significantly increased thioredoxin reductase activity of up to

1.6-fold compared to WT. Thioredoxin reductase activity in transgenic line OxNTRA-2 was not significantly different from thioredoxin reductase activity in WT plants. Similarly, thioredoxin reductase activity in transgenic lines transformed with the *AtNTRB* construct did not show a significant difference compared to WT levels. These results provide further evidence indicating that only transgenic lines OxNTRA-9, OxNTRA-12 and OxNTRA-15 are successfully overexpressing *AtNTRA* and furthermore demonstrate that the NTR protein encoded is functional. Similarly, these results provide further evidence of the lack of overexpression of *AtNTRB* in transgenic lines transformed with the *AtNTRB* construct. The reason why transgenic lines transformed with the *AtNTRB* construct failed to overexpress the *AtNTRB* gene is unclear. Both *AtNTRA* and *AtNTRB* constructs were developed with the same vector and *Agrobacterium* strain. Overexpression constructs were sequenced and transformed in parallel and no changes in nucleotide sequence were observed for either construct. Thus, it appears that the reason why overexpression of *AtNTRB* failed cannot be found in the method followed to develop these transgenic lines.

Following characterization of the developed *Arabidopsis* transgenic lines overexpressing NTRs, relative root growth in the presence of Al and Cd compared with controls was determined in order to test whether plants with enhanced thioredoxin reductase activity show increased tolerance to Al and Cd compared to WT. Although transgenic lines transformed with the *AtNTRB* construct failed to overexpress the *AtNTRB* gene, relative root growth in the presence of Al and Cd was also quantified and compared with controls. Transgenic lines transformed with the *AtNTRA* construct (OxNTRA-2, OxNTRA-9 OxNTRA-12 and OxNTRA-15) did not show a significant increase in

relative root growth in the presence of Al and Cd compared to WT. Similarly, in the presence of Al and Cd, *Arabidopsis* transgenic lines transformed with the *AtNTRB* construct did not show a statistically significant difference in relative root growth from WT. It can be concluded from these results that none of the transgenic lines overexpressing NTRs developed in this study show increased tolerance to Al or Cd compared to WT plants.

Previous studies have shown that boosting the plant's antioxidant defense system is an effective strategy to increase tolerance to Al (Ezaki *et al*., 2000; Basu *et al*., 2001; Ezaki *et al*., 2005; Yin *et al*., 2010). In these studies, it has been hypothesized that the antioxidant system protects plants from Al-induced oxidative damage. Thus, in the present study, lipid peroxidation induced by Al and Cd was measured in transgenic lines overexpressing NTRs and WT to test whether transgenic lines show decreased levels of lipid peroxidation. However, no significant differences were observed in lipid peroxidation levels between transgenic lines overexpressing NTRs and WT plants in the presence of Al or Cd. These results suggest that increased activity of NTRs does not enhance the plant's antioxidant system sufficiently to reduce lipid peroxidation and confer increased tolerance to Al and Cd.

In order to get further insight into the role of NTRs in Al and Cd tolerance, I quantified thioredoxin reductase activity in transgenic lines and WT plants treated with Al and Cd. Previously (Chapter 3), I observed that thioredoxin reductase activity is induced only at lower concentrations of Al and Cd, thus transgenic plants and WT were exposed to the same low concentrations of Al (25 µM) and Cd (2.5 µM). No significant

differences were observed in the increment of thioredoxin reductase activity between transgenic lines and WT. Thus, thioredoxin reductase activity does not appear to be further induced, beyond WT levels, by Al or Cd in transgenic lines overexpressing NTRs.

Since enhancing the plant's ability to synthesize NTRA does not result in an improvement in Al and Cd tolerance, we can conclude that increasing availability of mitochondrial and cytosolic NTRs is not an effective strategy to increase Al and Cd tolerance in plants. Thus, NTRs are not ideal candidate genes for genetic manipulations aimed at developing crops with increased Al or Cd tolerance. However, the lack of increased tolerance to Al and Cd in *Arabidopsis* transgenic lines overexpressing NTRs compared to WT does not completely reject the putative role of NTRs in Al and Cd tolerance. Results from my previous work (Chapter 2 and Chapter 3) suggested that NTRs play a role in Al and Cd tolerance in yeast and *Arabidopsis*. I observed that Al and Cd increased lipid peroxidation in mutants defective in thioredoxin reductases in both yeast and *Arabidopsis*, which suggests that thioredoxin reductases play a role in ameliorating Al- and Cd-induced oxidative stress. In the current study however, lipid peroxidation levels in transgenic lines overexpressing *AtNTRA* were not significantly different from those in WT plants. I hypothesized that overexpression of NTRs would enhance the plant's antioxidant system and that levels of lipid peroxidation would decrease in transgenic plants compared to WT in the presence of Al and Cd. My results suggest that NTRs may not have a direct effect in the plant's ability to scavenge reactive oxygen species produced during Al and Cd exposure in order to protect the plant from oxidative damage including lipid peroxidation.

