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Transcriptome and proteome based survey to identify aluminum-responsive genes in roots of *Arabidopsis thaliana*

by

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Dedication

This thesis is dedicated in memory of my father Late Mr. Gulzari L Bhuttay who always wanted a doctor in the family and to my husband Rajesh for his unconditional support.

Abstract

Aluminum (Al) stress is a major limitation to crop productivity on acidic soils. To help understand the cellular mechanisms underlying the toxicity and resistance of plants to Al, this thesis involved a large-scale, transcriptomic and proteomic analysis of roots of *Arabidopsis thaliana* and reports on comparative analysis of transcriptome and proteome of Al stress responses. Using a microarray representing ~93% of the predicted genes in Arabidopsis, a relatively small proportion (3%) of transcripts were detected as Al-responsive. More changes in the transcriptome were detected after long-term (48 h; 1,114 genes), than short-term (6 h; 401 genes) with relatively little overlap of transcripts detected for each time point. These results suggest that Al toxicity is progressive over time and poses some unique challenges to plants. Further, using two dimensional differential in gel electrophoresis (DiGE), 12 (6 h) and 17 (48 h) proteins were found differentially abundant after Al exposure. Most of the identified proteins were involved in primary metabolism and oxidative stress. Cytosolic-malate dehydrogenase (cyt-MDH) was one of the novel Al-responsive protein identified in this study. Transcript abundance of cyt-MDH correlated well with protein abundance, suggesting that cyt-MDH is regulated in part at transcriptional level. Furthermore, homozygous *mdh-1* and *mdh-2* mutants were more resistant to Al as compared to WT suggesting that regulation of cyt-MDH could play a role in Al resistance. In general, comparative analysis of proteomics data and transcriptomics data showed a poor correlation for both 6 h ($r^2 = 0.155$) and 48 h ($r^2 = 0.083$).

The potential role of five class III peroxidases (*PER2, PER27, PER34, PER42, PER69*) in resistance of roots to Al was explored using quantitative reverse transcriptase PCR and a reverse genetics approach. A diverse range of patterns of transcript abundance was detected using QRT-PCR in response to Al. Furthermore, *per2, per21, and per69*

mutants showed greater increases in root lengths as compared to WT after Al stress suggesting that regulation of *PER* might play a role in Al resistance. These results contribute to the identification of candidate genes for the generation of Al-resistant transgenic plants.

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List of Abbreviations

2-3BPGM,	bi-phosphoglycerate mutase
4CL,	4-coumarate:coa ligase
ABC,	ATP cassette binding transporters
AGI,	Arabidopsis Genome Initiative
AKT2,	protein kinase B
ALMT1,	aluminum activated malate transporter
ALAAC,	aluminum activated anion channels
ADH,	alcohol dehydrogenase
Al,	aluminum
AP2/EREBP,	apetala-2/ ethylene-responsive element binding proteins
ABRC,	Arabidopsis Biological Resource Center
APX,	ascorbate peroxidase
ABC transporters,	ATP binding cassette transporter
ATCIMS,	cobalamin-independent methionine synthase
BHLH,	basic helix-loop-helix
COMT,	Caffeic acid O-ethyltransferase
CEC,	cation exchange capacity
C2H2	Cys2- His2
cDNA,	complementary DNA
CS,	cysteine synthase
C2C2-GATA,	GATA sequence binding transcription factor
DEZ,	distal elongation zone
DAG,	diacylglycerol
DIGE,	differential in-gel electrophoresis

DLD, dihydrolipoamide dehydrogenase

DSP, dual Specific Phosphatases

DTT, dithiothreitol

ECA, ER-type Ca^{2+} pump

eFP, electronic fluorescent protein

EST, expressed sequence tag

FDR, false discovery rate

FBP, aldolase, fructose biphosphate aldolase

GSH, tripeptide glutathione

GH, glycoside hydrolases

HSP, heat shock protein

HAS, hydroxyaluminosilicates

IAA, indole acetic acid

IP_3, inositol-1,4,5-triphosphate

IEF, isoelectric Focusing

LTP, lipid transfer protein

ME, malic enzyme

MIAME, minimum information about microarray experiment

MT, microtubule

m-MDH, mitochondrial malate dehydrogenase

MDHAR, monodehydroascorbate reductase

OASTL, o-acetylserine thiol lyase enzyme

OA, organic anion

PER, class III plant peroxidase

PME, pectin methylesterase

PPP, pentose phosphate pathway

PPX-1, phosphoprotein phosphatase

PA, phosphatidic acid

PIP_2, phosphatidylinositol-4,5-bisphosphate

PLC, phospholipase C

PL, phospholipids

PM, plasma membrane

PMSF, phenylmethylsulfonyl fluoride

PP2C, protein phosphatase 2C

PDC, pyruvate dehydrogenase complex.

PVPP, polyvinylpyrrolidone

QRT-PCR, quantitative reverse transcription PCR

QTL, quantitative trait loci

RIL, recombinant inbred line

ROS, reactive oxygen species

ST, serine/threonine phosphatases

SL, steryl lipids

SCS, succinyl-coa ligase

SOD, superoxide dismutase

TCA, tricarboxylic acid

T-DNA, transfer-DNA

TEMED, tetramethylethylenediamine

TRX, thioredoxins

TZ, transition zone

UBC, ubiquitin-conjugating enzyme

UTR, un-translated region

WAK, wall-associated receptor kinase

WAK1,	wall associated kinase-1
WAKL,	WAK-like kinase
WU-BLAST,	Washington university BLAST
XTHs,	xyloglucan endotransglycosylases

1. Introduction

1.1. General

Abiotic and biotic stresses are integral components of natural ecosystems. Plants have unique abilities to cope with various stresses, despite being sessile. Although plants have evolved mechanisms to avoid or tolerate abiotic stresses, these still remain a major factor limiting crop yields (Boyer 1982, Bray *et al.* 2000). Fifty percent of world's agricultural soils are acidic (Von Uexküll and Mutert 1995). In these soils, acidity, aluminum (Al) toxicity, and reduced availability of some essential nutrients can reduce crop yields (Matsumoto 2000, Kochian *et al.* 2004). With changes in climate expected in the future, further reductions in crop yields may be anticipated (Olesen and Bindi 2002; Brouder and Volenec 2008). Therefore, development of stress resistant plants by engineering resistant genotypes has attracted much attention. Development of high throughput technologies such as microarrays has helped to characterise genome-wide screening of genes in responses to various stresses (Vij and Tyagi 2007). Similarly, proteomics based tools, such as 2D gel electrophoresis (DiGE), have helped to characterize proteins associated with stress and development processes in plants (Amme *et al.* 2006, Chivasa *et al.* 2006, Hotte and Deyholos 2008). In this thesis, these tools have been selected to identify transcriptomic and proteomic based responses to Al stress in roots of Arabidopsis (chapter 2 and chapter 3). These two data sets helped to focus on some candidate genes (chapter 3 and chapter 4) to better understand Al toxicity and resistance.

1.2. Aluminum: distribution and complex chemistry of ionization

Aluminum is the most abundant metal present in the earth's crust. Most Al in the earth's crust occurs naturally as aluminosilicates and bauxite and has low bioavailability.

However, upon acidification, Al ionizes to a toxic form Al^{3+} that is absorbed by plant roots. At pH 7.4 and above, $Al(H_20)_2(OH)_4^-$ dominates, upon acidification the relatively insoluble $Al(H_20)_3(OH)_3$ becomes predominant. As pH further decreases, $Al(H_20)_4(OH)_2^+$ and $Al(H_20)_5(OH)^{2+}$ predominate. At pH <5.0, Alexists as octahedral hexahydrate $Al(H_2O)_6^{3+}$, conventionally called Al^{3+} or monomeric Al (Kinraide 1991). When solution is partially neutralized, polynuclear species of Al can also form such as triskaideaaluminum $(AlO_4Al_{12}(OH)_{24}(H_2O_2)_{12}^{7+}$; commonly known as Al_{13} (Bertsch and Anderson 1989).

Kinraide and Parker (1989) demonstrated that Al solutions contain mononuclear species if $[Al^{3+}]/[H^+] \leq 10^{8.8}$. At values above $10^{8.8}$, significant formation of Al_{13} or precipitation occurs. Monomeric Al can bind to oxygen donor ligands such as carboxylate, phosphate, and sulfate groups. Thus, Al^{3+} forms low molecular weight complexes with organic acid anions (OAs), inorganic phosphate, polyphosphate, and sulfate. Equilibrium constants are available for many the prominent reactions involving Al. Computer programs such as eALCHEMI, GEOCHEM, MINEQL$^+$ (Schecher and Driscoll 1987, Schecher and McAvoy 1992, Parker *et al.* 1995) use these equilibrium constants to predict concentrations and activities of Al species in a defined solution.

A considerable body of research has sought to determine which form(s) of Al, is (are) the most rhizotoxic species (Alva *et al.* 1986, Parker *et al.* 1988, Kinraide and Parker 1989). Kinraide (1991) concluded that Al toxicity might be limited to polyvalent Al ions with charge ≥ 2 suggesting that Al^{3+} and Al_{13} are the most rhizotoxic forms of Al.

1.3. Aluminum toxicity: anatomical, physiological, and biochemical basis

Exposure and uptake of Al in plants results in interaction of Al with the cell wall,

plasma membrane and several symplastic targets including the cytoskeleton, proteins and DNA. These interactions result in rapid inhibition of root elongation.

1.3.1. Cellular uptake of aluminum: There has been considerable debate about the movement of Al at the cellular level. Several authors have speculated that Al may not appear in the protoplasm immediately upon exposure (Rengel and Elliot 1992, Marienfeld *et al.* 1993). The cell wall was considered to be a large sink for Al that restricted the access of polyvalent ions to the membrane surface. In addition, it was suggested that cationic Al species may not move readily across the plasma membrane. Thus, the importance of extracellular injury in plant response to Al was speculated (Reid *et al.* 1996). Also, there has been debate over the passive or active binding of Al in the apoplasm. Most authors suggest that Al binds passively. Uptake of Al, in contrast, has also been suggested to include metabolism dependent binding of Al in the apoplasm (Zhang and Taylor 1990).

Direct evidence of Al transport across a plasma membrane came from the pioneering work of Taylor *et al.* (2000). These authors reported that Al is capable of crossing biological membranes within minutes of exposure using the rare ^{26}Al isotope. Aluminum was reported to accumulate in to the vacuole after 30 min of exposure and Al uptake saturated in the cell wall, avacuolate protoplasm, and vacuole within 12 to 24 h of exposure. One hypothesis suggests that entry of Al into the symplast occurs through weakly selective ion channels. For instance cationic Al^{3+} is similar in ionic radius to Fe^{3+} and Mg^{2+}, therefore it has been postulated that Al can enter the symplasm through cation channels that would normally permeate Fe^{3+} or Mg^{2+} (Martin 1988). This hypothesis is supported by the observation that exposure to Al inhibits Mg^{2+} transport (MacDiarmid and Gardner 1998). In bacterial systems, Al is thought to be transported via siderophores

(high-affinity iron chelating compounds), which can absorb and transport Al^{3+} (Winkler *et al*. 1986). Another possible mechanism of uptake of Al is through endocytosis (Akenson *et al.* 1989a and 1989b). Aluminum can be adsorbed tightly to the surface of plasma membrane which may result in depolarization and flipping of membrane (Akenson *et al.* 1989a, 1989b). Ezaki *et al.* (2007) recently showed that inhibition of endocytosis in root hair cells of Arabidopsis results in Al resistance using an enhancer-tagged mutant line, over- expressing the F9E10.5 gene. F9E10.5 gene encodes an auxilin-like protein related to the clathrin uncoating process in endocytosis. These studies provide some insight into the mechanism of Al uptake; however in the absence of direct evidence, these mechanisms remain speculative. Previous reports have suggested that the majority of absorbed Al (45-99.9%) is located in the apoplast (Zhang and Taylor 1989 and 1990, Tice *et al.* 1992, Rengel and Reid 1997, Taylor *et al.* 2000). The greatest accumulation of Al is proposed to occur in the first 30 µm (two to three cell layers), and elevated levels of Al can reach at least 150 µm inward from root border (Lazof *et al.* 1994, 1996). Recently, Babourina and Rengel (2009) used fluorescent lifetime imaging analysis to demonstrate that the primary sites for Al^{3+} entry in roots of Arabidopsis are the meristem and distal elongation zones. They reported that intracellular concentrations of Al can increase from 0.4 µM to 100 µM Al within the first 3 h of exposure. Since substantial accumulation of Al in the root cell layers can occur as early as 30 min, these results correlate with direct measurement studies of Al uptake (Taylor *et al.* 2000) and with early effects of Al, such as inhibition of cell extension and cell division, that occur within several minutes after exposure.

1.3.2. Inhibition of root elongation: Aluminum begins to inhibit root elongation within minutes after exposure. In maize, Al-induced inhibition of root elongation occurs within

30 min (Llugany *et al.* 1995). Ryan *et al.* (1993) reported that exposure of the distal 2-3 mm of the root (root cap and meristem) is sufficient to inhibit root elongation. Although the root cap was hypothesized to be involved in signal perception and hormone distribution in response to Al (Bennet and Breen 1991), decapped roots were found to be equally sensitive to Al as intact roots (Ryan *et al.* 1993), suggesting the importance of the root meristem in Al toxicity (Delhaize and Ryan 1995). Sivaguru and Horst (1998) improved upon the experiments of Ryan *et al.* (1993), exposing 1 mm apical root zones of intact maize seedling to Al. In addition to measuring growth, they measured induction of callose formation and accumulation of Al in 1 mm sections along the root apex. They found that the 1 to 2 mm root zone had the highest levels of Al accumulation and Al-induced callose formation. Both observations were independent of whether Al was applied to individual apical root zones or uniformly to the whole-root apex (Sivaguru and Horst 1998). In maize, the 1-2 mm apical region corresponds to transition zone / distal elongation zone (TZ/DEZ) where the cells undergo a preparatory phase for rapid elongation (Baluska *et al.* 1996). Sivaguru and Horst (1998) concluded that the TZ/DEZ is the most sensitive region of root apex, however they did not rule out the importance of the meristematic zone in contributing to Al toxicity as suggested by Ryan *et al.* (1993), although it accumulated less Al. The reason for preferential Al accumulation in the TZ compared with the meristmatic zone (0–1 mm) and elongation zone is not known (Sivaguru and Horst 1998), however, reduced Al uptake by the meristmatic zone might be due to excretion of mucilage, which strongly binds Al (Archambault *et al.* 1996).

Considerable debate has focused on the question of whether Al mediated inhibition of root growth is due to inhibition of cell division or inhibition of cell elongation. Several reports indicate that Al inhibits cell division. For example, Clarkson (1965) reported that blockage of cell division is the primary mode of Al toxicity, which was suggested by the

close relationship between cessation of root elongation and disappearance of mitotic figures. Cell division, however, is a slow process (cell cycles in plant roots take about 24 h) and cell division accounts for only 1-2% of the overall root elongation. Since inhibition of root elongation occurs within 1-2 hours of Al treatment, it has been suggested that both cell division and cell elongation are affected. Sasaki *et al.* (1997) reported that cell length decreased and diameter of the roots increased after wheat roots were exposed to Al. This was accompanied by reduced viability of cells in the elongation zone. Also, lignin deposition and disruption of microtubules were detected in cells whose elongation was inhibited (Sasaki *et al.* 1996). These studies imply that inhibition of cell elongation is an important component of Al stress.

1.3.3. Cell wall: To better understand the mechanism of Al toxicity, it is important to determine whether the primary target of Al is apoplastic (cell wall and periplasmic space) or symplastic (plasma membrane and components of cytosol). The cell wall has a negative charge that contributes to cation exchange capacity (CEC) on the root surface. The carboxy group of pectin (Blamey *et al.* 1993, Horst *et al.* 1999) and apoplastic phosphate (Marienfeld and Stelzer 1993, Zheng 2005) thus act as barriers to the movement of Al into roots (Millard 1990). Van *et al.* (1994) reported an increase in the pectin-fraction of cell walls after Al exposure, suggesting a protective mechanism. In contrast, immuno-localization studies in maize (Eticha *et al.* 2005) and rice (Yang *et al.* 2008) detected higher proportions of low methylated pectins in Al-sensitive cultivars compared to Al-resistant cultivars. Furthermore, Yang *et al.* (2008) detected higher activity of pectin methylesterase (PME) in the cell wall of an Al-sensitive cultivar of rice compared to a resistant cultivar. Higher activity of PME indicates a higher degree of demethylesterification and thus the presence of higher proportion of free pectic acid

residues in the cell wall that should result in a binding of Al in sensitive cultivars. Blamey *et al.* (1990) reported that less Al accumulates in a resistant variety of *Lotus* (compared to a sensitive variety) that was characterised by low CEC of the cell wall. Blamey *et al.* (1993) reported an instantaneous decrease in water permeability of an artificial Ca-pectate membrane after exposure to Al. Binding of Al to Ca-pectate in cell walls makes the cell wall more rigid. Ma *et al.* (2004) reported that Al caused a significant decrease in both the viscous and elastic extensibility of cell walls of the root apices of Al-sensitive cultivar of wheat. Also, binding of Al made cell walls brittle as the "break load" of the cell was decreased with Al exposure. To reduce cell wall extensibility, it was concluded, that Al must interact with the cell walls of actively elongating cells. Slow elongating cells did not show Al-dependent decrease in cell wall extensibility (Ma *et al.* 2004). This supports the previous reports that exposure of Al to DEZ (distal elongation zone) alone is sufficient to reduce root elongation (Sivaguru and Horst 1998). Together these reports suggest that constituents of the cell wall, activity of cell wall modifying enzymes (PME) and maintenance of cell wall extensibility play important role in Al stress resistance.

1.3.4. Plasma membrane: Although most absorbed Al is associated with apoplastic regions of the root, a small proportion enters into the symplasm and interacts with the plasma membrane (PM) and other intercellular targets (Taylor *et al.* 2000). Binding of Al to phospholipids on the PM may decrease fluidity of membrane (Deleers *et al.* 1985, Shi and Haug 1988, Akeson *et al.* 1989b). Zhang *et al.* (1997) showed increases in phospholipids (PL), and decreases in steryl lipids (SL) in Al resistant plants after Al stress. These changes were reported as specific to the resistant genotype, thus suggesting that if a decrease in membrane lipid fluidity is one of the toxic lesions induced by Al,

then Al-induced changes that are capable of improving membrane fluidity (such as increase in PC and decrease in SL) may constitute an adaptive response to Al stress.

Exposure to Al is known to depolarize plasma membranes (Olivetti *et al.* 1995, Takabatake and Shimmen 1997). This may be due to binding of Al to membrane surfaces (Akeson *et al.* 1989b). For instance, 5 µM Al^{3+} was reported to neutralize the surface charge of the plasma membrane and cause a shift in surface potential from -30 to +11mV. Kinraide *et al.* (1994) developed a model to predict interactions between Al and other cations on the plasma membrane. This model suggests that the negatively charged membrane surface provides a strong attractive force for trivalent cations such as Al^{3+}. This altered charge of cell walls and membranes (due to binding of Al^{3+}) was proposed to decreased infux of cations (such as Ca_2^+, NH_4^+, and K^+) and increased influx of anions (such as NO_3^-, and $PO4_3^-$), which has been observed in an Al sensitive cultivar of barley after exposure to Al (Nichol *et al.* 1993). In contrast to accumulation of Al in the cell wall and protoplasm, (where accumulation of Al is strongly influenced by the concentration and speciation of Al in uptake solutions), no clear relationship was reported between the rate of transport across the plasma membrane and predicted Al^{3+} in exposure solutions (Taylor *et al.* 2000). However, increased rates of transport of Al reported in the presence of SO_4 and citrate suggest that their complexes with Al ($AlSO_4$, Al:citrate) may be readily transported across the plasma membrane (Taylor *et al.* 2000). Several authors have suggested that Al:citrate may provide a means by which Al may pass through biological membranes (Martin 1988, Kochian, 1995).

Interestingly, the lipid composition of the plasma membrane may play an important role in Al resistance. Changes in the spingholipid composition of cell membranes is proposed to protect plants from Al stress (Ryan *et al.* 2007). Recently, an Al sensitive cultivar of rice showed increased permeability of the PM and greater Al

uptake as compared to a resistant cultivar. However, comparison of the composition of the PM showed a lower ratio of phospholipids to delta(5)-sterols in the resistant cultivar (Khan *et al.* 2009). Altering this ratio using inhibitors of delta(5)-sterols biosynthesis makes resistant plants susceptible to Al stress, suggesting that lipid composition of the PM is a factor underlying variations in Al resistance among rice cultivars (Khan *et al.* 2009), which could not be explained by efflux of organic anions from root apices to chelate toxic Al^{3+} in the rhizosphere (Ma *et al.* 2002).

One prospective physiological marker for measuring Al toxicity is Al-induced callose ((1-3) β-D-glucan) formation in root apices (Wissemeier *et al.* 1992, Bhuja *et al.* 2004, Hirano *et al.* 2006). Callose is synthesized by (1-3) β-D-glucan synthase located on the plasma membrane. One of the prerequisites for the induction of callose formation is an increase in cytoplasmic Ca^{2+}, which acts as signal to activate (1-3) β-D-glucan synthase. Interestingly, exposure to Al increases cytoplasmic Ca^{2+} levels in root hairs of Arabidopsis (Jones *et al.* 1998) and roots apices of wheat (Zhang and Rengel 1999). Aluminum induced depolarization of the plasma membrane along with increased Ca^{2+} levels results in Al-induced callose synthesis in wheat (Bhuja *et al.* 2004) and tobacco (Sivaguru *et al.* 2005). Deposition of callose is a rapid process (Stass and Horst 1995), which is positively related to Al concentration under both laboratory and field conditions in coniferous trees (Wissemeier *et al.* 1998, Hirano *et al.* 2004, Nagy *et al.* 2004). Deposition of callose, in wheat root apices, was several fold higher in DEZ and was found localized in the plasmodesmata (PD), which could block symplastic transport and cell-to-cell communication (Sivaguru *et al.* 2000).

1.3.5. Cytoskeleton: Inhibition of root elongation and the swelling of root tips and root hairs indicate a possible alteration in cytoskeletal structures of root cells upon exposure to

Al. Cytoskeleton proteins include microfilaments (comprised of actin), intermediate filaments, and microtubules (MT; made up of tubulin). Microtubules orient transversely in elongating cells. Disruption of MTs (using MT blocking agent) results in lateral expansion, while inhibiting longitudinal expansion. Sasaki *et al.* (1997b) observed a disappearance of cortical MTs in elongating cells of wheat after exposure to Al in a time-dependent manner that was correlated with reductions in root growth. Cortical MTs help to orient cellulose microfibrils, therefore their disruption may affect the rate of cell growth due to changes in cell wall properties (Matsumoto 2000). Sivaguru *et al.* (1999a, b) found that alterations of MTs and microfibrils were most prominent in cells of distal transition zone. Interestingly, this is also the most Al sensitive region of the root apex (Sivaguru and Horst 1998) as meristematic cells leave the division phase and undergo actin-dependent rapid cell elongation (Baluska *et al.* 1992). It appears that the growth phase of cells is important in Al-mediated destabilization of components of the cytoskeleton. For instance, de-polymerization of spindle MTs and cortical MTs was observed in log-phase cells only and was correlated closely with Al-induced inhibition of growth and rapid callose formation (Sivaguru *et al.* 1999). Besides destabilization of microtubules, Al treatment results in increased rigidity within the microfilament network in soybean (Grabski and Schindler 1995). The effect of Al on rigidity and tension of microfilaments has been proposed to occur through displacement of Mg^{2+} by Al^{3+} in Mg^{2+}-ATP and Mg^{2+}-GTP to form complexes that hydrolyse poorly. *In vitro* measurements of $A1^{3+}$-GTP-microtubule complexes demonstrated that such complexes were stable and resistant to calcium mediated depolymerisation (Grabski and Schindler 1995). Since plant cells require dynamic actin- and tubulin-based networks for processes such as cell division and differentiation and cell-wall biosynthesis, Al mediated

disruption and rigidification of cytoskeleton may play important role in cell death (Amenos *et al.* 2009).