Decreasing NTR activity caused increased lipid peroxidation in the presence of Al and Cd (Chapter 3), but increasing NTR (NTRA) activity did not show an effect on lipid peroxidation levels compared to control in the presence of Al and Cd in this study. A possible explanation for these somewhat conflicting results is that NTR does not play a direct role scavenging ROS in order to avoid lipid peroxidation, but interacts with other thiol-disulfide oxidoreductases that would play a role as ROS scavengers. Thus, NTR could be playing a role in Al and Cd tolerance as a signal transducer. If NTRs play a role in Al and Cd tolerance by interacting with other molecules, we could hypothesize that elevated levels of NTR activity would not have an effect on Al- and Cd-induced lipid peroxidation since there would still be a limited amount of molecules that interact with NTRs. Interestingly, Miao *et al*. (2006) recently found that some glutathione peroxidases (GPXs) are oxidative stress sensors rather than direct antioxidants. Since both NTRs and GPXs are thiol-based oxidoreductases, it could be possible that NTRs also play a role as oxidative stress sensors in plants. This could explain why transgenic lines overexpressing NTRs do not seem to decrease Al- and Cd-induced oxidative damage compared to control. Moreover, Bashandy *et al*. (2010) recently reported that NTRs interfere with developmental processes trough modulation of auxin signaling. Bashandy *et al*. (2010) found that a triple mutant *ntra ntrb cad2* (defective in NTRs and a glutathione biosynthesis gene), shows loss of apical dominance, vasculature defects and reduced secondary root production. The role of NTRs modulating auxin signaling could partially explain why *ntr* mutants show increased inhibition of root growth in the presence of Al compared to WT.

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5. General discussion and conclusions

5.1. Discussion

Aluminum is the most abundant metal in the earths's crust and Al phytotoxicity is a major factor limiting plant growth on acid soils (Kochian 1995; von Uexküll and Mutert, 1995). Aluminum facilitates the formation of reactive oxygen species (ROS) and promotes peroxidation of phospholipids and proteins in cell membranes (Cakmak and Horst, 1991; Yamamoto *et al*., 2001). Since Al is a non-redox active metal, the exact mechanisms of Al-induced oxidative damage remain unclear. It has been suggested that much of the pro-oxidant activity of Al might be explained by the formation of an aluminum superoxide semireduced radical ion ($AlO_2^{\cdot 2+}$; Exley, 2004). The interaction of Al with membranes could facilitate the generation of ROS via redox-active metals such as iron (Fe; Oteiza, 1994; Yamamoto *et al*., 1997). Moreover, it has been suggested that Al increases the pro-oxidant activity of phenolic compounds (Sakihama and Yamasaki, 2002).

Another non-redox active metal of agricultural interest is Cd, which is a widespread toxic metal with no described biological function. Cadmium accumulates readily in plants, and consumption of crops grown in agricultural soils constitutes the major source of dietary Cd for humans (Wagner, 1993). Like Al, Cd is also capable of causing oxidative stress in plants as a mechanism of toxicity, even though Cd is also not a redox-active metal ion. The mechanisms of Cd-induced oxidative stress are also unclear, but it has been suggested that Cd-induced oxidative stress can be attributed mainly to depletion of glutathione (GSH) and binding of Cd to thiols of several antioxidant

enzymes (Schutzendubel and Polle, 2002). In the present study, I focused on these two non-redox active metals to gain further insight into whether the role of the antioxidant enzymes studied is specific to Al or whether these enzymes are also involved in oxidative stress induced by other metals.

Several studies have found that various components of the antioxidant system play a role in protecting plants from Al- and Cd-induced oxidative damage (Zhu *et al*., 1999; Ezaki *et al*., 2000; Basu *et al*., 2001; Schutzendubel *et al*., 2001; Ezaki *et al*., 2005; Yin *et al*., 2010). The present study was designed to examine the potential role of thiol-disulfide oxidoreductases (thioredoxin system, peroxiredoxins and glutaredoxins) in protecting plants from Al- and Cd-induced oxidative damage. These antioxidant enzymes have not been previously studied in the context of Al and Cd tolerance.

Since plants possess a complex antioxidant system that includes several isoforms for thioredoxins and glutaredoxins (~40 and 31 genes in *Arabidopsis thaliana*, respectively), I used yeast (*Saccharomyces cerevisiae*) as a model system to identify relevant genes that could play a role in Al and Cd tolerance to be further studied in *Arabidopsis*. Yeast has proven to be a useful model for the study of metal tolerance in the past (Schott and Gardner, 1997; Avery, 2001; Hamilton *et al*., 2001; Anoop *et al*., 2003; Basu *et al*., 2004). In the present study, I screened single and multiple mutants defective in genes of the thioredoxin system, peroxiredoxins and glutaredoxins for hypersensitivity to Al and Cd (Chapter 2). Before the present study, only one report has suggested a protective role for a yeast peroxiredoxin against metal-induced oxidative stress (Nguyen-nhu and Knoops, 2002). Nguyen-nhu and Knoops (2002) observed that a yeast mutant

lacking a thioredoxin peroxidase gene (*AHP1*) was more sensitive to several metals such as copper and cobalt than the WT strain. Lipid peroxidation levels in the yeast mutant lacking *AHP1* also increased significantly compared to WT upon exposure to several metals.

In the current study, a total of 15 disruption mutants affecting all of the genes of the yeast thioredoxin system, peroxiredoxins and glutaredoxins were screened for hypersentivitiy to Al and Cd. The cytosolic thioredoxin reductase (*TRR1*) showed the most hypersensitive phenotype to both Al and Cd, and this gene also showed the highest induction upon Al and Cd exposure, with transcript abundance increasing by up to 2.3-fold at 300 µM Al. Moreover, the *trr1*Δ yeast mutant was the only single mutant that showed a significant increase in lipid peroxidation compared to WT upon Al and Cd exposure. Complementation of the *trr1*Δ mutant with the WT *TRR1* gene restored growth of this mutant to WT levels in the presence of Al and Cd. These results clearly indicate that *TRR1* plays a role in Al and Cd tolerance in yeast, and suggest that it does so by protecting cells from Al- and Cd-induced oxidative damage.

Previously, Basu *et al*. (2004) studied several genes of the glutathione metabolic pathway and glutathione peroxidases, which are also thiol-disulfide oxidoreductase-based antioxidant enzymes, in relation to Al tolerance in yeast (*S. cerevisiae*). They found that glutathione peroxidase *phgpx1*Δ was the most hypersensitive single mutant compared to WT upon exposure to Al and demonstrated that complementation of the *phgpx1*Δ mutant with a functional gene (*PHGPX1*) restored the normal sensitivity to Al. They also observed that a triple mutant comprising 3 GPX genes in yeast (*PHGPX1*-3) showed

greater hypersensitivity to Al than any of the single mutants. Similar to what Basu *et al*. (2004) observed, I found that triple mutants defective in various genes of the thioredoxin system and glutaredoxins are more hypersensitive to Al and Cd than any of the single mutants (Chapter 2). This observation suggests that these genes may have complimentary and/or overlapping functions in yeast, since for most of these genes a hypersensitive phenotype is not observed upon Al and Cd treatment when working with single mutants. Redundancy of gene function observed in yeast supported the notion that it would be more difficult to observe a hypersensitive phenotype upon Al and Cd exposure by using single-gene knockouts in *Arabidopsis*.

Based on the results from my studies with yeast (Chapter 2), I decided to focus on thioredoxin reductases for my work with *Arabidopsis* (Chapters 3 and 4). The first step in my investigation was a screen of knockout lines defective in thioredoxin reductases for hypersensitivity to Al and Cd. I also decided to test lines defective in peroxiredoxins (PRXs), since a yeast single mutant defective in peroxiredoxin (*dot5*Δ) showed the second highest hypersensitivity to Al and Cd. All 11 *Arabidopsis* T-DNA lines defective in PRXs showed similar root growth inhibition to WT plants in the presence of Al and Cd. Similar to what I observed in yeast, the lack of phenotype in these mutants in the presence of Al and Cd also suggests that these genes may play an overlapping role, thus single-gene knockouts are not sufficient to produce a detectable phenotype.