1.3.6. Generation of reactive oxygen species (ROS): Aluminum is not a transition metal and therefore cannot directly catalyze redox reactions. Aluminum-induced lipid peroxidation and increased activities of enzymes related to ROS detoxification were first shown by Cakmak *et al.* (1991). Later, Al-induced lipid peroxidation was proposed to be mediated by Fe (Yamamoto *et al.* 2003). Further research suggested oxidative stress to be a component of Al toxicity because Al exposure triggered accumulation of ROS such as superoxide anions (O^{-}_{2}) and hydrogen peroxide (H_2O_2; Kobayashi *et al. 2004*, Hossain *et al.* 2005). Plants can scavenge ROS via enzymatic and non-enzymatic mechanisms. For example, reduced glutathione (GSH) can scavenge ROS non-enzymatically, whereas enzymatic scavenging of ROS is mediated by superoxide dismutase (reduction of O^{-}_{2} to H_2O_2), and catalase, peroxidase, and ascorbate peroxidase (detoxification of H_2O_2 to H_2O). Transgenic plants over-expressing genes of ROS-scavenging enzymes (peroxidase; Ezaki *et al.* 2000, superoxide dismutase; Basu *et al.* 2001) showed improved root elongation under Al stress. In addition, amelioration of Al toxicity by addition of antioxidants provided evidence that inhibition of root elongation could be due to Al-induced oxidative stress (Yamamoto *et al.* 2003). However, time-course experiments showed that inhibition of root elongation could be detected earlier than oxidative stress (Boscolo *et al.* 2003, Liu *et al.* 2008). Therefore, Al-induced oxidative stress could be a secondary response to Al toxicity (Liu *et al.* 2008).

1.4. Signal transduction

Responses to Al stress in roots can occur within minutes after Al exposure, but it remains unknown how an Al signal might be perceived and transduced. The cell wall-associated receptor kinase (WAK) and WAK-like kinase (WAKL) gene family members are good candidates as putative effectors of an Al signal, due to their anchorage between the cell wall and the cytoplasmic compartment (Horst *et al.* 1999, Sivaguru *et al.* 2003, Hou *et al.* 2005). Sivaguru *et al*. (2003) identified an Al-induced wall associated kinase-1 (WAK1) as an early Al-responsive gene whose over-expression enhanced Al resistance. Furthermore, elevated levels of WAK proteins were reported after 6 h Al stress and were found localized abundantly to peripheries of cortex cells within the elongation zone of the root apex. Other reports have suggested versatile roles for WAK-Like proteins in Arabidopsis mineral nutrition responses (Hou *et al.* 2005).

1.4.1. IP_3/DAG pathway: Phospholipase C (PLC) is involved in different signaling processes. This enzyme hydrolyzes membrane bound phosphatidylinositol-4,5-bisphosphate (PIP_2) to inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). While DAG remains bound to the membrane, IP_3 is released as a soluble substance into the cytosol where it binds to IP_3 receptors such as Ca^{2+} channels (Lemtiri-Chlieh *et al.* 2003), to aid in Ca^{2+} release from intracellular stores. Additionally, DAG is phosphorylated by diacylglycerol kinase to form phosphatidic acid (PA) that acts as lipid second messenger in both animal and plants. It has been suggested that Al affects the concentration of PIP_2 (Ramos-Díaz 2007). PIPs are required for the activation of plant shaker-type channels and Al applied to the cytoplasmic side of the membrane binds to PIPs to block the activation of these channels (Liu *et al.* 2005). Also, PLC activity was shown to be inhibited by Al^{3+}

(Jones and Kochian1995, Verstraeten *et al.* 2003). Several reports have suggested that alteration of cytosolic Ca^{2+} levels, as result of Al^{3+} exposure, occurs via PLC and IP_3 (Delhaize and Ryan *1995,* Jones and Kochian *1995,* Piña-Chable and Hernández-Sotomayor 2001; Martínez-Estevez *et al.* 2003). Recently, Ramos-Diaz *et al.* (2007) have shown inhibition of PA formation through the inhibition of PLC activity after Al exposure. One of the major PA binding proteins is PEPC (Testerink *et al.* 2004), whose over-expression results in Al resistance in rice (Begum *et al.* 2009).

1.4.2. MAPK pathway: Recently, Al^{3+} has been shown to activate a MAPK-like protein in coffee cells (Arroyo-Serralta *et al.* 2005). It has been speculated that membrane receptors can bind to Al and act as effectors of signal (Sivaguru 2003), and perhaps via protein phosphorylation this signal is transduced to activate efflux of organic anions (Osawa and Matsumoto 2001, Zhang *et al.* 2001), which is an important component of Al resistance.

1.5. Amelioration of Al toxicity

Toxic monomeric forms of Al bind to negatively-charged ligands such as organic compounds, PM, and the cell wall. Kinraide and Parker (1987) proposed two categories of effective amelioration treatment. The first is the addition of Al-complexing ligands and the second is amelioration by cations. Both of these reduce the activity of Al in solution. The toxicity of Al^{3+} is ameliorated by cations in the following order of effectiveness: $H+\approx C^{3+}>C^{2+}>C^{+}$. For instance, trivalent Al can displace Ca^{2+}, whereas other polyvalent cation ($\geq C^{3+}$) can compete and displace Al^{3+}. The exception is the monovalent cation, H^{+}. The higher effectiveness of H^{+} could be due in part to the small ionic radius of H^{+} as well as high-affinity binding for H^{+} to apoplast, which would further reduce negative surface

charges, thereby reducing $A1^{3+}$ enrichment on the cell surface. In amelioration by divalent cations, Ca^{2+} and Mg^{2+} are particularly effective.

1.5.1. Calcium (Ca) homoeostasis in the cytoplasm is disrupted upon exposure to Al (Rengel 1992) due to inhibition of Ca^{2+} influx (Huang *et al.* 1992) and blockage of Ca^{2+} channels (Rengel and Elliot 1992). In isolated cell walls equilibrated with 50 µM Ca^{2+} (pH 4.4), Al (100 µM) displaced more than 80% of bound Ca^{2+} within 25 min (Reid *et al.* 1995). However the displacement of Ca^{2+} and its reduced influx are not the main cause of Al toxicity (Matsumoto 2000). External supplementation of Ca^{2+} is known to alleviate Al toxicity in plants (Brady *et al.* 1993, Kinraide1998, Hossain *et al.* 2005) by maintaining $[Ca^{2+}]_{cyt}$, thus preventing disturbance of numerous ion transporters that are under Ca^{2+} control (e.g. K+ channels; Tester and MacRobbie 1990, Babourina *et al.* 2005). High supplementation of Ca^{2+} also decreases the negative charge on the surface of the root cell, which can decrease the interaction between Al and the apoplast (Kinraide 1998; Kinraide 2004).

1.5.2. Magnesium (Mg) Aluminum severely affects Mg uptake (Rengel and Robinson 1989, Fowler *et al.* 1999) and translocation in rapidly dividing and expanding cells of the root apex (Silva *et al.* 2001). Over expression of a magnesium transport gene in yeast (ALR; MacDiarmid and Gardner 1998) and tobacco (AtMGT1; Deng *et al.* 2006,) has been shown to alleviate Al toxicity. Silva *et al.* (2001b) argued that the superior effectiveness of Mg^{2+} over Ca^{2+} in terms of alleviating Al toxicity cannot be explained by competition with Al, and speculated presence of other mechanisms. Later, it was found that supplementation of Mg increased the production and exudation of citrate in roots of soybean (Silva *et al.* 2001a) and rice (Yang *et al.* 2007) after exposure to Al. Addition of Mg also increases the activity of PM H^{+}-ATPase, and helps to maintain the Mg and Ca contents in root apices (Yang 2007).

1.5.3. Silicon (Si) is the second most abundant element in the earth's crust. Some Al occurs as non-toxic hydroxyaluminosilicates (HAS; Exley *et al.* 2002, Excely 2003, Quintal-Tun *et al.* 2007). It has been reported that treatment with Si reduces Al uptake, thereby reducing inhibition of root elongation (Corrales *et al.* 1997). Supplementation with Si also increases the efflux of organic anions as well as flavnoid type phenolics (Barcelo *et al.* 1993, Kidd 2001). However, Wang *et al.* (2004) showed that Si supplements did not affect the Al-induced exudation of organic anions and phenols, but instead lead to the formation of HAS in the apoplasm, which would immobilise and detoxify Al.

1.5.4. Boron (B) is also an essential plant nutrient. Amelioration of Al toxicity by supplementation of B has been reported (LeNoble *et al.* 1996a, 1996b, Wojcik 2003, Hossain 2003, Yu *et al.* 2009). Recently, Corrales *et al.* (2008) reported supplementation of B enhanced levels of GSH in roots and reduced Al-induced cell death mediated by oxidative stress.

1.6. Aluminum resistance

Plants have developed resistance mechanisms against Al. Taylor (1991, 1995) proposed two broad categories of Al resistance mechanisms. The first category of resistance is based upon external mechanisms (those which operate within the apoplasm) and the second category of resistance is based upon internal resistance mechanisms (those which operate within the symplasm).

External resistance, also defined as exclusion mechanisms, include processes that limit the rate of entry of A1 into the cytosol including: 1) immobilization of Al at the cell wall or low cell wall CEC, 2) selective permeability of the plasma membrane, 3)

formation of a plant-induced pH barrier in the rhizosphere or root apoplasm, 4) exudation of Al-chelator ligands, 5) exudation of phosphate, and 6) Al efflux. On the other hand, suggested internal resistance mechanisms include: 1) chelation in the cytosol, 2) compartmentation in the vacuole, 3) evolution of Al-resistance enzymes, or 4) elevated enzyme activity. Detoxification of Al by secretion of organic anions (Al-chelator ligands) is the most well studied mechanism of Al resistance.

The first Al-resistance gene identified in plants was *TaALMT1*. This gene is expressed specifically and constitutively in the root apices of Al-resistant variety of wheat (Sasaki *et al.* 2004). The *ALMT1* gene was isolated by subtractive hybridization of cDNA prepared from Al-resistant (ET8) and Al-sensitive (ES8) cultivars of wheat (Sasaki *et al.* 2004). The cDNA was 1,517 bp long and the protein was predicted to be hydrophobic membrane protein with 8 putative trans-membrane regions. Sasaki *et al.* (2004) also reported differences in the sequences of the *ALMT* at both cDNA level (6 nucleotides) and protein level (2 amino acids) from ET8 (named *ALMT1*) and ES8 (named *ALMTl2)* cultivars of wheat**.** The association of *ALMT1* with inheritance of Al-resistance was clarified by analysis of F_2 progenies derived from a cross between near isogenic lines of ET8 and ES8. The Al resistance was measured as increased elongation of root lengths of seedlings in Al containing medium. This was followed by QRT-PCR to quantify *ALMT1* transcript levels in all seedlings. Interestingly, Al-resistant seedlings (showing increased root lengths) expressed either *ALMT1* or both *ALMT1* and *ALMT2,* but all Al-sensitive seedlings expressed only *ALMT2*, suggesting that Al-resistant phenotype correlate with *ALMT1* expression (Sasaki *et al.* 2004). Electrophysiological measurements of currents in *Xenopus laevis* injected with *ALMT1*cRNA provided evidence that ALMT1 is a membrane transporter. In these experiments, the addition of Al in bathing solution activated an inward current (consistent with anionic efflux) in oocytes that were injected

with *ALMT1* cRNA and malate, but not in control oocytes that were injected with water and malate. Further it was shown that except for Al, other trivalent cations did not activate this inward current. Also, replacing malate with citrate did not activate Al-dependent inward current (Sasaki *et al.* 2004). Together, these results provided evidence that *ALMT1* encodes and Al-activated malate transporter. Furthermore, the transgenic tobacco culture cells, over-expressing ALMT1, accumulated less Al than control cells and also had a greater capacity for re-growth after A1 treatment (Sasaki *et al.* 2004). Further, in transgenic barley (harboring the *ALMT1* gene), treatment with $A1^{3+}$ produced almost no lesions on the root apex (Delhaize *et al.* 2004). This was pioneering work in the field because it represented the identification of the first major Al resistance gene in crop plants and opened new avenues of research in the field (Kochian *et al.* 2004).

The second Al-resistance gene isolated in plants was an Al-activated citrate transporter in barley and sorghum (Furukawa *et al.* 2007, Magalhaes *et al.* 2007). This gene was identified using a fine mapping approach.

In barley, Ma *et al.* (2004) identified a major quantitative trait locus (QTL) for Al-induced secretion of citrate. This QTL was flanked by microsatellite markers Bmac310 and Bmag353 on the long arm of chromosome 4H. Additionally, this locus for Al-induced secretion of citrate was found to map to the same region that was previously identified as Al-resistance locus (*Alp*; Minella and Sorrells 1992). These data suggested that Al resistance in barley is primarily controlled by the secretion of citrate. By genotyping F_4 lines derived from a cross between sensitive and resistant plants of barley, Furukawa *et al.* (2007) delimited the region between microsatellite markers Bmac310 and Bmag353 to an equivalent to approximately 140 kb of the rice genome containing 21 annotated gene models. Further, they performed a microarray analysis with Barley 1 GeneChip (Affymetrix) that contained 25 mapped genes between the markers Bmac310

and Bmag353. Among these 25 genes only one gene was up-regulated by >20-fold in the resistant cultivar, irrespective of Al treatment. This gene was later cloned and named aluminum-activated citrate transporter (*HvAACT*). Using the approach of Sasaki *et al.* (2004), Furukawa *et al.* (2007) showed that oocytes expressing HvAACT1 had enhanced efflux activity for citrate compared with oocytes not expressing HvAACT1, and HvAACT1 had transport activity for citrate, but not for malate. Also, over-expression of this gene in tobacco resulted in enhanced Al-activated secretion of citrate and Al resistance.

In sorghum, a major aluminum resistance locus, AltSB, was mapped to the terminal region of chromosome 3 (Magalhaes *et al.* 2004). Using high-resolution mapping, Magalhaes *et al.* (2007) localized the AltSB locus to a 24.6-kb region of a sorghum BAC 181g10 where only three predicted candidate ORFs were present. One of the ORF represented members of the multidrug and toxic compound extrusion (MATE) transporter family. Sequence comparison showed that the *SbMATE* was not related to the *ALMT* family of membrane proteins and thus was a new candidate resistance gene. *SbMATE* was found to be Al inducible and expressed primarily in the root apices of Al-tolerant lines. Furthermore, over-expression of *SbMATE* in transgenic Arabidopsis plants conferred a significant increase in Al resistance and root citrate exudation.

In Arabidopsis, *AtALMT1* (a functional homologue of *TaALMT*) facilitates Al-activated malate exudation (Hoekenga *et al.* 2006, Magalhaes *et al.* 2006). A putative zinc finger transcription factor, STOP1, is found to regulate several Al responsive genes (Sawaki *et al.* 2009) and Al resistance (Iuchi *et al.* 2007). It has been demonstrated that both Al-activated citrate exudation (facilitated by AtMATE), and Al activated malate exudation (facilitated by AtALMT1) have evolved independently of each other in Arabidopsis, and together confer expression of Al resistance (Liu 2009). Previously, it

was speculated that Al-activated anion channels (ALAAC) are ABC proteins (Ryan *et al.* 2001; Zhang *et al.* 2001); however, recent reports suggest these are members of the MATE class of membrane proteins (Liu *et al.* 2009).

1.6.1. Detoxification of Al by secretion of organic anions: Detoxification of Al by secretion of OAs was first suggested by Kitagawa *et al.* (1986) and the first convincing data on the relationship between Al resistance and OA secretion was presented by Delhaize *et al.* (1993). Secreted organic anions are believed to chelate and detoxify Al^{3+} at the root apex, which is considered the most sensitive region for Al stress. Exudation of organic anions is cultivar and species-specific (Table 1.1) and the most common organic anions secreted in response to Al stress include citrate, malate, and oxalate (Basu *et al.* 1994, Ma *et al.* 1997, Ma 2000, Ma *et al.* 2001, Ryan *et al.* 2001, Kochian *et al.* 2004, Kinraide *et al.* 2005). These di/tri-carboxylic organic anions form stable complexes with Al^{3+}, thus reducing the availability, concentration, and toxic effects of Al. The complex of Al-citrate (1:1) and Al-oxalate (1:3) have been identified in Hydrangea and buckwheat; respectively (Ma 2000). Although oxalate can form three different complexes with Al (1:1, 1:2, and 1:3), it is the 1:3 Al-oxalate complex that is the most stable. Its stability constant (12.4) is higher than that of Al-ATP, meaning that the formation of the 1:3 Al-oxalate complex can prevent binding of Al to cellular components (Ma *et al.* 1998). Ma (2000) proposed two patterns of Al-induced secretion of OAs: In pattern I, organic anions are released immediately after exposure to Al. In pattern II, there is a lag between the addition of Al and the inception of OA release. The delay in external release of OA in pattern II could be due to transcriptional and post-transcriptional regulation of genes involved in synthesis of organic acids, whereas the immediate release of OA (pattern I) could reflect activation of OA transporters.

Although exudation of OAs is considered an important resistance mechanism, emerging evidence suggests that other uncharacterized mechanisms of Al resistance exist as well. For instance, Wenzl *et al.* (2001) demonstrated that OA secretion does not account for Al resistance in the highly resistant species of signal grass (*Brachiaria decumbens*). Similarly, citrate efflux could not explain the difference in Al resistance in some maize cultivars (Piñeros *et al.* 2005). In buckwheat, where oxalic acid secretion contributes to the high resistance to Al, the variation in Al resistance between sensitive and resistant cultivars of buckwheat is not dependent on oxalic acid (Zheng *et al.* 2005).

1.6.2. Genetics of aluminum resistance: Genetic inheritance of Al resistance in crop plants has been an important area of research for many years. Resistance to Al toxicity varies widely among cereal crops, with barley and durum wheat considered the most susceptible cereal crop (Garvin and Carver 2003). The Al resistance order, has been reported as maize>rye>triticale>wheat>barley (Polle and Konzak 1985), rye>oats>millet>bread wheat>barley>durum wheat (Bona *et al.* 1993), and rice>maize>pea>barley (Ishikawa *et al.* 2000).

1.6.2.1. Comparative genetics of aluminum resistance in cereals:

Wheat (*Triticum aestivum*): Aluminum resistance in wheat cultivars has been found to be under the control of a single dominant locus (Delhaize *et al.* 1993, Riede and Anderson 1996, Luo and Dvorak 1996) whereas in other crosses, segregation patterns suggest that two loci are responsible for resistance (Garvin and Carver 2003, Kochian 2004). The loci identified to date are *Alt1* and *Alt2*. Delhaize *et al.* (1993) demonstrated that the *Alt1* locus explains most of the differences in Al resistance between isogenic wheat lines. The *Alt2* locus had been mapped to the long arm of chromosome 4D (Luo

and Dvorak 1996, Riede and Anderson 1996). The recently identified *ALMT1* gene (Sasaki *et al.* 2004) is suggested to correspond to *Alt1* (Raman *et. al.* 2005, Jardim 2007). The majority of variation in Al resistance observed between wheat cultivars has been suggested to exist within a single physiological mechanism of root OA secretion (Kochian *et al.* 2004).

Rye (*Secale cereale*): Four separate loci are related to Al resistance in rye. These are: *Alt1, Alt2, Alt3, and Alt4* located on chromosome 6RS, 3RS, 4RL, and 7RS respectively (Aniol and Gustafson, 1984, Gallego *et al.* 1998, Miftahudin *et al.* 2005, Matos *et al.* 2005). Recently, a wheat *ALMT1* homolog in rye (*ScALMT1)* was cloned and mapped to the previously identified locus *Alt4* (Fontecha *et al.* 2007). In rye, the *ScALMT1* gene co-segregates with the Al resistance phenotype.

Sorghum (*Sorghum bicolour*): In sorghum, a major Al resistance locus, Alt_{SB} was mapped to chromosome 3. Its position is not orthologous to Triticeae chromosomal 4 (which is the region of the *Alt1* gene in wheat), instead it maps to a homeologous region of wheat chromosome 3, and chromosome 1 in rice (Jardim 2007). QTLs associated with Al resistance have also been mapped on these chromosomes (Magalhaes *et al.* 2004).

Barley (*Hordeum vulgare*): Barley also contains a Al resistance locus, *Alp*, on chromosome 4 (Minella and Sorrells 1992). However, unlike wheat and rye, barley is very sensitive to Al toxicity. The major Al resistance locus, *Alp* perhaps imparts resistance to only low levels of stress (Minella and Sorrells 1992). Although *Alp*, *Alt3* (rye), and *Alt2* (wheat) are suggested to be orthologous loci, it remains to be determined how a wide range of Al resistance phenotypes is achieved within these species (Kochian *et al.* 2004).

1.6.3. Quantitative inheritance of aluminum resistance

Rice (*Oryza sativa*): Molecular mapping of genes conferring Al resistance in rice suggested that Al resistance is a complex multigenic trait. Quantitative Resistance Loci (QTL) have been identified on all 12 rice chromosomes (Wu *et al.* 2000, Nguyen *et al.* 2001, 2002, 2003, Mao *et al.*2004). The major QTLs were detected on chromosomes 1 and 12 (Wu *et al.* 2000), on chromosomes 1, 2, and 6 (Ma *et al.* 2002), or on chromosomes 1 and 8 (Nguyen *et al.* 2002). However, chromosome 1, which was common to all these studies, does not contain Al genes that had been mapped in other species (Nguyen *et al.* 2001) mainly because chromosome 4 of the *Triticeae* (where Al resistant loci were identified) is homeologous to chromosome 3 in rice. However, in later work a major QTL explaining 24.9% of the phenotypic variation was found on chromosome 3 of rice, which is conserved across cereal species (Nguyen *et al.* 2003).

Oats (*Avena strigosa*): In oats, four QTLs were identified for Al resistance explaining 55% of phenotypic variation (Wight *et al.* 2006). The major QTL was associated with a marker linked to the *Alt2* locus in wheat. Therefore, it is likely that this genomic region in oats contains the gene that is ortholog to the main Al resistance gene identified in the Triticeae (Jardim 2007).

Arabidopsis *(Arabidopsis thaliana)*: Arabidopsis is a recent entry to the genetics of inheritance of Al resistance. Two independent studies (Kobayashi and Koyama 2002, and Hoekenga *et al.* 2003) used quantitative trait loci (QTL) mapping to identify genes important for Al resistance using L*er* x Col Recombinant Inbred Lines (RIL). Both used malate exudation and relative root growth as measures of physiological status. Kobayashi and Koyama (2002) reported two significant single factor QTLs detected by RRL on chromosomes 1 and 4. Howkenga *et al.* (2003) also identified a factor on chromosome 1 (QTL 1) as the major resistance locus, however, the remainder of the

QTLs did not agree with those identified by Kobayashi and Koyama (2002). The authors speculated that these differences were due to different physiological conditions of growth used in two different studies (Howkenga *et al.* 2003). More recently, Koyama's group again used QTL mapping to identify Al resistant loci in Arabidopsis. In this study they used RILs obtained from ecotypes carrying similar levels of Al resistance and malate release (Col x Kas) to possibly identify additional loci. They detected a QTL that overlapped the chromosomal position of a previously identified major QTL not associated with Al-responsive malate release (Ikka *et al.* 2008).

1.7. Proteins regulated by Al stress

Compared to transcriptome-based gene discovery, there has been slower progress in search of proteins responsive to Al stress. Basu *et al.* (1994) found two 51 kD root proteins induced in response to Al, which were later identified as the β-subunit of vacuolar H^+-ATPase (V-ATPase) and the α- and β-subunits of the mitochondrial ATP synthase (Hamilton *et al.* 2001). Sharma and Dubey (2007) identified increases in the activities of several antioxidant proteins, including superoxide dismutase (SOD), guaiacol peroxidase, ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase, and glutathione reductase, whereas activities of catalase and chloroplastidic APX decreased after in response to Al treatment.

Recently, proteomics-based approaches have been used in rice (Fukuda *et al.* 2007, Yang *et al.* 2007), soybean (Zhen *et al.* 2007), and tomato (Zhou *et al.* 2009) to identify differentially abundant proteins after exposure to Al. Yang *et al.* (2007) identified 16 proteins regulated in response to Al stress using a resistant cultivar of rice (Xiangnuo), whereas Fukuda *et al.* (2007) identified 31 proteins classified into mainly

carbon metabolism, nitrogen metabolism, nucleotide related and, oxidation-reduction related. Two of these studies (Fukuda *et al.* 2007, Zhen *et al.* 2007) identified several metabolism-related proteins indicating regulation of primary metabolism could be an important component to combat Al stress. However, there was a little overlap between the identified pools of proteins. For instance, Al-responsive proteins in soybean (Zhen *et al.* 2007) included thiamine pyrophosphokinase, sucrose synthase, monoglyceridelipase, acetone cynohydrin lyase, NAD(P)H dependent 6'deoxychalcone synthase, sulfotransferase, and entkaurene oxidase, whereas the majority Al-responsive proteins identified in rice (Fukuda *et al.* 2007) were related to primary energy metabolism (proteins of glycolysis and TCA cycle). These differences in the proteome profile may be partly due to the different plant species and exposure conditions. With an objective to study the proteome involved in long-term survival (10 d) of tomato plants under Al stress, Zhou *et al.* (2009) identified 88 protein spots where 61 spots increased and 27 spots decreased in abundance. Most of the identified proteins were associated with changes in oxidative stress, detoxification, organic acid metabolism, methyl flow, cell proliferation, and cell death (Zhou *et al.* 2009).

1.8. Genes regulated by Al stress

Several physiological- and reverse genetics-based approaches have identified genes involved in Al stress resistance. Several reports have identified genes or transcripts through analysis of expressed sequence tag (EST) libraries (Milla *et al.* 2002), cDNA libraries (Snowden and Gardner 1993, Cruz-Ortega *et al.* 1997, Hamel *et al.* 1998, Richards *et al.* 1998, Sasaki *et al.* 2004, Guo *et al.* 2007), differential display (Ermolayev *et al.* 2003), cDNA-AFLP (Mao *et al.* 2004), mutant populations, and transgenic over-

expression studies (Tesfaye *et al.* 2001, Anoop *et al.* 2003). Several of these genes (Table 1.2) have been previously linked with responses to other abiotic and biotic stresses. Thus it is difficult to determine if these responses are specific to Al stress and therefore responsible for Al resistance. However, it can be implied that Al stress shares common stress responsive pathways with other abiotic stresses (Cheong *et al.* 2002, Chinnusamy *et al.* 2004, Mahajan and Tuteja 2005).

Since OAs play an important role in detoxification of Al, several successful attempts have been made to engineer Al resistance by over expression of the key enzymes of TCA cycle (Table 1.3). For instance, over expression of citrate synthase (in tobacco, carrot, and canola) and malate dehydrogenase (in alfalfa) imparts Al resistance to transgenic plants. Arabidopsis seems to be a preferred choice for over-expression studies as several Al responsive genes (besides OA metabolism) have been over-expressed and shown to impart resistance (Table 1.4). These include blue copper binding protein (*BCB)*, Auxilin-like gene, GDP dissociation inhibitor (*GDI*), anionic peroxidase *(POX)*, Glutathione S-transferase, and wall-associated kinase (*WAK-1)*. Transgenic canola and tobacco harboring a superoxide dismutase and phosphatidylserine synthase, respectively; also showed increased resistance to Al stress.

During the past decade (Chandran *et al.* 2008, Houde and Diallo 2008, Kumari *et al.* 2008, Maron *et al.* 2008, Goodwin and Sutter 2009, Zhao *et al.* 2009), there has been a progressive increase in the characterization of Al-responsive genes, most recently exploring changes in expression that occur at the whole genome level. The emergence of high throughput technologies (such as microarrays) has provided a valuable tool to plant physiologists to identify and characterize individual genes and gene families (Wullschleger and Difazio 2003). Recently, microarrays have been used to identify Al responsive genes in various plant species such as Arabidopsis (Kumari *et al.* 2008, Zhao

et al. 2009, Goodwin and Sutter 2009), maize (Maron *et al.* 2008), alfalfa (Chandran *et al.* 2008) and wheat (Houde and Diallo 2008).

To conclude, a considerable amount of research during the last two decades has helped to understand Al toxicity and resistance in plants. Nonetheless, we have a better understanding of physiological aspects of Al toxicity than molecular basis of Al resistance in plants. As described above, several reports have used a transgenic approach (mostly focused on one aspect of resistance) to generate Al resistance in plants. However, some true limitations to develop Al-resistant transgenic plants is the poor understanding of other, but still unknown mechanisms of resistance.

To complement the current understanding, I set out to use high throughput technologies such as microarrays for genome-wide screening of differentially abundant genes in responses to Al stress (Chapter 2). This technique has been used successfully to identify genome wide screening of transcriptional profiling in response to various stresses (Vij and Tyagi 2007). Similarly, proteomics based tools such as 2D gel electrophoresis (DiGE), have been used to characterize proteins associated with stress and development processes in plants (Amme *et al.* 2006, Chivasa *et al.* 2006, Hotte and Deyholos 2008). I used DiGE technology to identify differentially abundant proteins in response to Al stress (Chapter 3). Further, I have compared these two data sets to detect if changes in transcript level are also reflected at biologically relevent level of proteins (Chapter 3). Based on the genes and proteins identified in my two screening approaches, I further focused on cyt-MDH (Chapter 3) and Class III plant peroxidases (Chapter 4) to help understand their role in response to Al stress.

This study will not only provide a platform to select gene (s) whose modified expression would help to generate Al resistant plants, but will also help to identify future research directions.

Table 1.1. Organic anions released from plant species in response to aluminum stress.

OA released	**Plant**	**References**
Citrate	Maize *(Zea mays)*	Pellet *et al.* 1996
Citrate	Tobacco *(Nicotiana tabacum)*	Delhaize *et al.* 2001
Citrate	Sickle senna *(Cassia tora)*	Ishikawa *et al.* 2000
Citrate	Soybean *(Glycine max)*	Silva *et al.* 2001
Citrate, malate	Oat *(Avena sativa)*	Zheng *et al.* 1998
Citrate, malate	Rye *(Secale cereale)*	Li *et al.* 2000
Citrate, malate	Triticale *(Triticale ssp)*	Ma *et al.* 2000
Citrate, malate	Sunflower *(Helianthus annuus)*	Saber *et al.* 1999
Citrate, malate	Radish *(Raphanus sativus)*	Zheng *et al.* 1998
Citrate, malate	Rape *(Brassica napus)*	Zheng *et al.* 1998
Citrate, malate	Leguminous shrub (*Lespedeza bicolor*)	Dong *et al.* 2008
Citrate, malate, aconitate	Sorghum *(Sorghum bicolor)*	Magalhaes *et al.* 2007, Goncalves *et al.* 2005
Malate,citrate	Wheat *(Triticum aestivum)*	Delhaize *et al.* 1993, Ryan *et al.* 2009
Malate, citrate	Arabidopsis *(Arabidopsis thaliana)*	Kobayashi and Koyama 2003, Liu *et al.* 2009
Malate, citrate	Cowpea *(Vigna unguiculata)*	Jemo *et al.* 2007
Oxalate	Taro *(Colocasia esculenta)*	Ma and Miyasaka 1998
Oxalate	Buckwheat *(Fagopyrum esculentum)*	Zheng *et al.* 1998
Oxalate, citrate	Poplar *(Populus tremula)*	Qin *et al.* 2007
Oxalate	Rice (*Oryza sativa*)	Begum *et al.* 2009

Table 1.2. Aluminum-responsive genes identified in various species.

Gene Description	**GENE**	**Plant**	**References**
Phosphoenolpyruvate carboxylase	*PEP CARBOXYLASE*	*Medicago sativa*	Tesfaye *et al.* 2001
Malate dehydrogenase	*MDH*	*Medicago sativa*	Tesfaye *et al.* 2001
Glutathione peroxidase	*PHGPX*	*Arabidopsis thaliana*	Sugimoto *et al.* 1997
Phototropic response transducer	*PEARLI8*	*Arabidopsis thaliana*	Richards *et al.* 1998
Pro-rich hydrophobic protein	*PEARLI1*	*Arabidopsis thaliana*	Richards *et al.* 1998
Unknown	*PEARLI2*	*Arabidopsis thaliana*	Richards *et al.* 1998
Aldolase chloroplast	*ALD*	*Arabidopsis thaliana*	Richards *et al.* 1998
Pro-rich hydrophilic protein	*PEARLI4*	*Arabidopsis thaliana*	Richards *et al.* 1998
Berberine bridge enzyme	*PEARLI5*	*Arabidopsis thaliana*	Richards *et al.* 1998
Blue copper-binding Protein	*BCB*	*Arabidopsis thaliana*	Richards *et al.* 1998
Peroxidase	*PER*	*Arabidopsis thaliana*	Richards *et al.* 1998
Alanine aminotransferase	*ALA*	*Arabidopsis thaliana*	Richards *et al.* 1998
Chlorophyll *A/B*-binding protein	*CAB*	*Arabidopsis thaliana*	Richards *et al.* 1998
Cu/Zn superoxide dismutase	*CZSOD*	*Arabidopsis thaliana*	Richards *et al.* 1998
Catalase	*CAT*	*Arabidopsis thaliana*	Richards *et al.* 1998
Tobacco glutathione s-transferase	*PARB*	*Arabidopsis thaliana*	Ezaki *et al.* 2000
Tobacco peroxidase	*NtPOX*	*Arabidopsis thaliana*	Ezaki *et al.* 2000
GDP-dissociation inhibitor	*NtGDI1*	*Arabidopsis thaliana*	Ezaki *et al.* 2000
Cell Wall-associated receptor kinase 1	*WAK1*	*Arabidopsis thaliana*	Sivaguru *et al.* 2003
Glutathione s-transferase	*AtGST1*	*Arabidopsis thaliana*	Ezaki *et al.* 2004
Glutathione s-transferase	*AtGST11*	*Arabidopsis thaliana*	Ezaki *et al.* 2004
Cell Wall-Associated Receptor Kinase	*WAKL4*	*Arabidopsis thaliana*	Hou *et al.* 2005
Aluminum Sensitive ABC Transporter-Like Protein	*ALS3*	*Arabidopsis thaliana*	Larsen *et al.* 2005
Glutamate Receptors	*AtGLR3.4*,	*Arabidopsis thaliana*	Meyerhoff *et al.* 2005

Sensitive to proton rhizotoxicity-1	*STOP-1*	*Arabidopsis thaliana*	Iuchi *et al.* 2007
Auxilin-like protein	*F9E10.5*	*Arabidopsis thaliana*	Ezaki *et al.* 2007
Mg Transporter	*ATMGT1*	*Arabidopsis thaliana*	Li, 2001
Malate transporter	*ALMT1*	*Hordeum vulgare*	Delhaize *et al.* 2004
Al activated citrate transporter	*AACT1*	*Hordeum vulgare*	Furukawa *et al.* 2007
Manganese super oxide dismutase-mito	*Mn-SOD*	*Brassica napus*	Basu *et al.* 2001
Citrate synthase	*CS*	*Brassica napus*	Anoop *et al.* 2003
Aconitase	*ACO*	*Brassica napus*	Anoop *et al.* 2003
Isocitrate dehydrogenase	*IDH*	*Brassica napus*	Anoop *et al.* 2003
mito-Citrate synthase	*CS*	*Daucus carota*	Koyama *et al.* 1999
Superoxide Dismutase	*SOD*	*Citrus reshni*	Chen *et al.* 2005
Ascorbate Peroxidase	*APX*	*Citrus reshni*	Chen *et al.* 2005
Monodehydroascorbate Reductase	*MDAR*	*Citrus reshni*	Chen *et al.* 2005
Glutathione Reductase	*GR*	*Citrus reshni*	Chen *et al.* 2005
Catalase	*CAT*	*Citrus reshni*	Chen *et al.* 2005
Similarity: tomato alcohol dehydrogenase	*SADA*	*Pisum sativum*	Brosché and Strid 1999
Similarity: tomato alcohol dehydrogenase	*SADB*	*Pisum sativum*	Brosché and Strid 1999
Similarity: tomato alcohol dehydrogenase	*SADC*	*Pisum sativum*	Brosché and Strid 1999
Alcohol dehydrogenase short-chain	*SAD*	*Pisum sativum*	Brosché and Strid 1999
Polyubiquitin	*PU1*	*Pisum sativum*	Brosché and Strid 1999
Chalcone synthase	*CHS*	*Pisum sativum*	Brosché and Strid 1999
Disease resistance protein 230	*PsDRR230*	*Pisum sativum*	Sävenstrand *et al.* 2000
Leu-rich repeat protein	*PsLRRP*	*Pisum sativum*	Sävenstrand *et al.* 2000
Extensin	*PsEXT*	*Pisum sativum*	Sävenstrand *et al.* 2000
mito- Citrate Synthase	*MCS*	*Paraserianthes falcataria*	Osawa and Kojima. 2006
Copper Chaperone	*CCH*	*Populus alba*	Lee *et al.* 2005
B. Napus Al-Activated malate transporter	*BnALMT1*	*Brassica napus*	Ligaba *et al.* 2006
B. Napus Al-Activated malate transporter	*BnALMT2*	*Brassica napus*	Ligaba *et al.* 2006
MT class II	*RICMT*	*Oryza sativa*	Yu *et al.* 1998
Dihydrolipoamide *s*-acetyltransferase	*OsAR1*	*Oryza sativa*	Mao *et al.* 2004

2-Oxoglutarate dehydrogenase	*OsAR2*	*Oryza sativa*	Mao *et al.* 2004
Aspartate aminotransferase	*OsAR3*	*Oryza sativa*	Mao *et al.* 2004
4-Coumarate:CoA ligase isoform 2	*OsAR4*	*Oryza sativa*	Mao *et al.* 2004
Phenylalanine ammonia-lyase	*OsAR5*	*Oryza sativa*	Mao *et al.* 2004
Cinnamyl-alcohol dehydrogenase-putataive	*OsAR6*	*Oryza sativa*	Mao *et al.* 2004
P-Coumarate 3-hydroxylase	*OsAR7*	*Oryza sativa*	Mao *et al.* 2004
Beta-1,3-glucanase	*OsAR10*	*Oryza sativa*	Mao *et al.* 2004
UDP-*N*-Acetylglucosamine pyrophosphorylase	*OsAR11*	*Oryza sativa*	Mao *et al.* 2004
O-Deacetylbaccatin iii-10-*o*-acetyl transferase	*OsAR12*	*Oryza sativa*	Mao *et al.* 2004
Quinone oxidoreductase	*OsAR13*	*Oryza sativa*	Mao *et al.* 2004
Proteinase inhibitor	*OsAR14*	*Oryza sativa*	Mao *et al.* 2004
Elongation factor EF-2	*OsAR15*	*Oryza sativa*	Mao *et al.* 2004
Sumo-1	*OsAR16*	*Oryza sativa*	Mao *et al.* 2004
MCT-1 protein-like	*OsAR17*	*Oryza sativa*	Mao *et al.* 2004
Rice KN1-like proteins	*OsAR18*	*Oryza sativa*	Mao *et al.* 2004
Putative Retroelement Pol Polyprotein	*OsAR19*	*Oryza sativa*	Mao *et al.* 2004
Histone H4	*OsAR20*	*Oryza sativa*	Mao *et al.* 2004
Tonoplast aquaporin	*AQP*	*Secale cereale*	Milla *et al.* 2002
Ubiquitin-Like Protein SMT3	*SMT3*	*Secale cereale*	Milla *et al.* 2002
Glutathione Peroxidase	*GPX*	*Secale cereale*	Milla *et al.* 2002
Glucose-6-phosphate-dehydrogenase	*GAPDH*	*Secale cereale*	Milla *et al.* 2002
Ascorbate peroxidase	*APX*	*Secale cereale*	Milla *et al.* 2002
Iron deficiency specific proteins	*IDS1, IDS3A,B*	*Secale cereale*	Milla *et al.* 2002
S-Adenosyl methionine synthase	*SAM*	*Secale cereale*	Milla *et al.* 2002
Methionine synthase	*MS*	*Secale cereale*	Milla *et al.* 2002
Pathogenesis-related protein 1.2,	*PR*	*Secale cereale*	Milla *et al.* 2002
Heme oxygenase	*HY*	*Secale cereale*	Milla *et al.* 2002
Epoxide hydrolase	*EH*	*Secale cereale*	Milla *et al.* 2002

Alunium Tolerance Locus	*ALT3*	*Secale cereale*	Miftahudin *et al.* 2004
	ALT_{SB}	*Sorghum bicolor*	Magalhaes *et al.* 2004
Multidrug and toxic compound extrusion family gene	*SbMATE*	*Sorghum bicolor*	Magalhaes *et al.* 2007
Auxin down-regulated ADR6	*SALI3-2*	*Glycine max*	Ragland and Soliman 1997
Auxin down-regulated adr6	*SALI5-4a*	*Glycine max*	Ragland and Soliman 1997
Phosphoenolpyruvate carboxylase	*PEPC*	*Glycine max*	Ermolayev *et al.* 2003
Translationally controlled tumour proteins	*TCTP*	*Glycine max*	Ermolayev *et al.* 2003
Inosine-5'-monophosphate dehydrogenases	*IMPDH*	*Glycine max*	Ermolayev *et al.* 2003
Plasma Membrane H+-Atpase	H^+*-ATPASE*	*Glycine max*	Shen *et al.* 2005
Auxin-regulated protein	*PAL111*	*Nicotiana tabacum*	Ezaki *et al.* 1995
Glutathione *S*-transferase	*PAL142*	*Nicotiana tabacum*	Ezaki *et al.* 1995
Cys-Rich Domain Protein	*PAL139*	*Nicotiana tabacum*	Ezaki *et al.* 1995
Unknown	*PAL141*	*Nicotiana tabacum*	Ezaki *et al.* 1995
Anionic Peroxidase	*PAL 201*	*Nicotiana tabacum*	Ezaki *et al.* 1995
GDP-dissociation inhibitor	*GDI*	*Nicotiana tabacum*	Ezaki *et al.* 1995
Manganese superoxide dismutase	*MnSOD*	*Nicotiana tabacum*	Devi *et al.* 2003
Arabidopsis Magnesium Transport Family	*ATMGT1*	*Nicotiana tabacum*	Deng *et al.* 2006
Glycero-phosphodiesterase-like protein	*NtGPDL*	*Nicotiana tabacum*	Choi *et al.* 2007
MT Class I	*WALI 1*	*Triticum aestivum*	Snowden and Gardner 1993
Cys-Rich Protein	*WALI 2*	*Triticum aestivum*	Snowden and Gardner 1993
Proteinase inhibitor	*WALI 3*	*Triticum aestivum*	Snowden and Gardner 1993
Phe-ammonia lyase	*WALI 4*	*Triticum aestivum*	Snowden and Gardner 1993
Proteinase inhibitor	*WALI 5*	*Triticum aestivum*	Snowden and Gardner 1993
Proteinase inhibitor	*WALI 6*	*Triticum aestivum*	Richards *et al.* 1994
Unknown	*WALI 7*	*Triticum aestivum*	Richards *et al.* 1994
S-adenosyl homocysteine hydrolase	*SHH*	*Triticum aestivum*	Richards and Gardner 1994
Histone H3	*H3*	*Triticum aestivum*	Richards and Gardner 1994
Histone H4	*H4*	*Triticum aestivum*	Richards and Gardner 1994

Heat shock protein 70	*HSP70*	*Triticum aestivum*	Richards and Gardner 1994
1,3-beta-glucanase	*GLC1*	*Triticum aestivum*	Cruz-Ortega *et al.* 1997
Cytoskeletal fimbrin-like actin bundling proteins	*WFIM1*	*Triticum aestivum*	Cruz-Ortega *et al.* 1997
Wheat aluminum-regulated (war): peroxidase	*WAR4.2*	*Triticum aestivum*	Hamel *et al.* 1998
Cysteine proteinase	*WAR5.2*	*Triticum aestivum*	Hamel *et al.* 1998
Phenylalanine-ammonia lyase	*WAR7.2*	*Triticum aestivum*	Hamel *et al.* 1998
Oxalate oxidase	*WAR 13.2*	*Triticum aestivum*	Hamel *et al.* 1998
Vacuolar H^+-ATPase	*V-ATPASE*	*Triticum aestivum*	Hamilton *et al.* 2001
Alpha-Beta subunits mito- ATP synthase	*F1F0-ATPASE*	*Triticum aestivum*	Hamilton *et al.* 2001
Multidrug resistance similar to ABC	*TaMDR1*	*Triticum aestivum*	Sasaki *et al.* 2002
Aluminum-Activated Malate Transporter	*ALMT1*	*Triticum aestivum*	Sasaki *et al.* 2004
Lupinus albus Multi Drug And Toxin Effux	*LaMATE*	*Lupinus albus*	Uhde-Stone *et al.* 2005
Uptake system for mg2+ and other divalent cations	*ALR1*	*Saccharomyces cerevisiae*	MacDiarmid, 1998
Uptake system for mg2+ and other divalent cations	*ALR2*	*Saccharomyces cerevisiae*	MacDiarmid, 1998
Blue copper binding	*BCB*	*Saccharomyces cerevisiae*	Ezaki *et al.* 1999
GDP dissociation inhibitor of tobacco	*NTGDI1*	*Saccharomyces cerevisiae*	Ezaki *et al.* 1999
Phosphatidylserine synthase	*TAPSS1*	*Saccharomyces cerevisiae*	Delhaize *et al.* 1999
Mg Transporter	*ATMGT10*	*Saccharomyces cerevisiae*	Li *et al.* 2001
Phospholipid hydroperoxide glutathione-peroxidase	*PHGPX*	*Saccharomyces cerevisiae*	Basu *et al.* 2004
Glutathione S-transferase	*GST*	*Saccharomyces cerevisiae*	Basu *et al.* 2004
Long-Chain Base Desaturase	*LCB8E/Z-DESATURASE*	*Saccharomyces cerevisiae*	da Silva *et al.* 2006

Table 1.3. Engineering aluminum resistance in plant species using TCA cycle enzymes

Gene	**Origin**	**Recipient**	**Al resistance**	**References**
Citrate synthase	*P. aeruginosa*	Tobacco *(Nicotiana tabacum)*	Increased	De la Fuente *et al.* 1997
Citrate synthase	*A. thaliana*	Carrot *(Daucus carota)*	Increased	Koyama *et al.* 1999
Citrate synthase	*P. aeruginosa*	Tobacco *(N. tabacum)*	No-change	Delhaize *et al.* 2001
Citrate synthase	*A. thaliana*	Canola *(Brassica napus)*	Increased	Anoop *et al.* 2003
Citrate synthase	*P. aeruginosa*	Alfalfa *(M. sativa)*	Increased	Barone *et al.* 2008
Malate dehydrogenase	*M. sativa*	Alfalfa *(M. sativa)*	Increased	Tesfaye *et al.* 2001

Table 1.4. Engineering aluminum resistance using aluminum induced genes.

Gene	Description	Origin	Recipient	Resistance	References
AtBCB	Blue copper binding protein	*A. thaliana*	*A. thaliana*	Increased	Ezaki *et al.* 2000
F9E10.5	Auxilin-like gene	*A. thaliana*	*A. thaliana*	Increased	Ezaki *et al.* 2006
NtGDI	GDP dissociation inhibitor	*N. tabacum*	*A. thaliana*	Increased	Ezaki *et al.* 2000
NtPOX	Anionic peroxidase	*N. tabacum*	*A. thaliana*	Increased	Ezaki *et al.* 2000
PARB	Glutathione S-transferase	*N. tabacum*	*A. thaliana*	Increased	Ezaki *et al.* 2000
WAK1	Wall-associated kinase	*A. thaliana*	*A. thaliana*	Increased	Sivaguru *et al.* 2003
ALR1	Mg transporter	*S. cerevisiae*	*S. cerevisiae*	Increased	MacDiarmid and Gardner 1998
Mn-SOD	Mangnese super oxide dismutase	*T. aestivum*	*B. napus*	Increased	Basu *et al.* 2001
PSS	Phosphatidylserine synthase	*T. aestivum*	*N. tabacum*	Increased	Delhaize *et al.* 1999
PEPC	Phosphoenolpyruvate carboxylase	*Z. mays*	*O. sativa*	Increased	Begum *et al.* 2009
TaALMT	Aluminum activated malate transporter	*T. aestivum*	*H. vulgare*	Increased	Delhaize *et al.* 2004

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2. Transcriptomic responses to aluminum stress in roots of *Arabidopsis thaliana*

2.1. Introduction

Aluminum (Al) is the most abundant metal and the third most common element in the earth's crust. When soil pH is less than 5.0, Al ionizes to form phytotoxic ions (Al^{3+}) that are readily absorbed by plant roots (Kinraide 1990), inhibiting elongation of roots and reducing yields of crops (Kochian 1995, Matsumoto 2000). Because approximately 50% of the world's potentially arable land is acidic (Von Uexküll and Mutert 1995), Al toxicity is one of the major limitations to global crop productivity.