The *Arabidopsis* genome contains three genes encoding NADPH-thioredoxin reductases (NTRs), *NTRA* (the major cytosolic isoform), *NTRB* (the major mitochondrial isoform) and *NTRC* (a chloroplastic isoform) (Gelhaye *et al*., 2005). In the present study,

T-DNA lines defective in *NTRA* and *NTRB* showed increased inhibition of root growth compared to WT plants upon exposure to Al and Cd (Chapter 3). To confirm this phenotype, another allele was isolated for each of the two mutants. All four *ntr* mutants, *ntra-1*, *ntra-2*, *ntrb-1* and *ntrb-2* showed greater root growth inhibition than WT plants. A significant reduction in thioredoxin reductase activity was also observed in all four *ntr* mutants compared to WT. Similar to the yeast mutant defective in *TRR1* (Chapter 2), *ntr* mutants in *Arabidopsis* also showed increased levels of lipid peroxidation (compared to WT) in the presence of Al and Cd. These results suggest that NTRs play a role protecting *Arabidopsis* plants from Al and Cd toxicity, and further suggest that they may do so by reducing Al- and Cd-induced oxidative damage.

I also tested whether NTRs transcript levels are induced in *Arabidopsis* upon treatment with Al and Cd. In contrast to *TRR1* in yeast cells exposed to Al and Cd, NTRs (*NTRA* and *NTRB*) in *Arabidopsis* did not show a strong induction upon Al and Cd exposure (Chapter 3). Transcript levels for *NTRA* and *NTRB* in *Arabidopsis* increased up to 1.5-fold (relative to control samples) upon Al and Cd exposure, where as in yeast *TRR1*, transcript levels increased by more than 2-fold. Unlike yeast *TRR1* transcript levels, *NTRA* and *NTRB* transcript levels did not increase in a dose-dependent manner upon Al and Cd exposure in *Arabidopsis*. I also tested whether thioredoxin reductase activity increases upon exposure of *Arabidopsis* to Al and Cd . Thioredoxin reductase activity was only induced (up to 1.4-fold relative to control) by low concentrations of Al and Cd and enzyme activity decreased to WT levels with the highest concentrations of Al and Cd (Chapter 3).

These results suggest that NTRs in *Arabidopsis* play a role in Al and Cd tolerance, however, the evidence is not as strong as that from my work with yeast.I did not observe such a clear induction of NTRs transcript levels in *Arabidopsis* as I observed in yeast, and thioredoxin reductase activity was only induced at low concentrations of Al and Cd. However, T-DNA lines defective in NTRs did show decreased tolerance to Al and Cd, even though these lines were single mutants. Due to the fact that complementation is a considerably longer procedure in *Arabidopsis* than in yeast, I did not perform complementation assays in *Arabidopsis ntr* mutants. Such complementation assays would be a fruitful topic for further research. Such research would allow a direct comparison of results with *Arabidopsis* and yeast and could provide additional confidence in the role of NTRs may play a role in Al and Cd tolerance in plants. Notwithstanding, the results from my work with *Arabidopsis* T-DNA lines (Chapter 3) do suggest that NTRs may play a role in Al and Cd tolerance in plants. Based on this evidence, I hypothesized that overexpression of *NTRA* and *NTRB* may confer increased tolerance to Al and Cd.

In order to test whether increased expression of *AtNTRA* and *AtNTRB* in *Arabidopsis* confers increased tolerance to Al and Cd, I developed overexpression lines for both *AtNTRA* and *AtNTRB* genes in *Arabidopsis* under the control of a constitutive promoter (CaMV). Four homozygous, single-insert, transgenic lines were tested for each construct. Only three transgenic lines overexpressing *AtNTRA* showed increased transcript levels, increased protein levels and increased thioredoxin reductase activity. Even though the same methodology was used to develop *Arabidopsis* transgenic lines overexpressing *AtNTRB*, none of the transgenic lines transformed with the *AtNTRB*

construct showed increased levels or transcript, protein or enzyme activity. The reason why *Arabidopsis* transgenic lines transformed with the *AtNTRB* construct failed to overexpress *AtNTRB* is unclear.

Root growth in the presence of Al and Cd was determined in all eight transgenic lines and control plants to investigate whether overexpression of NTRs confers increased tolerance to Al and Cd. Transgenic lines transformed with both the *AtNTRA* and *AtNTRB* constructs showed similar inhibition of root growth as control plants in the presence of Al and Cd. These results indicate that increased expression of *NTRA* in *Arabidopsis* does not confer increased tolerance to Al and Cd. The same conclusion cannot be made regarding *AtNTRB*, since transgenic lines transformed with the *AtNTRB* construct did not show increased *NTRB* expression.