Alterations of root architecture (Doncheva *et al.* 2005) and inhibition of root elongation (Matsumoto 2000) are considered primary symptoms of Al toxicity. The majority of Al associated with roots is bound in the apoplasm; only a relatively small fraction enters into the symplasm where it can interact with intracellular targets (Taylor *et al.* 2000). Aluminum exposure results in alterations to the plasma membrane surface, disruption of cytoskeletal dynamics (Sivaguru *et al.* 2003), changes in Ca^{2+} homeostasis and signaling (Jones *et al.* 1998), peroxidative damage to membranes, induction of reactive oxygen species (ROS), and mitochondrial dysfunction leading to Al-mediated inhibition of root growth (Yamamoto *et al.* 2002).

Although most plant species are sensitive to Al, genetic variation in resistance to Al has been reported for many crops. As proposed by Taylor (1991), Al resistance can be based on two types of mechanisms: one based upon exclusion of Al from the root symplasm, and the other based upon resistance to Al once it has entered the symplasm. One well-characterized exclusion mechanism depends on exudation of organic anions

(OA) from the root apex (Ma *et al.* 2001, Kochian *et al.* 2004). Other proposed exclusion mechanisms include secretion of proteins (Basu *et al.* 1994), increasing rhizosphere pH (Degenhardt *et al.* 1998), and efflux of phosphate (Pellet *et al.* 1996). Genetic mapping has identified loci contributing to Al resistance in many crop species including wheat, rye, barley, sorghum, rice, and maize. These studies have identified loci associated with increased exudation of organic anions. A major Al-resistance locus in Arabidopsis was mapped at the top of chromosome 1 by quantitative trait locus (QTL) analysis of recombinant inbred lines created using Al sensitive (Landsberg) and Al resistant (Columbia) ecotypes (Kobayashi and Koyama 2002, Hoekenga *et al.* 2003).

Several reports have identified genes or transcripts related to Al responses through analysis of expressed sequence tag (EST) libraries (Milla *et al.* 2002), cDNA libraries (Snowden and Gardner 1993, Cruz-Ortega *et al.* 1997, Hamel *et al.* 1998, Richards *et al.* 1998, Sasaki *et al.* 2004, Guo *et al.* 2007), differential display (Ermolayev *et al.* 2003), cDNA-AFLP (Mao *et al.* 2004), mutant populations, and transgenic over-expression studies (Anoop *et al.* 2003). Many of the previously identified Al-responsive genes can be grouped into the following functional groups: i) oxidative stress pathways (Sugimoto and Sakamoto 1997, Devi *et al.* 2003, Basu *et al.* 2004, Chen *et al.* 2005); ii) membrane transporters (Hamilton *et al.* 2001, Sasaki *et al.* 2004, Shen *et al.* 2005, Larsen *et al.* 2005, Deng *et al.* 2006); iii) energy and primary metabolism (Tesfaye *et al.* 2001, Anoop *et al.* 2003); iv) polysaccharide and cell wall metabolism (Mao *et al.* 2004); v) protein metabolism (Brosche and Strid 1999); vi) signaling, hormones and vii) transcription factors (Sivaguru *et al.* 2003).

Completion of genome sequencing projects and the commercial availability of unique oligonucleotide probes have given rise to the development of high throughput technologies (e.g microarrays) to study genome-wide transcriptomic responses to various

stresses (Vij and Tyagi 2007). To date, genome-scale microarray analysis of Al stress responses has not been reported, despite the potential for this technique to identify large numbers of stress-responsive transcripts (Deyholos and Galbraith 2001). Here, I present a comprehensive transcriptomic analysis of Al stress responses in plants. This gene discovery effort will help expand our understanding of cellular responses to Al treatment, and will identify candidate genes for enhancement of Al resistance in crops.

2.2. Materials and methods

2.2.1. Plant growth and exposure to stress

Seeds of *Arabidopsis thaliana* (Col-0, Lehle Seeds) were surface sterilized (2.25% NaClO, 0.05% Tween-20), rinsed, and sown on agar plugs (Murashige & Skoog medium, 0.3% phytagel, Sigma) within floating rafts. Each raft was fashioned by drilling approximately 100 holes (3-4 mm diameter) in a Sintra® plastic disk (130 mm diameter; 3 mm thick) and covering each hole with nylon mesh (0.5 mm). After stratification (2 days at 4^{o} C), rafts were transferred onto the surface of 1 liter of sterilized Richard's medium (pH 5.75; Richards *et al.* 1998) in black, polyethylene tanks. Plants were grown for 14 days at 22^{o} C day and 19^{o} C night temperature on a 16 h light/8 h dark cycle in a growth chamber with approximately 160 μmol m^{-2} s^{-1} photosynthetic photon flux density and 65% relative humidity.

Aluminum (Al) stock solutions (25 mM) were prepared by adding $AlCl_3$ to water that had been adjusted to pH 3.0 with HCl. Aluminum exposure solutions were prepared in $CaCl_2$ (200 μM, pH 4.33) to avoid precipitation of Al. The use of alkali was avoided while adjusting the pH of exposure solutions to prevent the formation of non toxic polymeric Al species (Kinraide and Parker 1987). Aluminum treatments were conducted

by transferring rafts containing 14 day old plants to exposure solutions containing $AlCl_3$ in 200 μM $CaCl_2$ and grown for two more days. For controls, plants were transferred to exposure solutions with no Al. For dose response analyses, $AlCl_3$ concentrations ranged from 0 to 100 μM. Increases in the root length were measured from day 14 to day 16 and a Relative Root Growth Increment (RRGI) was calculated as ($RL_{treated}$ Day16 - $RL_{treated}$ Day14) / ($RL_{control}$ Day16 - $RL_{control}$ Day14) × 100, where RL refers to root length. For microarray analyses, exposure solutions contained 0 or 25 μM $AlCl_3$ at pH 4.33. After 6 h and 48 h of exposure, roots were harvested using a sterile razor blade and immersed in liquid nitrogen. Roots were then ground with a mortar and pestle and stored at -80°C.

2.2.2. Isolation of RNA and microarray hybridization

Root tissues from more than 120 plants from three independently maintained hydroponic tanks were pooled together to comprise a single sample. Eight independent samples (grown at separate times) were analyzed for each time point and treatment condition. At each time point, untreated controls were harvested in parallel with treated roots to control for the influence of developmental or circadian effects. Total RNA (50 μg) was extracted from each sample using a silica membrane-based RNA isolation kit (Qiagen), and was labeled with Cy dyes using a 3DNA array 50 Kit (Genisphere), with oligo dT and Superscript III (Invitrogen). A total of 26,090 probes that were 70 oligonucleotides long (Array-Ready Oligo Set™ for *Arabidopsis thaliana* genome Version 1.0 from Qiagen Operon) were deposited with additional probes for quality control on superamine aminosilane-coated slides (Telechem). An SDS-based hybridization buffer was used to hybridize cDNA to spotted probes. After 12 hours of incubation at 45° C, slides were washed under high stringency conditions as per the manufacturer's protocol. Cy3 and Cy5 capture reagent, complementary to the capture

sequence incorporated in the cDNA, was hybridized to slides for 4 hours at 55° C. To avoid dye-based bias due to differences in labeling efficiencies as well as different efficiencies in the detection of the fluorescent signals, I adopted an experimental design where labeling of the dye was reversed. Specifically, for 4 out of the total 8 independent samples (replicates) at each time point, I reversed the labeling of cDNA samples. TM4 software suite (Saeed *et al.* 2003) was used for acquisition and processing of microarray data where background-subtracted spot intensities were measured by Spotfinder Ver 3.0, and normalized by the Loess method in MIDAS Ver 2.19. Spots were defined as detectable above background if their signal intensity was greater than two standard-deviations above the mean signal intensities of all blank and randomized negative control spots and were detectable in more than fifty percent of arrays for each time point. Fold change was defined as the ratio of treated:control spot intensities. Significance analysis of microarrays (SAM; Tusher *et al.* 2001) was applied to identify genes for which the fold change differed significantly from 1 at either one or two time points in a multiclass test (with computed values at time point 0), using a false discovery rate (FDR) of approximately 5%. Microarray data are available in the ArrayExpress database as accessions E-MEXP-1071. Microarray data has been submitted according to MIAME recommendations.

2.2.3. Quantitative reverse-transcription PCR (QRT-PCR) analysis

QRT-PCR primers were designed using Primer3 (Rozen and Skaletsky 2000) or PrimerExpress 2.0 software (Applied Biosystems) so that at least one primer of each pair covered an exon-exon junction to prevent amplification of genomic DNA. The specificity of each primer pair was checked against the Arabidopsis genome using WU- BLAST 2.0 (http://www.Arabidopsis.org/wublast/index2.jsp). The primer sequences were as follows:

At4g36430 F: CTAGCCCCAATGCCACTGA, R: GCCTCCACAAATGCATTGAA;

At3g01190 F: ACTCGGAGTACGCCGCTAAG, R: TTGAAACTCCCCGGATCCA;

At5g39580 F: TTTCCTTGGCATCGAACGTT, R: AGGCGAAAAGCGGAGAATTT;

At5g64100 F: CTCTTGTTGGCGGACACA, R: GTCGATTGATGGGTCAGGTT;

At1g05250 F: GATGCCGTGGCAGTGAT, R: CTTTATGTCGGCGAAAGGAG;

At1g02930 F: GCAAGGACATGGCGATCATA, R: GGTCAAACTCATGCGACTCA;

At2g29460 F: AGAAGGAAGTCACTGGAAAAGAT, R:
CCTGCGACCAAGTCCAAAA;

At1g07810 F: GAGAGAGTGAGGCAGTGAGCAA, R:
CCTGCAAACACCATGCATTT;

At5g54510 F: GCTTGGTCAGGAGTACGAGCTT, R:
ACCAGCCACGCTTAGGACAT;

At3g15360 F: GCTGCTCCGTCGGTTTCA, R: TCACCAAACTCCGCGAAGAC;

At5g25760 F: CTTAACTGCGACTCAGGGAAT, R: GGCGAGGCGTGTATACATTT.

All RNA samples were treated with DNA*free* (Ambion). I quantified cDNAs in an Applied Biosystems 7500 Real-Time PCR System, with a SYBR green dye detection assay, and ROXpassive reference, in 10 µl reactions as follows: Stage 1, 1 cycle at 95^{o} C for 2 min; Stage 2, 40 cycles at 95^{o} C for 0.15 min and 60^{o} C for 1 min; Stage 3, 95.0^{o} C for 0.15 min; Stage 4, 60.0^{o} C for 1 min; and Stage 5, 95.0^{o} C for 0.15 min. C_t values generated for each primer pair set over a range of dilutions were used to calculate the ΔC_t (C_t target – C_t reference). ΔC_t values were then plotted against log input amount. If the slope of the ΔC_t vs. log input was < 0.1 then the relative abundance of each transcript was estimated using the $\Delta\Delta$ C_t (Livak and Schmittgen 2001).

2.3. Results

I conducted microarray analysis to identify transcripts in Arabidopsis roots that changed in abundance following exposure to Al in a hydroponic system. In this system, plants were germinated on agar plugs within floating plastic rafts. The design of these rafts permitted rapid transfer of plants between treatment conditions, and allowed for measurement of pH and electric conductivity with minimal disturbance (Fig. 2.1). Unlike some previously described hydroponics systems, mine was capable of sustaining plant growth through a complete life cycle and avoided substrates such as rock wool or filter paper, which can sequester Al.

To select an appropriate concentration of Al for stress treatments, I conducted a dose response analysis (Fig. 2.2a). I observed that exposure of plants to 5 µM Al for 48 h reduced primary root elongation to 59% of control, but did not have marked effect on lateral root formation. When the concentration of Al was increased to 45 µM, primary root elongation was further reduced to 34% of control and lateral root formation was fully inhibited. Exposure of plants to 100 µM Al for 48 h resulted in complete inhibition of primary root elongation and lateral root formation. However, at 25 µM $AlCl_3$, I observed inhibition of primary root elongation, absence of lateral roots in zone of division and elongation, and a 39% reduction in root elongation with an overall induced reduction of growth of plants (Fig. 2.2b). I found that juvenile plants treated with 25 µM Al continued to grow to maturity and produce seeds. I therefore selected 25 µM Al as my exposure concentration because it induced physiological stress responses, but was not acutely lethal.

2.3.1. Microarray based expression profiling

I used microarrays to measure the effects of exposure to Al(6 h and 48 h) on the transcriptome of Arabidopsis roots. At each time point, untreated controls were harvested in parallel with treated roots to control for the influence of developmental or circadian effects. A total of 23,686 distinct genes were represented by the probes spotted on my microarrays. After filtering out spots with signal intensities below background cut-off criteria and probes that could not be associated with a specific AGI (Arabidopsis Genome Initiative) identifier, I defined 7,413 spots as detectable at the 6 h time point and 7,673 as detectable at the 48 h point. By applying the SAM algorithm (Statistical Analysis of Microarrays; Tusher *et al.* 2001) to these filtered data, I identified 5,693 genes in which the mean signal intensity of treated and control samples differed significantly (False Discovery Rate, FDR <5%) at either one or both time points. I focused my attention on the subset of these transcripts that differed by ≥ 1.5-fold between treated and untreated tissues, and that were also detectable above background intensity at each time point. In summary, I used following four criteria to select Al-responsive genes: above the background cut-off, AGI identifier, FDR <5%, and, fold change ≥ 1.5-fold.

By using these criteria, transcripts for a total of 401 distinct genes, where 170 increased and 231 decreased in abundance, were found Al-responsive after 6 h exposure (Fig. 2.3). Similarly, transcripts for 1,114 distinct genes, where 601 increased in abundance and 513 decreased in abundance; were found Al-responsive after 48 h exposure. Among the 401 distinct genes responding after 6 h, 42% (170) showed increased abundance of transcripts, while 54% (601) of the 1,114 distinct genes responding after 48 h showed increased abundance of transcripts. Similar trends were observed when a 2- fold cut off was used. A greater number of responsive genes were observed after 48 hours (733) compared to 6 h (127) and transcripts for 42% (53) and

57% (419) of responsive genes showed increased abundance after 6 h and 48 h (respectively).

I also calculated the proportion of common genes present at both time points (Fig. 2.3). Among the 170 genes that increased in transcript abundance after 6 h, only 12% of these also increased after 48 h treatment, compared to 20% that decreased in transcript abundance after 48 h treatment (1.5-fold cutoff). Similarly, among the 231 transcripts that decreased in transcript abundance after 6 h, only 15% of these also decreased in abundance after 48 h compare to 19% that increased in abundance after 48 h exposure. A similar pattern was observed for data from the 2-fold cutoff.

Although 70 mer sequences printed on arrays normally represent unique regions within each gene, transcripts belonging to genes from large gene families occasionally share high sequence identities and therefore could result in cross-hybridization. As a consequence, some of the genes identified by this research as Al-stress responsive may not in fact be associated with Al stress. Analysis of data from both the 1.5-fold and 2.0-fold cutoffs reveal relatively little overlap between the identities of transcripts that increased or decreased at each time point. Moreover, the proportions of transcripts that had the same pattern of expression at each time point were similar to the proportion of transcripts that had the opposite pattern of expression at subsequent time points. Thus, remodeling of the transcriptome following Al treatment appears to be a dynamic process with distinct features at early (6 h) and late (48 h) time points following exposure. Given the similar overall patterns of response detected using the 1.5-fold and 2.0-fold cut off thresholds, my subsequent analysis of results focuses only on 1.5-fold data.

2.3.2. Cellular pathways and functional categories of transcripts responsive to aluminum

I categorized my transcriptional profiling results according to inferred function or structural similarity to other genes, as defined by resources including the Gene Ontology consortium and The Arabidopsis Information Resource (TAIR), as well as previous descriptions of the physiological and molecular effects of Al treatment. The majority of Al responsive transcripts with known functions could be assigned to one of the following seven functional categories (Fig. 2.4) : i) oxidative stress responses; ii) transporters; iii) energy and primary metabolism; iv) polysaccharide and cell wall metabolism; v) protein metabolism; vi) signaling and hormones; and vii) transcription factors.

2.3.2.1. Oxidative stress responses

The metabolic balance of cells is disrupted by many biotic and abiotic stresses, resulting in enhanced production of reactive oxygen species (ROS; Mittler 2002). Increased ROS levels are an important component of Al stress (Richards *et al.* 1998, Basu 2001, Yamamoto *et al.* 2002). A set of 152 genes has been defined as comprising the ROS response network in Arabidopsis (Mittler *et al.* 2004). Among the 138 ROS response network genes spotted on my arrays, transcripts from 50 genes were detected above background for 6 h, and transcripts from 59 genes were detected above background for 48 h (Table 2.1). I observed increased abundance of transcripts for ascorbate peroxidase (At3g09640), glutathione reductase (At3g24170) and superoxide dismutase (At4g25100) after 48 h exposure. In contrast, I observed decreased abundance of transcripts for other genes nominally associated with oxidative stress responses, including alternative oxidase (At3g22370 and At5g64210), glutaredoxin (At1g03850), peroxiredoxin (At1g60740), monodehydroascorbate reductase (At5g03630; *MDAR*) and

thioredoxins (At4g35460). Interestingly, some genes (e.g. *MDAR* and thioredoxins) encoding similar protein sequences had opposite responses to treatment within or between the two time points measured. This may point towards the existence of different mechanisms of regulation of expression within the same class of genes, perhaps reflecting differential spatial expression or functional diversification.

Class III peroxidases are involved in diverse functions including the formation of ROS, cross-linking of cell wall constituents, and catabolism of lignin and certain hormones (Bakalovic *et al.* 2006). These heme-containing peroxidases constitute another family of genes for which I observed contrasting patterns of increased and decreased abundance of individual transcripts following 6 h and 48 h of treatment (Table 2.1). Among the 73 class III peroxidase genes predicted in the Arabidopsis genome, 71 were printed on my array, I detected transcripts of 36 and 41 genes above background for 6 h and 48 h respectively. In contrast to the genes in the ROS network, for which differences in transcript abundance were more commonly detected after 48 h of exposure, differences in transcript abundance for Class III peroxidases were more commonly detected after 6 h of exposure. Transcripts for ten Class III peroxidase genes increased after 6 h, while four of these decreased at the same time point. A nearly opposite pattern was observed for the 48 h profile, in which transcripts for only one gene increased and three others decreased. Because Class III peroxidases are a multifunctional class of proteins (Passardi *et al.* 2005), the various patterns of expression could represent a wide range of peroxide-related responses to Al stress.

Transcripts for Glutathione-S-transferase (GST) also showed diverse responses to Al stress. GST enzymes conjugate toxins to glutathione and form S-glutathionylated products, which are transported to the vacuole through the action of ATP Binding Cassette (ABC) transporters (Rea 1999). These enzymes also help to protect cells from

oxidative damage. Among the 53 GST genes in the Arabidopsis genome, I printed 49 different GST probes on my arrays and detected transcripts for 28 and 23 genes above background for 6 h and 48 h respectively. Transcripts for a total of three genes increased in abundance and eight decreased in abundance after 6 h exposure to Al (Table 2.1), whereas transcripts for two genes (At1g10370, At1g78380) were more abundant and transcripts for one gene (At5g62480) was less abundant at 48 hours. Approximately, 50% of the GSTs identified as Al responsive belonged to the Tau sub-family. This points towards the important role of Tau class of GSTs under stress conditions (Kilili *et al.* 2004).

2.3.2.2. Transporters

Regulated movement of solutes and various macro-molecules across cell membranes is an important component of stress responses. I observed differential abundance of transcripts for several types of transporters following Al treatment (Table 2.2). These included ABC transporters, aquaporins, sugar transporters, and antiporters. The ABC group of transporters, which belong to large gene family with diverse functions, are also known to facilitate the movement of glutathionylated toxins and other substrates across biological membranes (Rea 1999). Among the 94 ABC transporters in the Arabidopsis genome, I printed probes for 88 different transporter genes on my arrays (Table 2.2). I found reduced levels of transcript for a single gene after 6 h, whereas transcripts for a total of 3 genes increased in abundance and 4 genes decreased in abundance after 48 h stress. These results indicate that ABC transporters, which work in conjunction with other detoxifying systems, were associated primarily with later stages of Al stress.

Aquaporins transport water, gases, uncharged solutes, and micronutrients across the plasma membrane or tonoplast (Tyerman *et al.* 2002). Among the 35 aquaporin genes in the Arabidopsis genome, I printed 33 different probes on my arrays. I found transcripts for 9 genes increased in abundance after 6 h exposure to Al, whereas transcripts for one aquaporin gene increased (At2g21020) and transcripts for another aquaporin (At4g17340) decreased in abundance after 48 h (Table 2.2). These observations suggest that the regulation of water or solute movement by aquaporins may be an important early response to Al stress.

Plant sugar transporters are responsive to many stresses, including pathogen infection and wounding (Lalonde *et al.* 1999). I detected differences in transcript abundance for several sugar transporters following Al exposure. Among the 48 sugar transporter genes present in the Arabidopsis genome and 47 printed on my arrays, I detected increased abundance of transcripts for a hexose transporter (At5g61520) and an uncharacterized sugar transporter (At1g08900) after 48 h of Al treatment. Conversely, transcripts for a sugar transport protein (*STP13*; At5g26340) and sugar-porter family protein1 gene (*SFP1*; At5g27350) decreased in abundance after 6 h and 48 h exposure respectively. *STP13* is tightly related to programmed cell death where as *SFP1* is similar to a major monosaccharide transporter known to be induced by higher sugar concentration, and during leaf senescence (Quirino *et al.* 2001). More recently, over-expression of *STP13* has been reported to improve plant growth and nitrogen use in Arabidopsis (Schofield *et al.* 2009).

Among the 70 antiporter genes in the Arabidopsis genome, I printed 66 different antiporter probes on my arrays. I observed decreased abundance of transcripts encoding one Na^+/H^+ antiporter (At2g01980) after 6 h and 48 h exposures, while transcripts for a gene encoding a Ca^{2+}/Na^+ antiporter (*CAX7;* At5g17860) were decreased in abundance

after 6 h and then increased in abundance after 48 h of Al exposure. The Ca^{2+}/H^{+} exchanger family contains 11 members that have been named *CAX1* to *CAX11* and some members have been identified to play a role in sequestration of metals and calcium into the vacuole (Pardo *et al.* 2006).

I also observed differential regulation of two other genes encoding Ca^{2+}transporters. Transcript abundance patterns varied depending upon their sub-cellular location. Transcripts of a plasma membrane or plastid envelope Ca^{2+}-ATPase gene; *ACA1* (At1g27770), decreased by more than 50 fold; whereas transcripts for an ER-type Ca^{2+}-pumping ATPase; *ECA1* (At1g07810) increased by 50 fold. Since internal concentration of Ca^{2+} is a primary signal of a stress, transporters responsible for this Ca^{2+} influx may represent one sensor of stress (Xiong *et al.* 2002).

Exposure to Al reduces movement of ions across the plasma membranes of roots (Matsumoto 2000). I observed differential abundance of transcripts encoding various proteins involved in regulating intracellular concentrations of potassium, nitrogen, sulfur, sodium, and calcium. In addition to the genes identified in Table 2.2, I detected decreases in transcript abundance for three different K^{+} channels genes, namely *AKT2* (At4g22200), K^{+} channel beta subunit *KAB1* (At1g04690), and *KEA3* (At4g04850). However, I also observed a consistent increase in abundance of transcripts for a gene (At2g24240) encoding a K^{+} channel tetramerization domain protein. I found changes in transcript abundance for two NH_4^{+} transporters: ammonium transporter1, member3 (*AMT1.3;* At3g24300) increased in abundance, while transcripts for a similar protein (At3g24290) decreased following Al exposure. I also found increased levels of transcripts for three nitrate transporter genes (At1g08100, At3g45060 and At1g08090) after 6 h, and two sulfate transporter genes (At1g77990 and At5g10180) after 48 h. Sulfur is a constituent

of the amino acid cysteine, which is required for glutathione biosynthesis, which in turn is a major component of plant defenses against oxidative stress.

2.3.2.3. Energy and primary metabolism

Among the 70 glycolysis and 56 TCA genes in the Arabidopsis genome, I deposited probes for 64 glycolysis and 52 TCA cycle related genes on my array (Table 2.3). Seven glycolysis or TCA-related transcripts showed differential abundance following Al treatment. Within the glycolytic pathway, transcripts for fructose-bisphosphate aldolase (At2g16940) increased after 6 h of stress, while transcripts for another fructose-bisphosphate aldolase isoform (At2g36460) decreased after 48 h. Transcript levels for three genes encoding pyruvate kinase (At5g63680, At3g04050, At3g49160) increased after 48 h exposure, suggesting that pyruvate synthesis may be a favored process when roots experience Al stress. In addition to glycolysis related genes, I observed increased abundance of transcripts encoding pyruvate dehydrogenase (after 48 h Al treatment) that carries out oxidative decarboxylation of pyruvate to form acetyl CoA (an initial substrate for the TCA cycle). Among all TCA cycle genes, transcripts for only one gene, a mitochondrial malate dehydrogenase [Nicotinamide Adenine Dinucleotide] (*MDH*, At1g53240), increased in abundance after 48 h. A role for MDH in Al resistance is consistent with the Al-resistant phenotype observed (Tesfaye *et al.* 2001) in a transgenic line of alfalfa (*Medicago sativa*) over-expressing *MDH*.

The pentose phosphate pathway (PPP) is the major generator of reducing power (NADPH) in roots (Esposito *et al.* 2003). Among the 32 PPP genes present in the Arabidopsis genome, I deposited probes for 31 genes on my array (Table 2.3). Glucose-6-phosphate-1-dehydrogenase (G6PDH) and 6-phophogluconate dehydrogenase are two important components of the PPP. I found increased abundance of transcripts for two

genes encoding 6-phosphogluconate dehydrogenase (At1g64190 and At5g41670) at 6 h and 48 h exposures respectively, whereas, transcripts for a similar protein (At1g17650) decreased in abundance after 6 h and then increased after 48 h exposure. It is possible that because Al generates a burst of ROS, roots enhance the generation of NADPH to maintain a high ratio of reduced forms of antioxidants such as glutathione for the proper scavenging of ROS in cells.

2.3.2.4. Polysaccharide and cell wall metabolism

Glycoside hydrolases (GH) are a diverse group of enzymes organized into 31 families. These enzymes hydrolyze specific bonds involving one or more saccharides (Coutinho and Henrissat, 1999). Among the 289 GH probes printed on my microarrays, I detected hybridization to 78 of these above background (Table 2.3). I detected increased abundance of transcripts for six GH genes and decreased abundance of transcripts for seven GH genes after 6 h of exposure. However, after 48 h of stress, transcripts for more genes increased (14) than decreased (3) in abundance. I observed a predominance of families GH16 (containing xyloglucan endotransglycosylases; XTHs) and GH28 (containing polygalacturonases) among the Al-responsive transcripts related to glycoside hydrolases. Notably, transcripts for three Al-responsive XTH (GH16) genes decreased in abundance, after 6 h exposure including *ATXTH19* (At4g30290), *ATXTH18* (At4g30280) and *MERI5B/BRU* (At4g30270). In contrast, all of the Al-responsive transcripts of polygalacturonases (GH28) increased in transcript abundance after 48 h. Decreased abundance of XTH at 6 h and increased abundance of polygalacturonases at 48 h suggest a role for physiological and structural changes in the root cell wall during early and late phases of Al stress responses.

Glycosyl transferases (GTs) catalyze the transfer of sugar moieties from activated donor molecules to specific acceptor molecules, thereby forming glycosidic bonds (Coutinho *et al.* 2003). Among the 279 GT-related genes in the Arabidopsis genome, I printed probes for 269 genes on my arrays. I observed differential abundance of transcripts for 11 genes encoding GTs, primarily after 48 h (Table 2.3). This indicates that glycosylation processes, including synthesis of cell wall components, post-translational modification of proteins, and conjugation of other molecules could be an important component of cellular response towards Al stress. The activity of GTs is required for cell wall synthesis and hence may affect the thickening and rigidification of cell walls, and the inhibition of longitudinal cell expansion that have been reported under Al stress (Sasaki *et al.* 1996, Jones *et al.* 2006).

Among the other 152 cell wall-related genes represented on my arrays, I detected transcripts for 48 and 56 genes (6 h and 48 h respectively; Table 2.3), and the abundance of 4 and 7 genes were affected by Al stress after 6 and 48 h respectively. Caffeic acid O-methyltransferase (COMT) and 4-coumarate:CoA ligase (4CL) play important roles in lignification of cell walls. I observed increased abundance of transcripts for a COMT-like gene (At1g76790) and decreased abundance for a 4CL family gene (Table 2.3). Among three expansin genes detected in my data, transcripts for two genes (At3g45970 and At4g01630) increased whereas transcripts for another gene (At3g45970) decreased in abundance. Some Lipid Transfer Proteins (LTP) also play a role in cell wall extension (Nieuwland *et al.* 2005). I found increases (At3g18280 and At2g48140) and decreases (At3g22600; 6 h and At1g32280; 48 h) in the abundance of transcripts for LTP-encoding genes. Collectively, these results may highlight the importance of dynamic changes in cell wall structure during the Al-stress response.

2.3.2.5. Protein metabolism

Among the 228 ribosome proteins in the Arabidopsis genome, I printed probes for 200 genes on my arrays (Table 2.4). I observed increase in transcript abundance for five ribosomal genes after 6 h treatment, and did not observe any decrease in transcript abundance for ribosomal genes during this time period. However, after 48 h exposure, transcripts for five ribosomal genes increased and transcripts for three genes decreased in abundance, indicating that there may be increased demand for specific ribosomal components during Al exposure. This contrasts with reports of more generally decreased abundance of transcripts for ribosomal proteins in roots of Arabidopsis under conditions of salt stress (Jiang and Deyholos 2006).

Heat Shock Proteins (HSPs) are important in stabilizing, folding, and degrading damaged proteins. Among the 70 HSP-related genes found in the Arabidopsis genome, I printed probes for 68 on my arrays. I found increased abundance of transcripts for a gene encoding a chloroplastidic form of *HSP101* (At5g57710) after 48 h, whereas, transcript for two other genes (*HSP101*; At1g74310 and Aha-1 domain containing protein; At3g12050) decreased in abundance (Table 2.4).

Nutrient recycling and proteome modification require peptidases. The Arabidopsis genome contains at least 678 endo-peptidases, which have been grouped into seven super families (Rawlings *et al.* 2004). I printed probes for 524 genes on my arrays. Four endopeptidase super families were predominately represented on my arrays: aspartic peptidases, cysteine peptidases, metallopeptidases and serine peptidases (Table 2.4), with serine peptidases most frequently detected, followed by aspartic acid peptidases. Among the four aspartic acid peptidase encoding genes whose transcripts increased after Al exposure, transcripts for one gene (At3g61820) increased by over 200-fold after 48 h.

The ubiquitin proteosome pathway confers specificity to protein degradation (in contrast to the less specific peptidases described above) by tagging targeted proteins with ubiquitin (Moon *et al.* 2004). The 26S proteasome is a multi subunit complex that consists of a cylindrical 20S core protease with a 19S regulatory particle cap on both ends. I found transcripts from three genes encoding for 19S regulatory subunit to be differentially abundant in Al stressed roots after 48 h (Table 2.4). Approximately 90% of the genes specific to the Ub/26S pathway encode subunits of E3 ubiquitin ligases, which confer substrate specificity to the pathway (Smalle and Vierstra 2004). A large number of E3 ligases were found Al responsive after 48 h of stress. Interestingly, transcripts for an E3 ligase-RING encoding gene (At1g19310) decreased after 6 h, and increased over 100 fold after 48 h. However, transcripts for another E3 ligase-RING encoding gene (At2g34000) increased for both 6 h and 48 h exposures, with over 50 fold increase after 48 h stress. My data suggest that degradation of specific proteins may be regulated by transcriptional control of E3 ligases upon perception of Al stress (Scarafia *et al.* 2000).

2.3.2.6. Signaling and hormones

Perception and transmission of stress signals are likely to be important aspects of plant response to Al toxicity. Several components of signaling pathways detected on my arrays were responsive to Al, including protein kinases, protein phosphatases and genes related to hormone synthesis or perception, as well as transcription factors.

The Arabidopsis genome contains an estimated 979 protein kinases classified into 5 classes and 81 families. Of the 921 genes (protein kinases and protein phosphatases) represented on my arrays, I detected transcripts of 271 and 275 genes above background after 6 h and 48 h of stress, respectively (Table 2.5). I found transcripts of 1 gene increased in abundance, and transcripts of 11 genes decreased in abundance at after 6 h

exposure. After 48 h of Al exposure, transcripts for 21 kinase or phosphatase genes increased and transcripts for 15 decreased in transcript abundance. Most of the kinases that I detected belonged to the two largest classes namely, Class 1/ PPC 1(transmembrane receptor kinase and related non-transmembrane kinase), and Class 4/ PPC 4 (non-transmembrane protein kinase). Increased abundance of transcripts for a transmembrane receptor gene (At1g29740) belonging to S-domain kinase family were detected after 6 h as well as 48 h of exposure. Transcripts for three additional transmembrane receptor genes belonging to the leucine-rich repeat protein kinase family (At5g59660 and At2g24230) and CRPKIL-1(At2g23200) protein kinases were also abundant after 48 h of Al stress. Transmembrane receptor kinases can play an important role in stress perception and transmission from one cell to another. Differential abundance of transcripts for a number of different transmembrane receptor kinases after 48 h exposure to Al suggested that multiple receptors belonging to different classes may have unique regulatory mechanisms.

Protein phosphatases are divided into three groups: Serine/Threonine phosphatases (ST), Dual Specific Phosphatases (DSPs), and Protein Phosphatase 2C (PP2C; Tchieu *et al.* 2003). Among the 125 protein phosphatase specific genes in the Arabidopsis genome, I printed 116 on my arrays and detected 40 and 42 above background after 6 and 48 h respectively. I did not observe any protein phosphatase transcripts that increased in abundance after 6 h of stress treatment (Table 2.5). However, after 48 h I found increased abundance of transcripts for three protein phosphatases, namely: a ST phosphatase (phosphoprotein phosphatase, PPX-1; At4g26720), a PP2C gene (At2g33700), and a calcineurin-like phosphoesterase family protein (At3g09970). Interestingly, PPX-1 has been reported to be localized in the epidermal plastids of roots (Pujol *et al.* 2000).

Hormones play an important role in signaling. My results indicated that transcripts putatively related to auxin, ethylene, and cytokinin metabolism were affected under Al treatment (Table 2.5). Among the 20 IAA (auxin) biosynthesis genes present in genome of Arabidopsis, I printed probes for 19 genes. I detected transcripts encoding an auxin biosynthesis gene, *NITRILASE* 2 (At3g44300), decreased in abundance after 48 h of stress. Transcripts for *PIN*7 (At1g23080), an auxin efflux carrier, decreased sharply after 48 h of stress. Also, transcripts for IAA amido synthase (At5g54510) increased by approximately 130-fold. IAA-amido synthase conjugates Ala, Asp, Phe, and Trp to auxin and controls auxin homeostasis. These changes at the transcript level may reduce auxin activity and transport and may be part of the mechanism limiting lateral root growth under Al stress. I found decreases in transcript abundance for two genes involved in the ethylene biosynthesis pathway after 48 h of exposure to Al. Specifically, transcripts for two genes encoding a key enzyme for ethylene biosynthesis, 1-aminocyclopropane-1-carboxylate synthase (ACC synthase, At4g08040, At5g64330) decreased in abundance by approximately 200 fold after 48 h of Al-exposure.

2.3.2.7. Transcription factors

The Arabidopsis genome contains approximately 1,922 genes encoding transcription factors (TFs) divided into 62 groups (Guo *et al.* 2005). Among the 1,613 transcription factors represented on my microarray, 426 and 445 genes were detected above background for 6 h and 48 h respectively (Table 2.6). The majority of Al responsive transcripts belonged to Apetala-2/EREBP (AP2/EREBP), Myeloblastosis (MYB), basic helix-loop-helix (bHLH) and Cys2- His2 (C2H2) families. These are also some the largest TF families present in the Arabidopsis genome. Among TFs represented by transcripts that were highly enriched following Al-treatment (primarily after 48 h of

treatment), I observed members of the following families: C2C2-GATA (At4g24470 and At5g56860); TAZ (At5g63160); E2F/DPE2FC (At5g03415); GARP-G2-like (At1g13300) and SBP (At2g33810). Transcripts for some of the genes found highly abundant belonged to GRAS family (At1g50600, 250 fold) and bZIP family (At1g32150, 28 fold). Also, some TF encoding genes showed opposite transcript abundance patterns for 6 h and 48 h Al treatment. For example, transcripts for AP2/EREBP family gene (At5g67180) and MYB family gene (At1g17950) decreased by 2 fold at 6 h but increased by over 250 fold after 48 h of Al exposure.

2.3.3. Validation of microarray data

To validate the results of my microarray analysis, I conducted quantitative reverse transcription PCR (QRT-PCR). I selected ten transcripts representing a variety of expression patterns and biological functions, including five closely related members of the class III peroxidase family (Table 2.7). In addition, I selected ubiquitin-conjugating enzyme (UBC; At5g25760) as a reference gene (Czechowski *et al.* 2005) and confirmed its stable expression pattern in root tissue from eight independent samples collected after 6 h and 48 h exposures (data not shown). I measured the abundance of these transcripts in each of three biologically independent replicates for each time point (6 h or 48 h) and each treatment (0 or 25 μM Al), where at least one replicate in each case was independent of samples used for microarray hybridization. For nine of the ten transcripts analyzed, the general pattern of Al-treatment responses I observed was the same whether measured by QRT-PCR or microarray, although the magnitude of the changes in transcript abundance differed in most cases between the analytical techniques. Since most of the genes selected for validation of microarray belonged to large gene families, the selected primer

pairs for some of these gene may also to bind to related genes. A WU-BLAST analysis predicted that while *PER69*, *PER*62, *PER27*, *GSTF6*, IAA-amido synthase, and thioredoxin primers are gene specific, other primers: *PER2*, *GSTU4*,and *ECA1* primers could bind at least one other closely related gene. However, the dissociation curve of QRT-PCR for all of the products reported was consistent with a homogeneous amplification product. Also, primer pairs designed originally for *PER49* aligned with sequence *PER34*/*PER33* (At3g49110/At3g49120) instead of *PER49* indicating perhaps an error in naming the primer. Notwithstanding these limitations the changes in transcript abundance that I described using microarray analysis appeared to be reproducible independent of the analytical technique (Table 2.7). The sole exception was At3g15360 (thioredoxin) for which different responses were observed at 6 h using the microarray and QRT-PCR techniques.

To further detect dynamic changes in transcriptome beyond the two time points (6 h and 48 h) selected for microarray, I exposed hydroponically grown roots of Arabidopsis to 1 h, 3 h, 6 h, 12 h, 24 h, and 48 h of Al treatments. I measured the transcript abundance of five peroxidase genes (*PER2*, *PER27*, *PER34*, *PER62*, and *PER 69*) in three biologically independent samples for each time point using QRT-PCR. Interestingly, I detected dynamic changes in the transcript abundance for all peroxidases with changes detected as early as 1 h for most of them (Fig. 2.5). To determine if sequence identity correlated with similar transcript abundance patterns, I looked for the similarities and the differences in temporal expression profiles of peroxidases and compared them with available sequence information. I found no correlation between sequence identity and temporal expression profiles, e.g., despite 78% identity (highest among all five peroxidases), *PER62* and *PER69* displayed differences. Overall, all

peroxidases I studied followed a general trend of increased transcript abundance soon after exposure to Al followed by decline after 48 h exposures with sole exception *PER62*.

2.4. Discussion

I conducted microarray analysis to identify transcripts in Arabidopsis roots that changed in abundance following exposure to Al. I have focused on roots because soil is the site of Al exposure, and inhibition of root elongation is primary and most dramatic effect of Al toxicity (Kochian 1995). As a prerequisite to transcriptional profiling, I optimized a hydroponic growth system that allowed efficient sampling of roots from plants that had been treated with a well-defined activity of Al^{3+}.

I present here a large scale, transcriptomic analysis of root responses to Alusing a microarray with probes representing approximately 93% of the predicted genes in the Arabidopsis genome. I observed a broader range of statistically significant changes in transcript abundance after 48 h (1,114 genes) as compared to 6 h (401 genes) of Al treatment. There was relatively little overlap in the complement of Al-responsive transcripts detected at each time point (Fig. 2.3). These two trends were conserved in each of the eight biologically independent samples I analyzed at each time point, and also when comparisons were made for genes where transcript abundance changed by ≥ 1.5-fold or ≥ 2-fold following Al treatment. Because I detected hybridization signal intensities above background for an almost equal number of probes at each time point, the differences between 6 h and 48 h samples were not due to systematic biases in measurement. Neither could the different transcript abundance patterns be attributed to circadian effects, considering that each Altreated sample was compared to an untreated control sample harvested at the same time point. Thus, distinct sets of transcripts appear

to be expressed following 6 h and 48 h of Al treatment, with a much larger set of responsive transcripts observed after 48 h of treatment. These observations contrast with most previously published microarray analyses of other abiotic stress responses, in which the bulk of transcriptomic changes were generally detected at early (e.g. 6 h) rather than later (e.g. 24 h, 48 h) intervals after the imposition of stress (Kreps *et al.* 2002, Wang *et al.* 2003, Jiang and Deyholos 2006). This suggests that treatment with Al imposes some unique challenges to plants that are reflected in the transcriptomic response. This could reflect the pervasive nature of Al injury, which accumulates over time.

My results are in agreement with previous reports of Al-induced expression of specific components of the ROS response network, including ascorbate peroxidase, superoxide dismutase, MDAR, glutathione reductases, and GSTs (Richards 1998, Basu 2001, Basu 2004, Chen 2005). Although oxidative stress is commonly presumed to be a major component of Al stress, I observed increased transcript abundance for only a small proportion of ROS network genes (Table 2.1). These ROS network genes play a role in responding to increased production of ROS under conditions of Al stress. Alternatively, regulation of gene expression could initiate a transient change in redox state that may act as signal for activation of other stress responsive genes.

The majority of ROS-related transcripts that I observed to increase in abundance following Al treatment belonged to the multifunctional, Class III family of peroxidases (Table 2.1) which can either generate ROS, or detoxify them (Passardi *et al.* 2005). Modulating the expression and activity of peroxidases could affect plant growth via regulation of multiple processes including cell wall formation and loosening. I used QRT PCR to confirm transcript abundances of five peroxidases (*PER2, PER27, PER49, PER62,* and *PER69)* after 6 h and 48 h of Al exposures (Table 2.7).

Sequence similarities among members of class III peroxidases suggest occurrences of genomic rearrangement resulting in extensive duplication and perhaps functional diversification in Arabidopsis (Tognolli *et al.* 2002). To determine if duplication events in Class III peroxidases have any relevance to functional diversification within this class, I studied a comparative transcript profiling for selected peroxidases under different duration of Al stress for (Fig. 2.5). Even though *PER62* and *PER69* have high protein sequence similarity (Tognolli *et al.* 2002), each peroxidase had a distinct transcript profile. Overall, all peroxidases I studied followed a general trend of increased transcript abundance soon after exposure to Al followed by decline after 48 h exposures with sole exception *PER62*. This may point towards a unique role of Class III peroxidases when roots were exposed to Al. Further characterization of these peroxidases using promoter deletion, reporter fusion, and knock out based expression studies would help to elucidate their unique role under Al stress conditions.

Recent studies have demonstrated that some metabolic enzymes are regulated by enzymes of the ROS network, including thioredoxins (TRX; Hisabori *et al.* 2005, Hara *et al.* 2006), and via glutathionylation (Dixon *et al.* 2005, Zaffagnini *et al.* 2007). I detected increased transcript abundance of TRXs and components of the glutathione antioxidant system. For example, transcripts of a thioredoxin gene increased by 1.5-fold after 48 h Al exposure (confirmed by QRT-PCR). This gene is similar to thioredoxins reported to activate a cytosolic form MDH (Hara *et al.* 2006). In addition to their roles in mitigating oxidative damage within the cells, TRXs and other components of the ROS network may therefore control steps of metabolic pathways (Dixon *et al.* 2005), including OA exudation.

Exudation of organic anions (OA) is a strategy for alleviating Al toxicity that has attracted considerable attention in recent years. Some OAs are capable of forming a stable

complex with Al, thereby preventing the binding of Al to extra- and intracellular ligands. Ma *et al.* (2001) proposed two patterns of OA release. In Pattern I, secretion of OAs is rapid, due to the activation of anion channels and does not require transcription. In Pattern II, secretion of OA occurs after a lag of several hours, and is proposed to arise from the induction of various genes encoding biosynthetic enzymes and or transport proteins. I did not detect a significant increase in transcript abundance for any of the genes of the TCA cycle, except for MDH (malate dehydrogenase). MDH is also differentially abundant in response to various other abiotic stresses including NaCl (Zimmermann *et al.* 2004, Jiang and Deyholos 2006). Previous reports have failed to detect increased activity of several wheat TCA cycle enzymes (*in vitro*) upon Al exposure, even though OA exudation was seen to increase (Hayes and Ma 2003). My results also indicate that transcript levels for these enzymes are unaffected by Al stress. Nonetheless, overexpression studies of citrate synthase in canola (Anoop *et al.* 2003) and malate dehydrogenase in alfalfa (*Medicago sativa*; Tesfaye *et al.* 2001) have shown enhanced resistance to Al stress. At first sight, data such as these appear to be contradictory. However, experiments indicating a lack of regulation of TCA cycle enzymes have been largely based upon transcript abundance or *in-vitro* assays of enzymatic activity, both of which would not be expected to provide insight into post-transcriptional regulation. Increased abundance of proteins or increased activities of proteins as a result of post translational modification, along with enhanced efflux via transporters might be the important points of regulation of organic anion secretion. Recently, aluminum activated malate transporter (ALMT1) has already been identified as an OA transporter (Sasaki *et al.* 2004).

Among several transporters responsive to Al treatment, ABC transporters have drawn my attention because of the broad range of substrates that are transported (Rea

1999), and because of speculation that Al-activated anion channels (ALAAC) could be ABC proteins (Ryan *et al.* 2001; Zhang *et al.* 2001). Recently, an ABC transporter-related gene (*ALS3*) has been suggested to impart Al resistance, presumably by redistributing Al away from sensitive tissues (Larsen *et al.* 2005). They observed that, the expression of *ALS3* increases in roots after Al treatments and the immunogold studies showed that ALS3 predominantly accumulates in the plasma membrane. Since mutants of als3 mutants exhibited extreme root growth inhibition in the presence of Al, *ALS3* was proposed to be an Al resistance gene.