Lipid peroxidation levels were also determined in overexpression lines and control plants treated with Al and Cd. No significant differences were observed in lipid peroxidation levels between overexpression lines and control plants upon exposure to Al and Cd. Similarly, no differences were observed between NTR overexpression lines and WT in terms of the increase in thioredoxin reductase activity upon exposure to Al and Cd. Determination of levels of H_2O_2 in overexpression lines compared to control plants upon Al and Cd exposure and measuring another indicator of oxidative damage, such as oxidative DNA damage (Watanabe *et al*., 2006; Yin *et al*., 2010) would provide further information regarding the antioxidant capacity of NTR-overexpressing lines. However, these experiments could not be completed in this project due to time limitations.

All together, these results indicate that increased activity of thioredoxin reductase in *Arabidopsis* is not sufficient to increase tolerance to Al and Cd. The implication is that changes in thioredoxin reductase activity *per se* cannot be invoked as an explanation for the hypersensitivity of mutants to Al and Cd. My studies with yeast mutants (Chapter 3) and *Arabidopsis* T-DNA lines defective in NTRs (Chapter 3) provide evidence that NTRs do play a role in Al and Cd tolerance. The lack of increased tolerance to Al and Cd in NTR-overexpressing lines does not completely reject the hypothesis that NTRs play a role in Al and Cd tolerance. However, the lack of improved root growth and the unchanged levels of lipid peroxidation compared to control plants observed in transgenic lines suggests that the role that NTRs play in Al and Cd tolerance might not be direct scavenging of ROS in order to avoid Al-and Cd-induced oxidative damage such as lipid peroxidation. The lack of hypersensitivity to oxidant stresses shown by Reichheld *et al*. (2007) in the double mutant *ntra ntrb* also suggests that NTRs may not play an important role as ROS scavenging molecules.

Although in the present study, overexpression of NTRs did not confer increased tolerance to Al or Cd, Li *et al*. (2010) recently found that overexpression of a thioredoxin gene (*PTrx*) in barley increased tolerance to Al. A possible explanation for these results is that the increase in sulfhydryl content observed in transgenic barley is more important to alleviate Al-induced oxidative damage than increasing NTR levels, which seem to be not a limiting factor.

A possible hypothesis to explain the increased levels of lipid peroxidation in *ntr* mutants is that NADPH-thioredoxin reductases indirectly regulate the plant's antioxidant

capacity and the lack of NTRs disrupts the plant's ability to effectively protect itself from Al- and Cd-induced oxidative damage. Miao *et al*. (2006) found that an *Arabidopsis* glutathione peroxidase (*AtGPX3*) functions as both a redox transducer and a scavenger of ROS in abscisic acid (ABA) and drought stress responses. AtGPX3 appears to play dual roles in H_2O_2 homeostasis, acting as a general scavenger and specifically relaying the H_2O_2 signal as an oxidative signal transducer in ABA and drought stress signaling (Miao *et al*., 2006). Since both GPX and NTR are thiol-disulfide-based oxidoreductases, it would be possible that NTR might also play also a role as a signal transducer in Al and Cd tolerance. The role of NTR in Al and Cd tolerance could be indirectly regulating the plant's antioxidant capacity. Interestingly, Miao *et al*. (2006) also found that the physiological electron donor system for AtGPX3 is thioredoxin and not GSH. It has also been shown previously that the electron donor for some other GPXs is thioredoxin and not GSH (Herbett *et al*., 2002). It could also be possible that enzymes such as GPXs in *Arabidopsis* act as direct scavengers of ROS upon Al and Cd exposure and that NTRs provide the source of reduced thioredoxin necessary to reduce GPXs.

New lines of evidence provide further support for a role of NTRs in signaling, particularly in auxin signaling. Bashandy *et al*. (2010) found that NTRs are involved in auxin homeostasis, and thus are able to modulate various aspects of plant growth and development including root growth. Moreover, inhibition of root growth induced by Al has been found to be mediated by ethylene and auxin (Sun *et al*., 2010). Although the *Arabidopsis ntr* mutants in the present study do not show differences in root growth compared to WT in normal conditions, altogether, this new evidence suggests that it is possible that auxin homeostasis is more affected when *ntr* mutants are treated with Al

than when WT plants are treated with Al. According to this new evidence, the disruption of auxin homeostasis would be greater in *ntr* mutants treated with Al compared to WT treated with Al, which could explain the greater Al-induced root growth inhibition in *ntr* mutants compared to WT. Based on this new evidence and the findings from the present study, a model is proposed to show the possible link between NTRs and Al in root growth inhibition (Figure 5-1).