Interactions between Al and Ca^{2+} have been previously implicated in the Al stress response. I observed increased abundance of transcripts for various Ca^{2+} transporting pumps, including an ER-type Ca^{2+} pump (ECA), which is known to confer resistance to Mg^{2+} stress (Wu *et al.* 2002). I have also confirmed the transcript abundance of this gene using QRT-PCR (Table 2.7). Transcripts for several Calcium Response Kinases (CRKs) were also significantly responsive to Al treatment in my study. Sivaguru *et al.* (2003) previously identified a Calcium Response Kinase, WAK1 (cell wall-associated receptor kinase 1), that was induced in response to Al stress in roots. I also identified potential Al stress responsive membrane receptor kinases belonging to S domain kinase, CRPKIL-1 and leucine-rich repeat families. Changes in cytosolic calcium concentration and differential abundance of various receptor kinases suggest the role of these proteins in Al stress signaling.

Many other uncharacterized Al resistance mechanisms have been suggested to operate in plants. Interestingly, a large number of genes with unknown biological functions were found to be increased or decreased by more than 2 fold in response to Al exposure. These unknown genes may constitute novel targets of Al toxicity and

resistance. Further characterization of these Al-responsive genes using T-DNA insertion lines, siRNA knock down lines and over expression studies would be helpful.

Even though genomics-based approaches help to identify novel cellular target of stress at transcriptome level, further integration of these data with parallel proteomics-based studies would help to uncover post-transcriptional processing in response to Al stress. A comparative analysis of large sets of available transcriptomics data from various abiotic stresses would further help to elucidate unique target specific for Al.

Figure 2.1. Growth of roots a) and shoots b) of 14 day old plants of *Arabidopsis thaliana* in the hydroponic system.

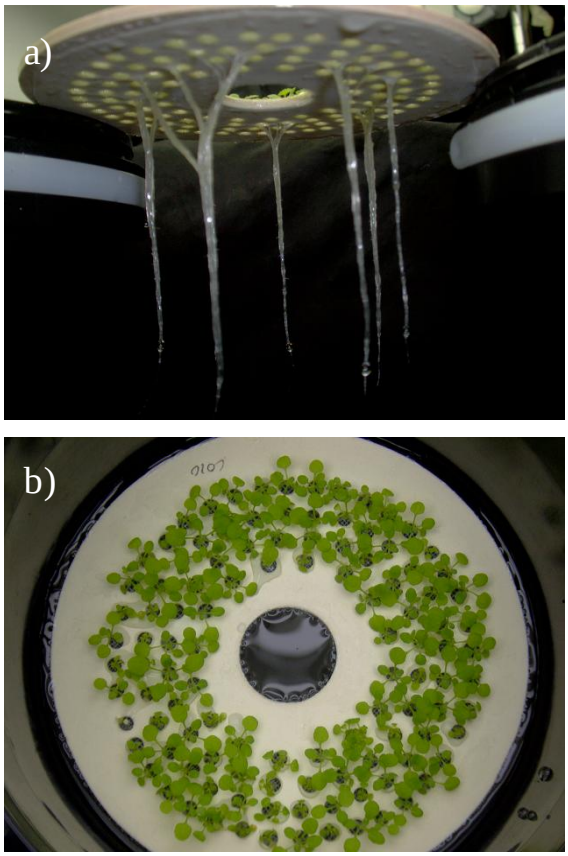
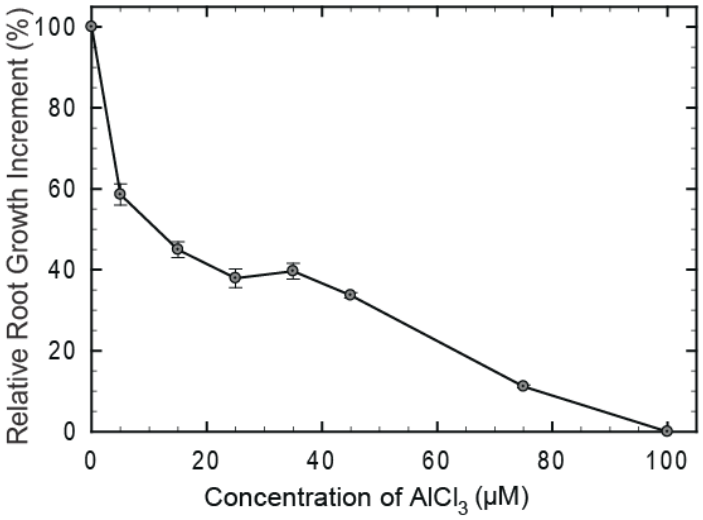


Figure 2.2. Relative root growth increment (RRGI) of roots of *Arabidopsis thaliana* after exposure to aluminum.

a) Relative root growth increment (RRGI) in response to various concentrations of aluminum after 48 h exposures; error bars are the standard error of the mean.

b) Phenotype of roots of Arabidopsis treated with 25 µM $AlCl_3$ for 48 h.

a)



b)

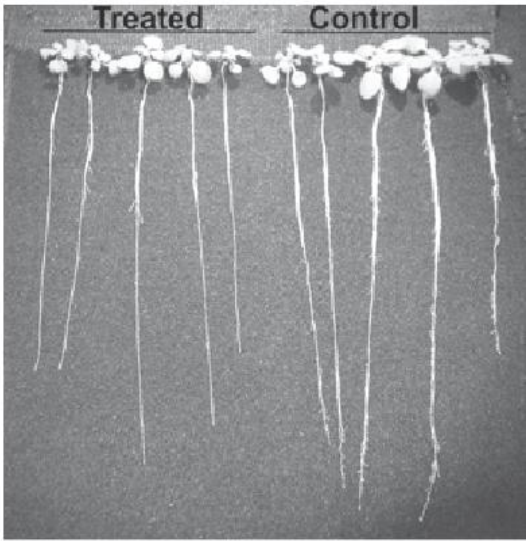
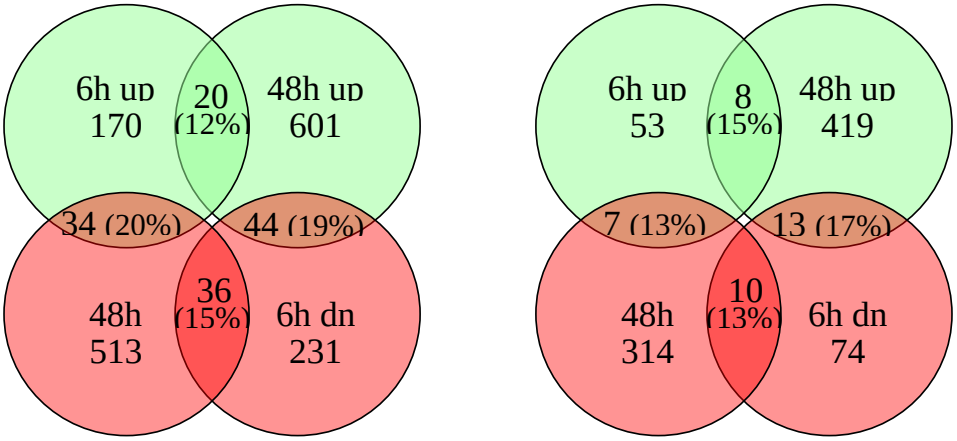


Figure 2.3. Number of genes for which transcript abundance increased (up) or decreased (dn) by >1.5-fold or >2.0-fold compared to controls when roots of *Arabidopsis thaliana* were exposed to 6 h and 48 h of aluminum stress (FDR 5%).



1.5 Fold 2.0 Fold

Figure 2.4. Functional categorization of genes for which transcript abundance increased (up) or decreased (down) by >1.5 fold compared to controls when roots of *Arabidopsis thaliana* were exposed to 6 h and 48 h of aluminum stress.

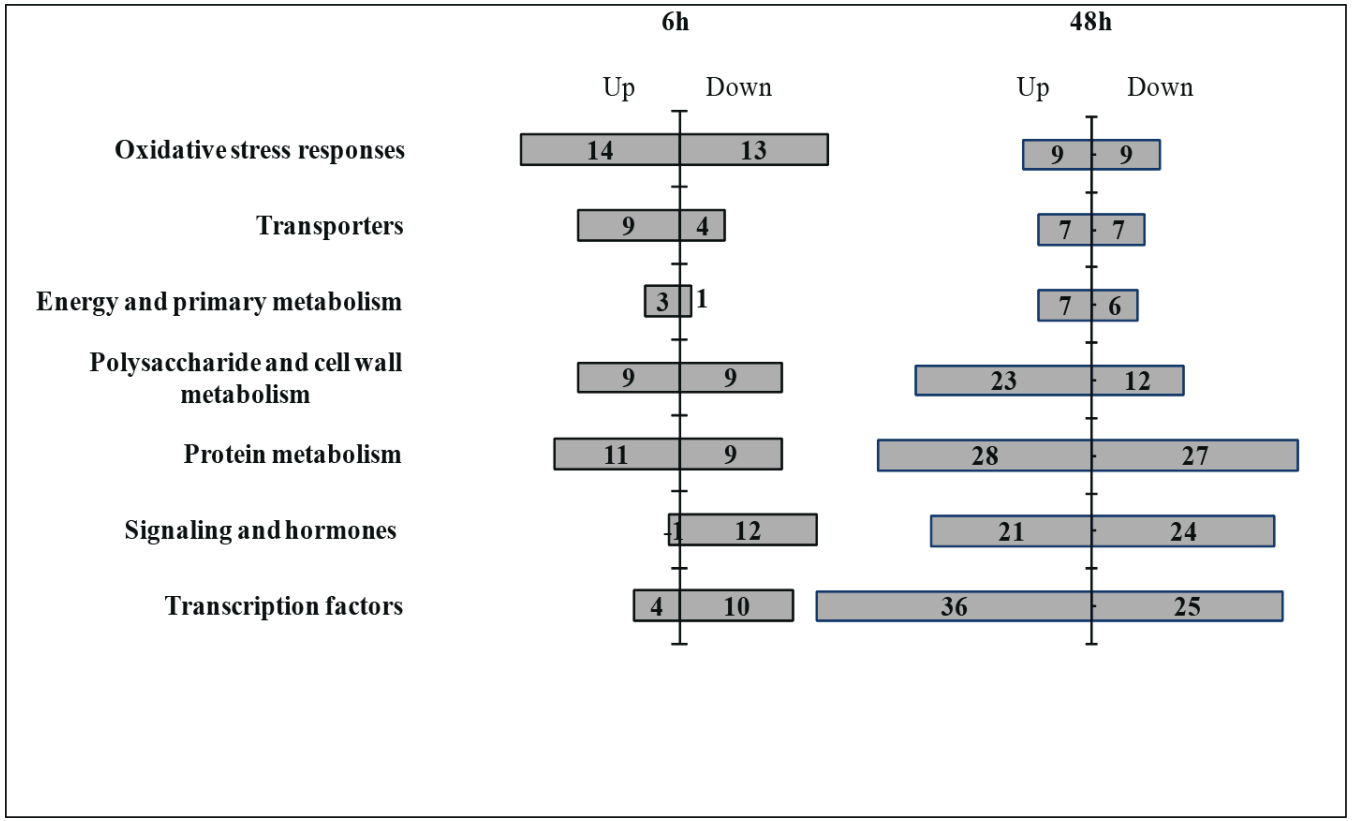


Figure 2.5. Time course study of transcript profiling of five class III peroxidases in roots of *Arabidopsis thaliana* exposed to 25 µM $AlCl_3$ in hydroponics measured by QRT-PCR. The relative expression profile for each peroxidase is labeled accordingly. Error bars are standard error of mean.

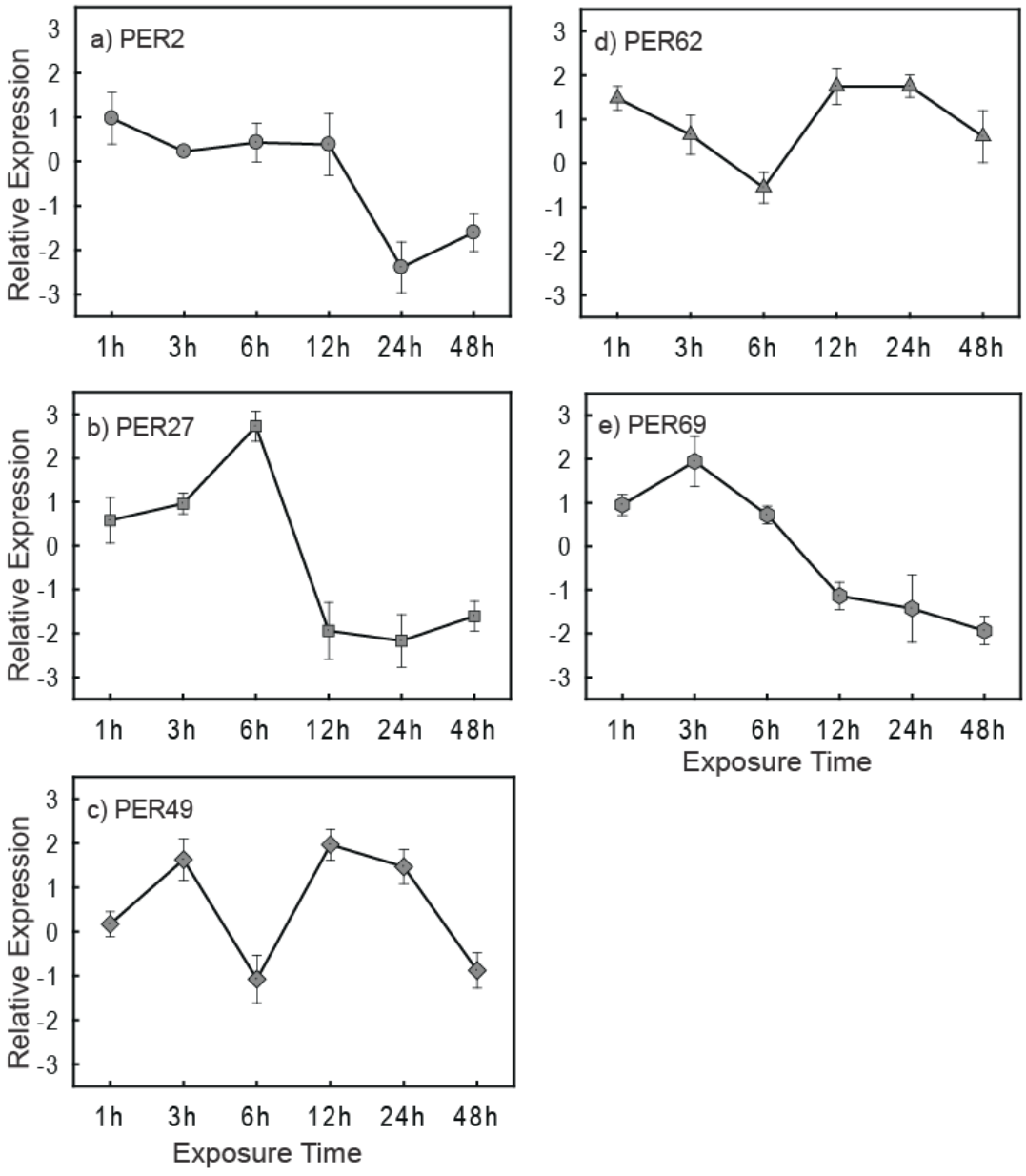


Table 2.1. Aluminum-responsive transcripts related to oxidative stress responses. Functional categories of genes, total number of genes found within the Arabidopsis genome, numbers of genes present on and detected on arrays, and numbers of genes showing significant differences (5% FDR) in transcript abundance are shown in rows and columns labeled accordingly. *Only subsections that have more proportion of genes are shown.

				6 h			48 h		
	Functional categories*	**in genome**	**on array**	**detected**	**increased**	**decreased**	**detected**	**increased**	**decreased**
1	**Reactive Oxygen Species (ROS) network**	152	138	50	At1g63940	At3g22370	59	6	5
	ROS-Ascorbate Peroxidase (APX)	9	8	2	0	0	3	At3g09640	0
	ROS-Glutathione Reductase (GR)	5	4	3	0	0	3	At3g24170	0
	ROS- (SOD)	8	8	3	0	0	4	At4g25100	0
	ROS-Alternative Oxidase (AOX)	6	6	4	0	At3g22370	4	0	At5g64210
	ROS-Glutaredoxin (GLR)	27	26	4	0	0	8	0	At1g03850
	ROS-Peroxiredoxin (PrxR)	11	10	4	0	0	4	0	At1g60740
	ROS- (MDAR)	5	5	5	At1g63940	0	5	At3g09940	At5g03630
	ROS-Thioredoxins (Trx)	32	31	13	0	0	12	At3g20560A t5g42980	At4g35460

2	**Class III peroxidase**	73	71	36	At5g67400 At5g64100 At5g42180 At5g39580 At5g19890 At4g30170 At4g21960 At3g01190 At2g37130 At1g05250	At4g37520 At4g36430 At3g49120	41	At5g15180	At5g39580 At3g01190 At2g39040
3	**Glutathione-S-Transferase (GST)**	53	49	28	3	8	23	2	1
	GST::Tau family	28	26	12	At3g43800	At3g09270 At1g78380 At1g78340	11	At1g78380 At1g10370	At5g62480

Table 2.2. Aluminum-responsive transcripts related to transporters. Functional categories of genes, total number of genes found within the Arabidopsis genome, numbers of genes present on and detected on arrays, and numbers of genes showing significant differences (5% FDR) in transcript abundance are shown in rows and columns labeled accordingly.

			6 h			48 h		
Functional categories	**in genome**	**on array**	**detected**	**increased**	**decreased**	**detected**	**increased**	**decreased**
1 **ABC transporters**	94	88	18	0	At1g30400	18	At1g04120 At2g28070 At5g03910	At3g60160 At2g37360 At2g29940 At1g71330
2 **Aquaporins**	35	33	19	At5g47450 At4g17340 At4g01470 At3g61430 At3g53420 At3g16240 At2g37180 At2g37170 At2g36830	0	23	At2g21020	At4g17340
3 **Sugar transporter**	48	47	17	0	At5g26340	18	At5g61520 At1g08900	At5g27350
4 **Antiporters**	70	66	19	0	At5g17860 At2g01980	20	At5g17860	At2g01980

Table 2.3. Aluminum-responsive transcripts related to energy, primary metabolism, polysaccharide and cell wall metabolism. Functional categories of genes, total number of genes found within the Arabidopsis genome, numbers of genes present on and detected on arrays, and numbers of genes showing significant differences (5% FDR) in transcript abundance are shown in rows and columns labeled accordingly. TCA: Tricarobxylic acid pathway. *Only subsections that have more proportion of genes are shown.

				6 h			48 h	
Functional categories*	**in genome**	**on array**	**detected**	**increased**	**decreased**	**detected**	**increased**	**decreased**
1 **Glycolysis**	70	64	38	At2g16940	0	41	At5g63680 At3g49160 At3g04050	At2g36460 At2g21170
2 **TCA**	56	52	20	0	0	25	At1g53240	0
3 **Pentose Phosphate Pathway**	32	31	11	At5g13420 At1g64190	At1g17650	11	At5g41670 At1g17650	At1g71100
4 **Polysaccharide and cell wall**								
Glycoside Hydrolases (GH)	306	289	78	6	7	78	14	3
GH Family 16	27	26	7	0	At4g30290 At4g30280 At4g30270	7	0	0
GH Family 28	52	46	6	0	0	8	At3g49170 At1g80140 At1g70500	0
Glycosyl Transferases (GT)	279	269	73	0	At2g38650	74	At5g12890 At3g45100	At1g20570 At2g38650

							At2g22590	At5g16190
							At1g71990	At5g64740
							At1g24170	At3g06440
							At1g12990	
other cell wall related	166	152	48	At1g76790	At3g22600	56	At4g01630	At3g45970
				At3g45970			At3g19450	At4g19010
				At3g18280			At2g48140	At5g48930
								At1g32280

Table 2.4. Aluminum-responsive transcripts related to protein metabolism. Functional categories of genes, total number of genes found within the Arabidopsis genome, numbers of genes present on and detected on arrays, and numbers of genes showing significant differences (5% FDR) in transcript abundance are shown in rows and columns labeled accordingly. *Only subsections that have more proportion of genes are shown.

				6 h			48 h	
Functional categories*	**in genome**	**on array**	**detected**	**increased**	**decreased**	**detected**	**increased**	**decreased**
1 **Ribosomes**	228	200	156	4	0	162	5	3
ribosome-Large	121	100	78	At3g49010 At2g39460 At1g27400	0	81	At5g27770 At3g04400	At5g60670
ribosome-Small	94	87	66	At5g59850	0	70	At3g43980 At5g52650 At5g63070	At3g02560 At1g04270
2 **Heat Shock Protein (HSP)**	70	68	34	0	0	33	At5g57710	At3g12050 At1g74310
3 **Peptidase**	678	524	165	4	5	174	10	8
peptidase:A	76	69	18	1	0	19	4	1
peptidase:A:01 aspartic	61	57	17	At3g20015	0	17	At3g61820 At3g51350 At3g51340 At1g66180	At3g20015
peptidase:C cysteine	102	102	30	0	At4g32940 At3g48340 At1g47128	35	At1g79310	At5g06600
peptidase:M	85	78	27	At1g79560	0	24	At1g44350	At4g22720

	metallopeptidase								
	peptidase:S serine	261	244	73	At5g58820 At3g01690	At1g64670 At5g58840	78	At5g67090 At5g42230 At3g51000 At3g14240	At1g29840 At1g32970 At5g39220 At5g47040 At5g58840
	peptidase:S:08 subtilisin	56	53	14	At5g58820	At5g58840	15	At3g14240 At5g67090	At1g32970 At5g58840
4	**Ubiquitin proteasome pathway**								
	19S proteasome	33	31	13	0	0	19	At2g20140	At5g43010 At5g09900
	E3:RING	430	414	107	At2g34000 At2g46160	At5g07040 At4g26400 At1g68820 At1g19310	119	At5g48655 At3g19910 At2g39100 At2g38920 At2g34000 At1g63840 At1g57800 At1g19310 At1g05120	At1g62370 At1g65430 At2g20650 At2g22120 At2g25410 At3g48070 At3g54360 At4g09110 At4g32600 At5g41350
	E3:Ubox	61	53	19	0	0	24	At2g33340 At5g64660	At3g52450 At1g20780
	E3:Ubox::Class III	12	11	4	0	0	5	At5g64660	At3g52450

Table 2.5. Aluminum-responsive transcripts related to signaling and hormones. Functional categories of genes, total number of genes found within the Arabidopsis genome, numbers of genes present on and detected on arrays, and numbers of genes showing significant differences (5% FDR) in transcript abundance are shown in rows and columns labeled accordingly. *Only subsections that have more proportion of genes are shown.

				6 h			48 h		
	Functional categories*	**in genome**	**on array**	**detected**	**increased**	**decreased**	**detected**	**increased**	**decreased**
1	**Protein Phosphatase/Kinase (PPC)**	979	921	271	1	11	275	21	15
	PPC:1 Transmembrane-receptor kinase and related	564	530	155	At1g29740	At1g05700 At3g45860 At4g05200	149	At5g59660 At4g23270 At4g21370 At2g24230 At2g23200 At1g29740 At1g11300	At1g29730 At1g65800 At3g45860 At3g51550 At3g51990 At3g56100 At3g57750 At4g39400 At5g16900
2	**PPC:2** ATN1/CTR1/EDR1/GmPK6 like	52	51	17	0	0	21	At5g50180 At3g06620 At3g01490 At1g16270	At3g50720
3	**PPC:4 Non-transmembrane-kinases**	279	265	75	0	At3g53030 At3g29160 At3g20410 At2g19400 At1g18890	82	At1g09840 At1g10940 At1g50700 At2g19400 At2g34290 At3g53030	At3g44850 At2g34180 At3g23310 At1g18890

								At5g14640	
	Calcium Response Kinase	123	119	32	0	At3g29160 At3g20410 At2g19400 At1g18890	39	At2g19400 At1g50700 At1g10940	At2g34180 At1g18890 At3g23310
4	**PPC:6 Protein phosphatase**	125	116	40	0	0	42	At4g26720 At3g09970 At2g33700	0
5	**Hormones**								
	IAA biosynthesis	20	19	4	0	0	6	0	At3g44300
	Auxin transport	8	8	2	0	0	3	0	At1g23080
	AUX/IAA inducible	29	28	9	0	0	9	At5g54510	At2g22670
	ARF	23	20	7	0	0	7	0	At1g34390 At2g28350
	Ethylene biosynthesis	28	27	9	0	0	9	0	At5g64330 At4g08040

Table 2.6. Aluminum-responsive transcripts related to transcription factors. Functional categories of genes, total number of genes found within the Arabidopsis genome, numbers of genes present on and detected on arrays, and numbers of genes showing significant differences (5% FDR) in transcript abundance are shown in rows and columns labeled accordingly.

			6 h			48 h		
Functional categories	**in genome**	**on array**	**detected**	**increased**	**decreased**	**detected**	**increased**	**decreased**
Transcription Factors	1992	1613	426	4	10	445	36	25
AP2/ EREBP	146	138	43	At5g25810	At5g67180	43	At4g27950 At5g47230 At5g50080 At5g52020 At5g67180	At5g25190 At2g28550 At1g80580
MYB	203	188	53	0	At1g17950	59	At1g17950 At2g26960 At5g40330 At5g58900 At5g65230	At3g55730 At3g48920
bHLH	163	148	35	0	0	44	At4g36930 At4g34530 At1g10610 At1g09250	At2g28160 At4g05170 At5g04150
C2H2	130	122	39	0	0	41	At1g75710 At2g28200 At3g49930	At5g40710

C2C2-GATA	29	27	14	0	0	15	At1g08010 At4g24470 At5g56860	0
TAZ	9	9	2	0	0	2	At5g63160	0
E2F/DPE2FC	8	8	4	0	0	5	At5g03415	0
GARP-G2-like	43	43	9	0	0	12	At1g13300 At1g69580	0
SBP	16	15	2	0	0	3	At2g33810	0
GRAS	32	30	10	0	0	11	At1g50600 At3g50650	At1g14920
bZIP	75	68	23	At5g65210	At1g42990 At1g32150	26	At1g32150	At4g36730
ARF	23	20	7	0	0	7	0	At1g34390 At2g28350
C2C2-co-like	31	31	8	0	0	6	0	At1g28050
GeBP	16	14	5	0	0	5	0	At2g36340
HSF	24	23	7	0	0	7	0	At4g11660 At3g63350
PcG	4	3	1	0	0	1	0	At4g02020
PHD	11	10	2	0	0	3	0	At5g63900
TCP	24	23	7	0	At1g35560	4	0	0
Trihelix	28	28	12	0	0	11	0	At3g10000
VOZ	2	2	1	0	0	1	0	At1g28520
WRKY	72	65	18	0	At5g13080 At2g38470	23	0	At4g01250
ABI3/VP1	13	13	6	0	0	6	At3g11580	0
AS2	42	39	14	At2g23660	0	10	At4g37540	0
BES1	5	5	2	0	0	1	At1g19350	0
PLATZ	9	8	4	0	0	6	At1g43000	0
CCAAT-HAP3	11	11	7	0	At1g09030	6	At5g47640	0

HB	94	92	32	At4g17460	0	29	0	At4g17460
MADS	107	96	24	0	0	23	At5g51870 At3g05860	At5g26630
NAC	113	107	30	0	At5g13180 At3g49530	30	At4g10350	0

Table 2.7. Validation of microarray with QRT-PCR. Selected genes and corrosponding log_2 fold change values from microarrays and QRT-PCR are shown here. se: standard error.

		6 h			48 h		
		microarray	**QRT-PCR**		**microarray**	**QRT-PCR**	
AGI	**Gene name**	**log_2 fold change**	**log_2 fold change**	**se**	**log_2 fold change**	**log_2 fold change**	**se**
At4g36430	Peroxidase (*PER49*)	-1.01	-1.97	± 0.55	-0.26	-0.80	± 0.42
At3g01190	Peroxidase (*PER27*)	2.89	1.12	± 0.05	-0.59	-1.06	± 0.38
At5g39580	Peroxidase (*PER62*)	-0.91	-3.20	± 0.35	-0.03	-1.45	± 0.44
At5g64100	Peroxidase (*PER69*)	1.52	1.37	± 0.43	-0.47	-0.43	± 0.03
At1g05250	Peroxidase (*PER2*)	2.86	1.02	± 0.09	-0.25	-0.85	± 0.19
At1g02930	Glutathione transferase (*GSTF6*)	-1.83	-4.79	± 0.25	-0.28	-0.53	± 0.09
At2g29460	Glutathione transferase (*GSTU4*)	-0.90	-2.73	± 0.16	0.18	1.27	± 0.11
At1g07810	ECA1	-2.22	-2.65	± 0.64	5.74	2.28	± 0.64
At5g54510	IAA-amido synthase	-0.51	-0.94	± 0.50	7.05	1.65	± 0.57
At3g15360	Thioredoxin M-type 4	0.25	-0.58	± 0.45	2.01	1.64	± 0.63

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3. Analysis of changes in the root proteome of Arabidopsis under aluminum stress.

3.1. Introduction

Use of high throughput technologies such as microarrays (Chandran *et al.* 2008, Houde *et al.* 2008, Kumari *et al.* 2008, Maron *et al.* 2008) has led to an increase in the characterization of Al-responsive genes. More recently, proteomics-based approaches have been used to identify differentially abundant proteins after exposure to Al (Fukuda *et al.* 2007; Yang *et al.* 2007, Zhen *et al.* 2007). For example, Yang *et al.* (2007) identified 16 proteins regulated in response to Al stress in a resistant cultivar of rice (Xiangnuo), whereas Fukuda *et al.* (2007) identified 31 proteins responding to Al and classified these proteins as proteins primarily involved in carbon metabolism, nitrogen metabolism, and oxidative stress. Two of these studies (Fukuda *et al.* 2007, Zheng *et al.* 2007) identified several proteins related to metabolism, indicating that regulation of primary metabolism may be an important part of strategies to combat Al stress. However, there is little overlap between the pools of proteins identified in these studies. For instance, differentially expressed proteins in soybean (Zheng *et al.* 2007) include thiamine pyrophosphokinase, sucrose synthase, monoglyceridelipase, acetone cynohydrin lyase, NAD(P)H dependent 6'deoxychalcone synthase, sulfotransferase, and entkaurene oxidase, whereas the majority of differentially proteins identified in rice (Fukuda *et al.* 2007) are related to primary energy metabolism (e.g. proteins of glycolysis and TCA cycle). These differences in the proteome profile may be due in part to the use of different plant species and exposure conditions.

Previous research has identified proteins that are differentially abundant or activated in response to Al. Basu *et al.* (1994) found two 51 kD root proteins that were

more abundant in response to Al. These proteins were later identified as the β-subunit of vacuolar H^+-ATPase (V-ATPase) and the α- and β-subunits of mitochondrial ATP synthase (Hamilton *et al.* 2001). Sharma and Dubey (2007) identified increases in the activities of several antioxidant proteins including superoxide dismutase (SOD), guaiacol peroxidase, ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase and glutathione reductase, whereas activities of catalase and chloroplastidic APX decreased after in response to Al treatment. Recently, transport proteins such as AtALMT1 and AtMATE have been shown to play a role in resistance to Al by gating transport of organic anions such as malate and citrate (Liu *et al.* 2009). The role of mitochondrial malate dehydrogenase (m-MDH) is well characterized in response to Al stress (Tesfaye *et al.* 2001); however the role of the cytosolic, chloroplastidic, and peroxisomal isoforms of MDH is unknown. My previous transcriptomic data (Kumari *et al.* 2008) showed increased abundance of cyt-MDH after 48 h exposure and I have further explored its possible role under Al stress and in this chapter.

The availability of full genome sequence, transcriptomic data, and sequence-indexed insertion mutants in Arabidopsis provides a useful system to identify differentially expressed proteins after Al stress. To complement these resources, I present a survey of changes in the root proteome in response to Al, using DIGE (Differential In-Gel Electrophoresis). This technique has been used to characterize proteins associated with stress and development processes in plants (Amme *et al.* 2006, Chivasa *et al.* 2006, Hotte and Deyholos 2008). The system uses spectrally resolvable CyDye™ fluors (size and charge-matched) to differentially label three samples that can be simultaneously separated and scanned on a single 2-D gel. To determine whether root proteome data complement or contrast with my previous transcriptome data (Kumari *et al.* 2008), I performed a comparative analysis between data sets arising from plants grown and treated under the same conditions. This is the first reported comparison of transcriptome and

proteome data to identify Al stress responses that are regulated at the transcriptional and post transcriptional level.

3.2. Materials and methods

3.2.1. Plant material and growth conditions

Wild type seeds of *Arabidopsis thaliana* (Col-0) were obtained from Lehle seeds. *Arabidopsis thaliana* T-DNA insertion mutants, *mdh-1* (SALK_021840) and *mdh-2* (SALK_125011) in Col-0 background, were obtained from the Arabidopsis Biological Resource Center (ABRC). Seeds were surface sterilized (2.25% NaClO, 0.05% Tween-20), rinsed, and sown on agar plugs (1/2 X MS medium, 0.3% phytagel, Sigma) within floating rafts and grown in hydroponics as described previously (Chapter 2; Kumari *et al.* 2008). Plants were grown in Richards medium (pH 5.75) for 14 d in hydroponic tanks in a growth chamber with 16 h light/8 h dark cycle at 65% humidity. Plants were transferred after 14 d to medium containing 200 µM $CaCl_2$ (pH4.33) with or without 25 µM $AlCl_3$. Plants were exposed to Al for 6 h and 48 h and roots tissue was collected and frozen in liquid nitrogen. Control and treated plants were blocked and grown in parallel. Tissue from three independently grown hydroponics tanks were pooled to make a single replicate. Three independent control replicates and treatment replicates constituted a total of three paired replicates each for 6 h and 48 h. Dry weight measurement of roots and shoots of hydroponically grown control and treated plants was performed by drying plants at 80^{o} C for 3 days in a hot air oven. A two-way ANOVA was used to test for differences in biomass between genotypes, concentrations of Al, and the genotype x Al interaction, and a Tukey pairwise multiple comparison was used to identify differences in means using Sigma Stat ver. 3.5.

3.2.2. Root elongation

Plate assays and assays using double layer agar in magenta vessels were used to compare differences in root elongation of WT and *mdh* mutants. The exposure medium (Snowden *et al.* 1995) containing 0.15 mM NH_4NO_3, 0.3 mM KNO_3, 0.2 mM NaCl, 0.1 mM $MgSO_4$, 5 µM $(NH_4)H_2PO_4$, 5 µM H_3BO_4, 1 µM $MnSO_4$, 0.2 µM $CuSO_4$, 1 µM $ZnSO_4$, 0.2 µM $CoCl_2$, 5 µM $FeCl_3$ and 500 µM $CaSO_4$ was prepared. Prior to autoclaving, pH was adjusted to 4.3. Aliquots of $AlCl_3$ stock solution (filter sterilized) were added after autoclaving. To obtain 0 µM, 100 µM, 150 µM and 200 µM $AlCl_3$, 0 ml, 4 ml, 6 ml or 8 ml of 25 mM stock of $AlCl_3$ was added to one litre media (respectively) followed by plating 25 ml in 15 mm square plates or 180 ml in magenta vessels. To prepare a top layer of germination medium in magenta vessels, 1/2X MS medium (pH 5.75) with 6 g phytagar was prepared and 50 ml was poured over the solidified exposure medium contained in magenta vessels. Seed of WT and *mdh* mutant plants were sown on the germination medium and root elongation was monitored for 72 hours.

3.2.3. Quantitative Reverse Transcriptase-PCR (QRT- PCR)

Wild type (WT) and mutant plants were grown and treated as described above and RNA from roots of both control and treated plants were extracted in parallel after 1 h, 6 h, 12 h, 24 h, 48 h and 78 h exposures. Total RNA from roots were extracted using an RNeasy Plant Mini kit (Qiagen). All RNA samples were individually treated with DNA*free* (Ambion) and reverse transcribed into cDNAs using MMLV reverse transcriptase (Fermentas). For QRT-PCR analysis Ubiquitin-conjugating enzyme (*UBC*; At5g25760) was used as a reference gene (Kumari *et al.* 2008, Czechowski *et al.* 2005). QRT-PCR primers were designed using PrimerExpress 2.0 software (Applied Biosystems).

Cyt-MDH: F-5'CTTTGAACGGTGTTAAGATGGAGTT3',

R-5'TACATCCCTCAACGGCATCA3';

UBC: F-5'CTTAACTGCGACTCAGGGAAT3',

R-5'GGCGAGGCGTGTATACATTT3'.

I quantified cDNAs in an Applied Biosystems 7500 Real-Time PCR System, with a SYBR green dye detection assay and ROX passive reference, in 10 µl reactions as follows: 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 1 min; Stage 3, 95.0° C for 0.15 min; Stage 4, 60.0° C for 1 min; and Stage 5, 95.0° C for 0.15 min. C_t values generated for each primer pair set over a range of dilutions were used to calculate the ΔC_t (C_t target – C_t reference). ΔC_t values were then plotted against log input amount. If the slope of the ΔC_t vs. log input was < 0.1, the relative abundance of each transcript was then estimated using the $\Delta\Delta$ C_t method (Livak and Schmittgen 2001).

3.2.4. PCR to determine homozygous insertion mutations

To identify homozygous plants harboring T-DNA insertions, seeds of WT (col-0) and two SALK-lines (*mdh-1*, *mdh-2*) were surface sterilized (2.25% NaClO, 0.05% Tween-20) and sown on separate petri plates containing 1/2X Murshe and Skoog medium pH 5.75 (Sigma) and Phytagar (Sigma). Whole plant tissue was ground in liquid nitrogen and genomic DNA was extracted (Genomic DNA isolation kit; Fermentas) for PCR. PCR was performed using gene-specific and T-DNA-specific primers as described at http://signal.salk.edu/T-DNAprimers.2.html. The gene specific primer sequences were:

SALK_021840: LP- 5'CAGTATTTTGCTCCCCAAATG3',

RP- 5'GCTGAGGAGATCTAAAGTCACAAC3';

SALK_125011: LP- 5'TGCCCTCTTCAATTGCATAAC3',

RP- 5'AACGGTTTCTAAATCAGAGTTCG3';

T-DNA specific primer: LBb1.3- 5'ATTTTGCCGATTTCGGAAC3'.

3.2.5. Protein Extraction

Plant material was ground in liquid nitrogen using a mortar and pestle. Total protein extractions were made using the protocol of Martinez-Garcia *et al.* (1999). Frozen tissue

was mixed with buffer containing 125 mM Tris-HCl pH 8.8, 1% (w/v) SDS, 10% (w/v) glycerol, and 50 mM $Na_2S_2O_5$. Plant material was then ground and mixed at room temperature in microfuge tube and immediately transferred to ice. Tubes were warmed to room temperature to solubilize SDS and centrifuged at 13,000 g for 10 min. The supernatant was diluted with 1/10 volume of buffer Z (125 mM Tris–HCl pH 6.8, 12% SDS (w/v), 10% glycerol (w/v), 22% β-mercaptoetahnol and 0.001% bromophenol blue). Samples were processed using a 2-D clean up kit (GE Healthcare) and protein concentration of each sample was determined by using the 2D-Quant-Kit (GE Healthcare) BSA as a standard.

3.2.6. Protein labeling with Cye dyes

Protein labeling was performed using CyDye fluors for DIGE as per the manufacturer's protocol. CyDyes were reconstituted in anhydrous N, N-dimethylformamide (DMF, 99.8%, Sigma-Aldrich) to create 1 nmol μl^{-1} CyDyeDIGE fluor stock. Protein and fluor were mixed (400 pmol of fluor to label 50 µg of protein sample) by vortexing and centrifuged for 30 second followed by incubation in the dark for 30 min on ice. The reaction was terminated with the addition of 10 mM lysine (Sigma-Aldrich), which reacts with free NHS esters of the cyanine dyes. Each protein sample was labeled either with Cy3 or Cy5. A mixture of equal amount of control and treated protein sample was labeled with Cy2 fluor dye to serve as an internal control (Fig. 3.1)

3.2.7. 1st dimension and 2nd dimension gel electrophoresis.

To prepare samples for loading on the first dimension, Immobillin™ DryStrip, 50 µg of each of three differently labeled protein samples (Cy2, Cy3 and Cy5) were mixed together in an equal volume of 2X sample buffer (8M Urea, 2% DTT, 4% CHAPS and 2% pharmalyte). Prior to sample application, DryStrips (24 cm, pH 3-10 NL) were rehydrated overnight with DeStreak™ rehydration buffer (GE biosciences) as per manufacturer's protocol. Proteins were separated in the first dimension using Ettan

IPGphor™ II Isoelectric Focusing (IEF) system (GE biosciences) at the following settings 1 h 500 V, 3 h gradient from 500-1000 V, 3 h gradient from 1,000 V to 8,000 V for a total of about 64 kVh. Strips were then equilibrated with equilibration solution (TrisCl 50 mM, Urea 6 M, Glycerol 30% (v/v), SDS 2%, and bromophenol blue) containing 0.5% DTT for 15 min followed by 15 min in 4.5% Iodoacetamide (IAA)-containing equilibration solution. Strips were mounted on top of 12.5% SDS–polyacrylamide gels (Acrylamide/Bis 40% (w/v), TrisCl 1.5 M (pH 8.8), SDS 10% (w/v), APS 10% (w/v) and TEMED 10% w/v) followed by 2nd dimension separation with the Ettan™ Dalt *six* electrophoresis unit (GE biosciences) at 2 W per strip for 30 min, then 8 W per strip thereafter.

3.2.8. Gel scanning, data acquisition and processing

Gels were scanned using Fujifilm FLA-5000 imager (Fujifilm, Japan). All images were acquired at a scanning resolution of 50 µm and a photon multiplier tube voltage between 50,000 and 63,558 V. Scans for Cy2 used the 435 nm excitation laser and LBP emission filter (510 nm), scans for Cy3 used 532 nm laser with a BPG1 filter (570 nm), and scans for Cy5 used the 635 nm laser with the DRG1 filter (665 nm). I stained pick gels with Deep Purple™ fluorescent stain (GE biosciences) and scanned them with the 532 nm laser and LGP filter (575 nm). Scanned image files (.img) were converted to tagged information file format (.tiff/.tif) using ImageGauge and further converted to gel files (.gel) using ImageQuant V 5.2 software package. Gel images were analysed for detection of differentially abundant protein spots using DeCyder™ software V5.1 (GE healthcare). DeCyder™ Batch processor was used for spot detection and inter-gel matching of multiple gel images, including the post-stained preparative gels. Statistical analysis of data was performed in BVA module contained in DeCyder™ software. Each individual control spot was compared with treated spot in all three replicates using the student's t-

test. Coordinates of interesting spots were determined using two reference stickers and spot picking was performed using an Ettan™ Spot-picker (GE biosciences).

3.2.9. Spot identification

Gel pieces containing spots of interest were washed in HPLC grade water and then dehydrated with 100% acetonitrile and de-stained by washing twice with 1M NH_4HCO_3 and 100% acetonitrile for 10 min each. Protein spots were dehydrated, reduced and alkylated using 100% acetonitrile (15 min), 0.1M NH_4HCO_3/10 mM DTT (37° C; 30 min), 55 mM iodoacetamide / 0.1M NH_4HCO_3 (20 min in dark). Protein spots were digested (37° C in dark) with TrypsinGold (GE Healthcare). Digestion was stopped using 30 µl of 0.2% formic acid. Peptides were extracted using 20 µl 0.1% formic acid and 10% acetonitrile with three repeats of 30 min each.

Peptide analysis was carried out using an Agilent 1100 series LC/MSD TrapXCT (Agilent Technologies) and accompanying ChemStation software using the manufacturer's instructions for identification of peptide mixtures. The autosampler injected 15 µl of tryptic digest onto the first of two C-18 columns (Zorbax 300SB-C18 5 µm 5 x 0.3 mm and Zorbax 300SB-C18 5 µm 150 x 0.3 mm; Agilent Technologies) to trap, concentrate, and elute the samples in conjunction with a solvent gradient to separate the peptides. The peptide-separation gradient started at 85% solvent A (0.1% formic acid in H_2O) and ended at 55% solvent B (0.1% formic acid, 5% H_2O in ACN) over a 42-minute span. Columns were cleansed with 90% solvent B before returning to solvent A for the next sample. The ion trap mass spectrometer collected data using a MS 300-2200 m/z scan followed by a MS/MS analysis of the most intense ions. Raw spectral data were processed into mascot generic file format (.mgf) using the default method in the ChemStation data analysis module. Data files were used to identify proteins by searching against the Swiss-Prot, TREMBL, MS database, and NCBI databases using MASCOT

search engine (Matrix science) in taxonomy "Viridiplantae" with the search parameters of: peptide resistance of 2 Da, parent ion resistance of 0.8 m/z, ion charge of +2 and +3.

3.2.10. Cytosolic fractionation and enzyme activity

Wild type (WT) plants were grown and treated in hydroponic culture as described above. Roots of both control and treated plants were extracted in parallel after 1 h, 3 h, 6 h, 24 h, and 48 h exposures. Tissue from three independently grown hydroponics tanks were pooled to make a single replicate. Three independent control samples and treatment samples constituted a total of three paired replicates each for 1 h, 3 h, 6 h, 24 h, and 48 h. Root tissue was frozen in liquid nitrogen immediately after extraction. Frozen tissue (300 mg) were ground in a pre-chilled mortar and pestle with a pinch of sand, pinch of PVPP and 1.5 ml of extraction buffer containing 1M Tris-HCl (pH 7.8), 5 mM EDTA, 1 mM DTT, 10 mM cysteine, and 0.1 mM PMSF. The ground slurry was centrifuged at 3,000 rpm for 5 min and resulting supernatant was centrifuged at 18,000 g for 15 min to remove organelle contaminants (such as mitochondria). Portions of 18,000 g supernatant were aliquoted (to serve as cytosol fraction without organelles) and the remaining portions were ultra-centrifuged at 110,000 *g* for 1.5 h to remove membranes from the cytosolic fractions. All centrifugations were carried at 4° C. Protein concentrations were determined by using the 2D-Quant-Kit (GE Healthcare) with BSA as a standard.

Cyt-MDH enzyme activity was assayed by measuring decrease in absorbance at 340 nm resulting from oxidation of NADH to NAD in a BioTek 96-well plate reader. The reaction mixture contained 0.1 M potassium phosphate buffer (pH 7.8), 250 µmoles NADH, and enzyme extract. The reaction was started by addition of a total of 200 µmoles of oxaloacetate in a reaction mixture. For each reaction, 5 min of spectrophotometrical change at A340 nm was monitored automatically at 1 minute intervals. All the solutions were made fresh in 0.1 M potassium phosphate (pH 7.8). Fresh enzymatic extracts were used for all enzyme activity assays.

3.3. Results

3.3.1. DIGE based expression profiling of proteins after aluminum stress

The DIGE based 2D gel electrophoresis technique reproducibly detected 1,750 and 1,232 spots in 6 h gels and 48 h gels respectively. Using the statistical test Biological Variation Analysis contained in DeCycder (5.1) software, I selected a subset of 91 spots (55 significant + 36 random) from 6 h pick gel, and 63 (26 significant + 37 random) spots from 48 h pick gel (Table 3.1). I identified a spot as significant if the fold change difference was at least 13% in treatment as compared to control at $\alpha = 0.10$. Using these criteria, 55 spots from 6 h gel, and 26 spots from 48 h gel were identified as significant and differentially abundant after Al stress. I also picked some spots randomly (36 for 6 h, and 37 for 48 h). Among the 55 significant protein spots for 6 h treatment, a total of 13 spots increased in abundance and 42 spots decreased in abundance in treated roots compared to untreated controls. Among the 26 significant protein spots for 48 h treatment, a total of 22 increased in abundance and four decreased in abundance. The protein identities were determined by extracting spots from gels, followed by trypsin digested and identification using mass-spectrometry. I focused only on those protein spots where identities were confirmed with a Mascot score of at least >42. Using this criterion, I present here individual peptides, p*I*, molecular weight and the respective Mascot scores for 12 successfully identified, differentially expressed proteins for 6 h (Table 3.2) and 17 protein spots for 48 h gels (Table 3.3). I marked the position of these proteins on a 6 h gel image (Fig. 3.2a) and 48 h gel image (Fig. 3.2b).