In order to better understand the putative role of NTRs in Al and Cd tolerance, further studies could focus on the interactions of NTRs with other thiol-disulfide oxidoreductases such as GPXs. An approach that could yield valuable information would be using multiple mutants in *Arabidopsis* defective in NTRs and other thiol-disulfide oxidoreductases. The redox status in the mutant backgrounds could be studied using redox-sensing green fluorescent protein constructs (Jiang *et al*., 2006). These constructs could also provide information about the antioxidant capacity of overexpression lines.

In summary, the present study has provided the first evidence that NADPH-thioredoxin reductases are involved in the response of yeast and *Arabidopsis* to Al and Cd stress. This study has also shown that the role of NTRs is not unique to Al-induced stress, since NTRs in yeast and *Arabidopsis* respond in a similar manner to both Al and Cd stress. The evidence, however, is not conclusive as to what the exact role of NTRs is in protecting both yeast and *Arabidopsis* from Al and Cd stress. In yeast it seems plausible that the role of NTRs might include direct scavenging of ROS to avoid or reduce Al-and Cd-induced oxidative damage such as lipid peroxidation. Such a direct role in scavenging of Al- and Cd- induced ROS does not appear to be important in

Arabidopsis. I hypothesized that overexpression of NTRs would increase the antioxidant capacity of *Arabidopsis*, however, this does not seem to occur when comparing the levels of lipid peroxidation between transgenic lines and control plants. These results indicate that NTRs are not ideal candidates for an overexpression approach when attempting to engineer plants with increased antioxidant capacity and increased tolerance to Al and Cd. The findings from the current study, however, increase our understanding of the complex nature of the plant's response to Al and Cd stress and may help in designing future strategies to understand the role of antioxidant enzymes, particularly those based on thiol-disulfide oxidoreductases, in Al and Cd tolerance.

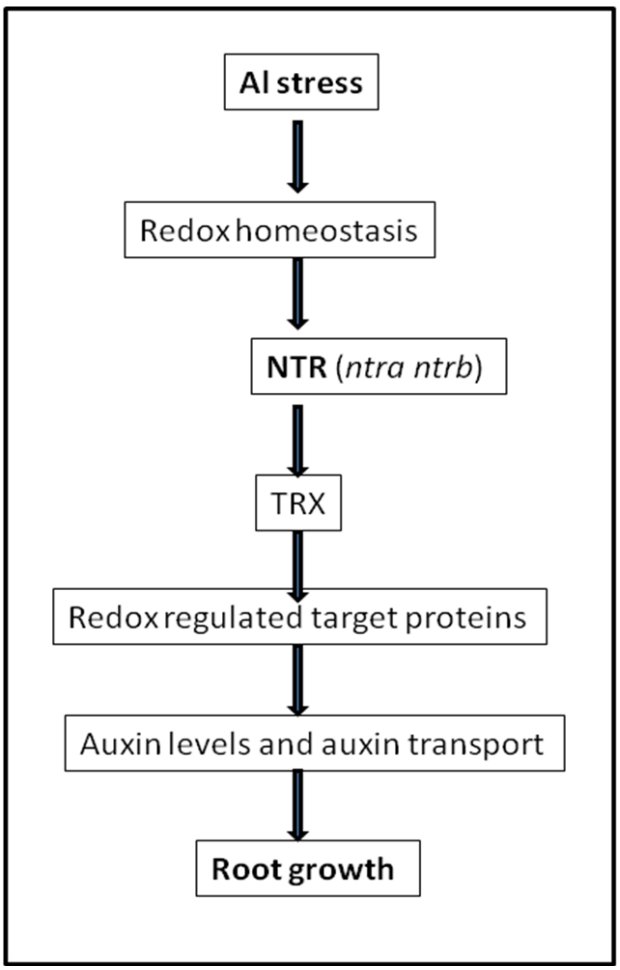

Figure 5-1. Hypothetical model illustrating the link between Al and NTRs in controlling root growth through modulation of auxin homeostasis (adapted from Bashandy *et al*. 2010 and Sun *et al*. 2010). In this model, modification of redox homeostasis induced by Al is relayed by NTR. Inactivation of NTR results in decreased reduction of TRX and subsequently of TRX target proteins. Mis-reduction of target proteins in *ntr* mutants may lead to perturbation of auxin metabolism and affect root growth. Al-induced disruption of auxin polar transport leads to increased inhibition of root growth.

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