For both 6 h and 48 h data, the majority of identified proteins were classified into the following broad categories: 1) energy and primary metabolism, 2) oxidative stress, 3) amino acid and protein metabolism.

3.3.1.1. Energy and primary metabolism

One of the most well-studied mechanisms of Al resistance has been the Al-induced release of organic anions from roots of Al resistant genotypes (Miyasaka *et al.* 1991, Ma 2000). Organic anions that are part of TCA cycle have previously been defined as Al-inducible and different species have been shown to exude different organic anions (malate, citrate, oxalate; Kochian *et al.* 2004). Manipulation of primary metabolism to achieve Al resistance has also been successfully demonstrated (Tesfaye *et al.* 2001, Anoop *et al.* 2003).

I detected the differential abundance of several proteins involved in glycolysis, and the TCA cycle after Al exposure (Fig. 3.3). Within the glycolytic pathway, I detected an increase in fructose biphosphate aldolase (FBP aldolase; EC 4.1.2.13), 2-3, bi-phosphoglycerate mutase (BPGM; EC 5.4.2.4) and phosphopyruvate hydratase/enolase (EC 4.2.1.11; Table 3.3 and 3.4). I detected a 1.13-fold increase in FBP aldolase after 6 h (spot 4). I detected no significant change in protein level for enolase after 6 h, however, after 48 h enolase (spot 7) increased in abundance by 1.5 fold. Increases in the abundance of glycolysis-related proteins after Al exposure are consistent with an increased requirement of pyruvate synthesis under Al stress. Pyruvate later undergoes oxidative decarboxylation to yield acetyl CoA that enters TCA cycle. This conversion is carried out by pyruvate dehydrogenase complex (PDC). Dihydrolipoamide dehydrogenase 1 (DLD; EC 1.8.1.4), which is one of the important components of PDC, increased in abundance by 1.5 fold after 48 h (spot 5) exposure to Al.

Within the TCA cycle, I detected increased abundance of succinyl-CoA ligase (SCS; EC 6.2.1.4), and mitochondrial malate dehydrogenase (m-MDH; EC 1.1.1.37; Table 3.3). Succinyl-CoA ligase catalyzes the substrate level phosphorylation step in TCA cycle by conversion of succinate to succinyl-CoA with a concomitant hydrolysis of GTP to GDP and phosphate. I detected a 1.5 fold increases in abundance (spot 2) after 48

h exposure to Al. Of four organelle-specific isoforms of MDH, I detected two (mitochondrial and cytosolic) that increased in abundance after 6 h and 48 h exposures. MDH typically catalyzes the reversible conversion of malate into oxaloacetate. For m-MDH, I detected an increased abundance of 1.2 fold (spot 12) after 48 h exposure. I noted that cyt-MDH decreased in abundance (-1.3 fold) after 6 h (spot 5). Over-expression of m-MDH gene is known to confer resistance to Al stress (Tesfyne *et al.* 2001). However, the role of cyt-MDH is unknown in response to Al.

In addition to changes described above, I also detected a 1.2 fold increase in the abundance of malic enzyme (ME; EC 1.1.1.38) after 48 h (spot 3). Malic enzyme de-carboxylates malate to produce pyruvate and CO_2 using NAD(P) to produce NADPH (Wedding 1989). Spots representing alcohol dehydrogenase (ADH; EC 1.1.1.1) also increased in intensity after 6 h (1.4 fold; spot 2). Alcohol dehydrogenase facilitates inter-conversion between alcohol and aldehyde, oxidizing alcohol to produce formaldehyde and ethylene glycol to ultimately yield glycolic acid and oxalate. Interestingly, wheat is known to exude oxalic acid from roots in response to Al stress and confers resistance to plants against Al stress (Ma 2000). Therefore, increased abundance and activity of ADH might be an adaptive response to increase the production of oxalic acid.

3.3.1.2. Oxidative stress

Oxidative stress is an important component of Al toxicity (Richards *et al.* 1998, Basu *et al.* 2001, Yamamoto *et al.* 2002, Basu *et al.* 2004). I identified several proteins involved in oxidative stress that were differentially abundant following Al exposure. These included glutathione-S-transferases (GST; EC 2.5.1.18), ascorbate peroxidases (APX; EC 1.11.1.11) and thioredoxin.

Glutathione-S-transferases conjugate toxins to the tripeptide glutathione (GSH) and form S-glutathionylated products, which are transported to the vacuole through the action of ATP Binding Cassette (ABC) transporters (Rea 1999, 2007). These enzymes

also help to protect cells from oxidative damage by reducing organic hydroperoxides that are formed during oxidative stress (Dixon *et al.* 2002). I detected decreased abundance (1.2 fold) of ATGSTF2 (Arabidopsis thaliana Glutathione S-Transferase-phi class; spot 7) and ATGSTF10 (spot 11) after 6 h. I detected contrasting abundance of two spots both identified as ATGSTF8 where one increased (1.3 fold; spot 15) and other decreased (1.13; spot 16) in abundance after 48 h exposures. All the GSTs identified in this study belonged to Phi class, which is also one of the most numerous in the Arabidopsis genome (Dixon *et al.* 2009).

Ascorbate peroxidases play a role in ROS scavenging by catalyzing the conversion of hydrogen peroxides to water and ascorbate to dehydroascorbate (Tarantino *et al.* 2005). I detected increased abundance of APX after 48 h (1.3-fold; spot 14) exposure to Al. I also detected decreases in protein abundance for thioredoxin (TRX) after 6 hr (1.3 fold; spot 12) exposure. TRXs are a group of small enzymes that participate in redox reactions, via the reversible oxidation of disulfide bond.

3.3.1.3. Amino acid and protein metabolism

Amino acids serve as precursors for a large array of metabolites involved in plant growth and response to various stresses (Less and Galili 2008). I detected increased abundance of two catalytic proteins involved in biosynthesis of cysteine, methionine and serine, but only after 48 h stress (Table 3.3).

Cysteine biosynthesis represents the final step of sulfate assimilatory reduction and is the sole entry point of reduced sulfur into an organic form in plants. The last and key step of cysteine biosynthesis is catalyzed by cysteine synthase (CS; EC 2.5.1.47); biochemically O-acetylserine (thiol) lyase enzyme (OASTL), that incorporates sulfide into *O*-acetyl-L-serine to yield L-cysteine. I detected an increase in abundance of CS after 48 h (1.6 fold; spot 13) exposure. Recent studies (Sugimoto *et al.* 2004, Yang *et al.* 2007) have documented increases in the cellular level of cysteine synthase in response to Al.

Another sulfur containing amino acid is methionine. I detected increased abundance of cobalamin-independent methionine synthase (ATCIMS; EC 2.1.1.14) after 48 h (1.4 fold; spot 1) exposure. ATCIMS catalyzes the transfer of a methyl group from methyltetrahydrofolate to L-homocysteine, the terminal step in the biosynthesis of methionine (Pejchal and Ludwig 2005). This suggests an important role of sulfur containing amino acids under Al stress, perhaps as a substrate for the synthesis of GSH.

The ubiquitin proteosome pathway confers specificity to protein degradation by tagging targeted proteins with ubiquitin. The 26S proteasome is a multi-subunit complex that consists of a cylindrical 20S core protease with a 19S regulatory particle cap on both ends. I detected decreased abundance of the 20S proteasome beta subunit PBF1 after 6 h (-1.6 fold; spot 8) exposure, and increased abundance of the 19S Proteosome subunit 9 after 48 h (1.4 fold; spot 10) exposure. Identification of these novel proteins from the processes of protein degradation and synthesis suggests protein remodeling occurs in response to Al stress.

3.3.2. Comparison of proteomic data with transcriptomic data

I compared my proteomic data with my previous transcriptomic data (Chapter 2; Kumari *et al.* 2008) obtained from plants grown under same conditions. I used all proteins spots identified as significant in this study (12 for 6 h, 17 for 48 h) to compare to transcriptomic data. Among the 12 protein spots identified after 6 h stress, probes for three corresponding genes (AtCg00490, At2g27285, At1g45145) were not printed on my microarrays and probes for genes corresponding to two spots failed to pass the background cut off (Supplemental Table 3.1a). For the remaining 7 spots, I compared mRNA and protein abundance data (Supplemental Table 3.2a). Similarly, for the 17 identified proteins after 48 h treatments, five protein spots failed to pass the background cut off (Supplemental Table 3.1b), thus mRNA and protein abundance data were

compared for 12 spots (Supplemental Table 3.2b). In these comparisons I found a poor correlation between mRNA abundance and protein abundance for both 6 h ($r^2 = 0.155$) and 48 h ($r^2 = 0.083$) treatments (Fig. 3.4). These results could suggest post-transcriptional regulation of the stress proteome, although the comparative analysis was based on small subset of proteins that I could successfully identify in this study. This could explain the differences observed in data obtained from two different techniques of profiling stress responses in roots.

3.3.3. Characterization of the role of cyt-MDH under aluminum stress

Aluminum-activated malate exudation (Hoekenga *et al.* 2006, Magalhaes *et al.* 2006) is an important mechanism of Al resistance in Arabidopsis. Increased exudation of organic acid anions (Ma 2000) by modulation of TCA cycle enzymes such as m-MDH (Tesfye *et al.* 2001) have shown to increase Al resistance in plants. Besides m-MDH, cyt-MDH was detected as one of the significant proteins in response to Al after 6 h. The role of cyt-MDH in response to Al is unknown. Although cyt-MDH was also detected after 48 h exposure (1.8 fold; p=0.38) this proteins failed to pass my criteria for significance (α=0.10). To determine whether this change was due to transcriptional or post transcriptional regulation, I used QRT-PCR to measure transcript abundance of cyt-MDH in WT roots after 1, 6, 24, 48, and 78 hours of exposure (Fig. 3.5). My data showed that cyt-MDH transcript levels decreased in abundance after 1 h and 6 h, followed by an increase after 24 and 48 h, which later decreased again after 78h exposure. The 6 h and 48 h transcript abundance is consistent with my proteomics data where the abundance of cyt-MDH protein decreased significantly after 6 h but increased after 48 hours, suggesting that regulation of cyt-MDH in response to Al occurs in large part at the level of transcription.

To further clarify the relevance of cyt-MDH to Al-stress I used a reverse genetics approach. I obtained two T-DNA insertion lines (*mdh-1*, and *mdh-2*) for cyt-MDH, confirmed their homozygosity (Fig. 3.6), and measured cyt-MDH transcript abundance in each using QRT-PCR. For each allele, cyt-MDH transcript abundance was decreased >64 fold compared to wild type (Fig. 3.7).

I compared the Al-sensitivity of root elongation in *mdh-1*, *mdh-2* and WT seedlings, using three separate techniques. To begin with, I conducted root elongation assays in agar plates using exposure medium containing 0, 100, 150, and 200 µM $AlCl_3$. Under control conditions, WT plants were healthy compared to either mutant allele. I detected no differences in primary root elongations between WT and either mutant allele after 12 h, 24 h, and 48 h (data not shown). However, after prolonged exposure (14 d) both *mdh-1* and *mdh-2* showed greater root elongation than WT under conditions of Al stress (150 µM, 200 µM).

I also compared root biomass of insertion lines and WT under hydroponic conditions. My hydroponics system allowed me to measure inhibition of root elongation over a period of time, and view root architecture and growth. In this experiment I treated 21 d old plants with 25 µM $AlCl_3$ for 48 h. I extracted the longest roots from each hydroponic tank and photographed them (Fig. 3.8). WT plants showed reduced root elongation under treatment conditions as compared to *mdh-1* and *mdh-2*, although roots of *mdh-2* were shorter than WT under control conditions. This observation was short-lived and prolonged exposures (>2 weeks) to Al made *mdh-1* and *mdh-2* lines flower early and appeared stressed compare to WT.

I also measured the dry weights of roots and shoots to calculate root/shoot ratios to assess to the physiological status of plants. An ANOVA indicated no significant affects due to Al ($p=0.227$), genotype ($p=0.098$), or the Al x genotype interaction ($p=0.390$; Fig. 3.9.)

To further test whether *mdh* mutants are more resistant than WT under conditions of Al stress, I grew plants in a double layer agar medium, where the top layer was a germination medium and bottom layer was an exposure medium containing 150 µM $AlCl_3$ (Fig. 3.10). Interestingly, WT plants did not penetrate the Al exposure medium (n=20), whereas roots of almost all *mdh* mutants (90% of individuals tested, n=20) grew into the exposure medium, although root elongation within the exposure medium stopped after approximately 48 h.

To help understand why decreased protein and transcript abundance of cyt-MDH may be beneficial to WT plants after 6 h exposure to Al, I measured the activities of cyt-MDH in WT plants exposed to 1 h, 3 h, 6 h, 12 h, 24 h and 48 h of Al stress. In these experiments, I reduced contamination from other organelle-specific isoforms of MDH (such as m-MDH) by sedimentation of organelles by centrifugation. I also obtained cytosol-enriched fractions by ultracentrifugation. Differences in kinetic and regulatory properties of soluble cyt-MDH, membrane bound cyt-MDH, and m-MDH have been previously reported (Sukalovic *et al.* 1999, Hanss *et al.* 2002, 2008). I measured the cyt-MDH activity in two different types of fractions of the cytosol. In my total cytosolic fraction (from which organelles were removed,) increased MDH activity was detected after 1 h, 3 h and 6 h with no change in activity after 24 h, and 48 h of exposure to Al (Fig. 3.11a). On the other hand, in cytosol-enriched fractions,enzyme activities in both control and treated plants followed a similar pattern, with activities declining after the first hour of transfer to $CaCl_2$ (+/- Al; Fig. 3.11b).). This could perhaps reflect transfer of plants to an incomplete nutrient solution. It is important to recognize that cyt-MDH activity reflects the sum of three different isoforms of cyt-MDH that cannot be distinguished in these data.

3.4. Discussion

I exposed Arabidopsis plants to Al and sampled roots after 6 and 48 h to detect changes in the whole root proteome. I assigned identities to only 12 spots that had changed significantly (α=0.10) in abundance at 6 h time point (Table 3.5) and 17 significant spot at 48 h (Table 4.5). Many of the differences in fluorescence intensities were not considered significant at α=0.05; nevertheless, several of these protein have been previously reported to be differentially expressed at either the transcript, protein, or phenotypic level after exposure to Al stress (Fukuda *et al.* 2007, Kumari *et al.* 2008, Yang *et al.* 2008, Zhang *et al.* 2007). For instance, I detected that cysteine synthase (p=0.091) was differentially abundant after Al exposure. The role of this gene in Al stress response has been recently described in detail (Yang *et al.* 2008).

Most of the proteins I identified (83% for 6 h; 53 % for 48 h) belong to one of two functional categories: primary metabolism or oxidative stress. Most of the identified proteins involved in primary metabolism belonged to glycolysis or the TCA cycle. Fakuda *et al.* (2007) also detected several glycolysis-related proteins after rice roots were exposed to Al. In particular, I detected increased abundance of BPGM, enolase, SCS, and m-MDH after 48 h exposure. One of the important intermediates of the TCA cycle is malate, which is also a chelator of Al for internal and /or external detoxification (Ma 2000). Malate is synthesized in the mitochondria and then transported to cytosol, where it could be converted to either pyruvate or oxaloacetate via cyt-MDH or ME, respectively (Fig. 3.12). It was interesting to detect decreased abundance of cyt-MDH after 6 h. I observed a similar pattern of transcript abundance in my QRT-PCR data, which suggests that cyt-MDH is regulated at the transcript level under Al stress (Fig. 3.5). I used a reverse genetics approach to test the possible role of cyt-MDH. My two T-DNA insertion lines, *mdh-1* and *mdh-2* were healthy after exposure to Al as compare to WT. This

observation was short-lived and prolonged exposures (>2weeks) to Al made *mdh-1* and *mdh-2* lines flower early and appeared stressed compare to WT. My double layer agar assays were interesting because WT plants did not penetrate the Al containing exposure medium, whereas roots of almost all *mdh* mutants grew into the exposure medium. This may suggest that mutants are more resistant to Al. I speculate that cells may be able to control two aspects of Al stress by regulating activities of cyt-MDH and ME. First, by maintaining cytosolic levels of malate for transport across the membrane to chelate Al^{3+} and second, by generation of NADPH that serves as a source of reducing power for reducing agents such as glutathione. This hypothesis is supported by differences in protein abundances. For instance, I detected decreased abundance of cyt-MDH after 6 h exposure with no change in ME. This would suggest that conversion of malate to oxaloacetate is slowed down during the initial periods of stress, which may help to maintain a pool of malate for external exudation. However, after prolonged exposures it appears that flow of carbon is towards the synthesis of pyruvate, because I saw a concomitant increase in protein abundance for both m-MDH and ME after 48 h. Therefore, I speculate that cyt-MDH could play an important role in Al stress by, 1) increasing the ratio of NADPH/NADP in association with ME (increased in abundance in this study) and, 2) maintaining a pool of cytosolic malate for external/ internal detoxification of Al (Fig. 3.12). The protein abundance and activity of ME has been shown to increase under salt stress (Valderrama *et al.* 2006) and over-expression of cyt-ME confers salt resistance which correlates with significant increase in the cytosolic NADPH/$NADP^+$ ratio in transgenic Arabidopsis (Cheng and Long 2007). Notably, several transcriptomic and proteomic screens have identified enzymes of primary metabolism as being stress-related proteins (Fakuda *et al.* 2007, Maul *et al.* 2008, Popova *et al.* 2008).

Among proteins involved in oxidative stress, I detected decreased abundance of 2 GSTs after 6 h exposure and increased as well decreased abundance of GSTs after 48 h as described previously under salt stress (Jiang *et al.* 2007). In my microarray, most of the transcripts encoding GSTs belonged to the Tau class, whereas, most of proteins identified in proteomics data belonged to Phi class. For some spots identified as GSTs, I noticed that the same ID was detected for two distinct spots (for instance spots 15 and 16 as ATGSTF8) although their position in respective gels was distinct. This suggests post-translational protein modifications may be involved in the regulation of activity. Ryue *et al.* (2009) showed that transcript levels of AtGSTF10 were not induced by plant growth regulators or abiotic stress (except drought). Nonetheless, over-expression of AtGSTF10 conferred higher resistance to salt and disturbed redox status of transgenic plants.

A role of sulfur in plant response to abiotic stress is emerging (Nocito *et al.* 2006). As a component of the tripeptide glutathione; γ-Glu-Cys-Gly, sulfur could represent a limiting factor for GSH biosynthesis and for resistance to metals (Nocito *et al.* 2006). I detected increased abundance of CS (previously reported to be differentially expressed after Al stress; Yang *et al.* 2008) and increased abundance of GST's after 48 h of stress. GSH serves important functions in plants as a reductant that transiently accumulates under stress conditions as oxidized disulfide (GSSG; Dixon *et al.* 2005). Glutathione transferases play a protective role by glutathionylation (Klatt and Lamas 2000) of toxic products which can be transported to the vacuole through the action of ATP Binding Cassette (ABC) transporters (Rea 1999). The involvement of these thiol exchange reactions in redox signaling has been postulated (Klatt and Lamas 2000, Shelton *et al.* 2005, Biswas *et al.* 2006, Dalle-Donne *et al.* 2007). Several reports have suggested increased abundance of ABC transporters under stress conditions. Taken together with my data, I speculate that sulfur metabolism, accumulation of GSH and increased abundance of GSTs are important components of the plant stress management strategy

after exposure to Al. Although I previously observed induction of transcripts for ABC transporter, proteins from this family were not among those detected in my present experiment. In future, the proteomic analysis of membrane-enriched fractions might prove useful to identify these and other membrane proteins that are differentially expressed after Al stress.

In summary, this study: 1) presents the proteomic analysis of response of plant roots to Al stress after 6 h and 48 h, 2) presents the comparison between the root transcriptome and proteome after 6 and 48 h exposure to Al in any plant species, 3) identified several novel proteins and confirmed some other previously reported proteins as differentially abundant, 4) provided evidence that protein abundance of cyt-MDH (one of the novel Al related proteins) is regulated in part at the level of transcription after Al stress, 5) showed that besides decreased abundance at the level of transcripts (detected by QRT-PCR) and protein (detected by DiGE), the activities of cyt-MDH also decreases after 6 h exposure to Al, 6) showed that insertion mutants (*mdh-1* and *mdh-2*) were more resistant to Al as compared to WT plants, 7) suggests that sulfur metabolism and increased abundance of GSTs play a role in resistance to Al.

Figure 3.1. Experimental design for analysis of the stress proteome in roots of Arabidopsis. Three independent replicates were used where control and treated samples were grown in parallel. Control, treated, and pooled samples were differentially labeled using CyDyes (Cy5, Cy3, and Cy2). For one of the three replicates (Rep 2), dye labeling was flipped where protein control and treated plants were labeled with Cy3 and Cy5 instead of Cy5 and Cy3; respectively.

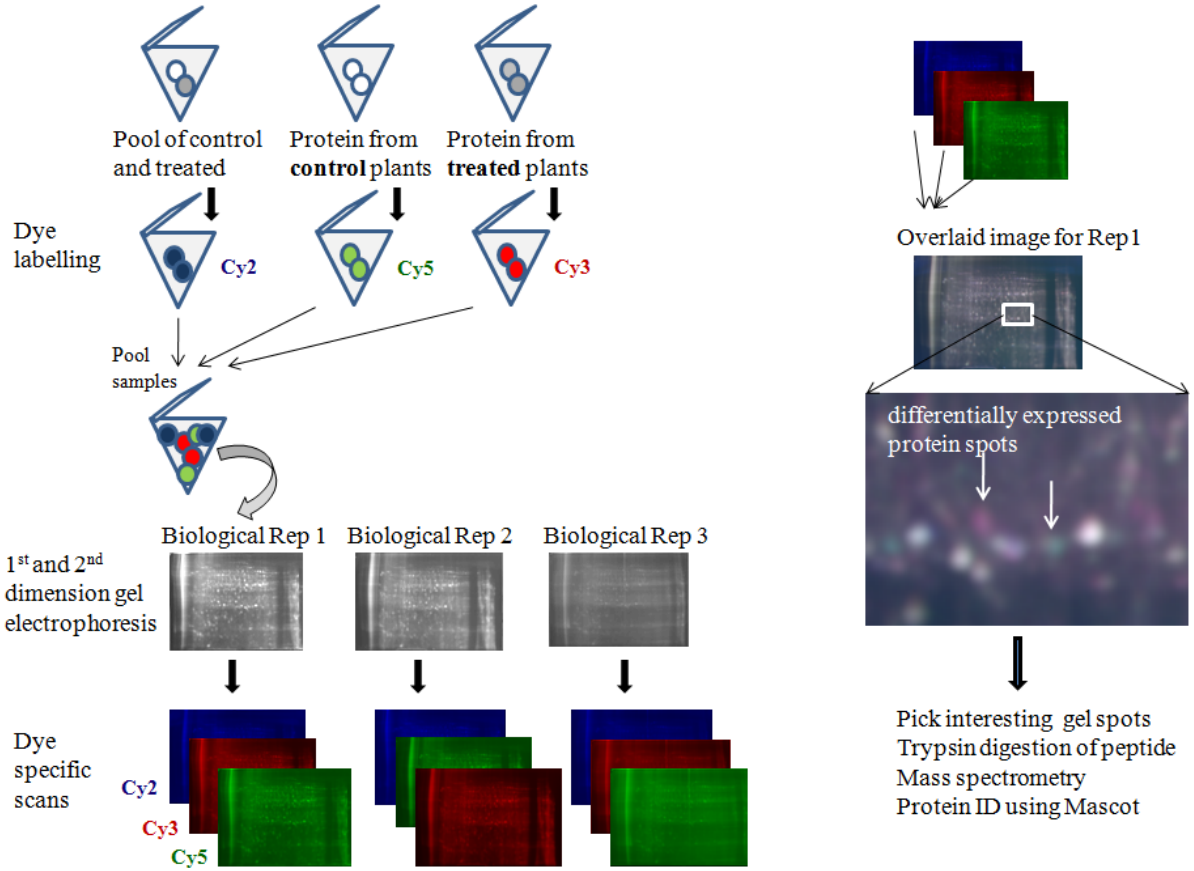
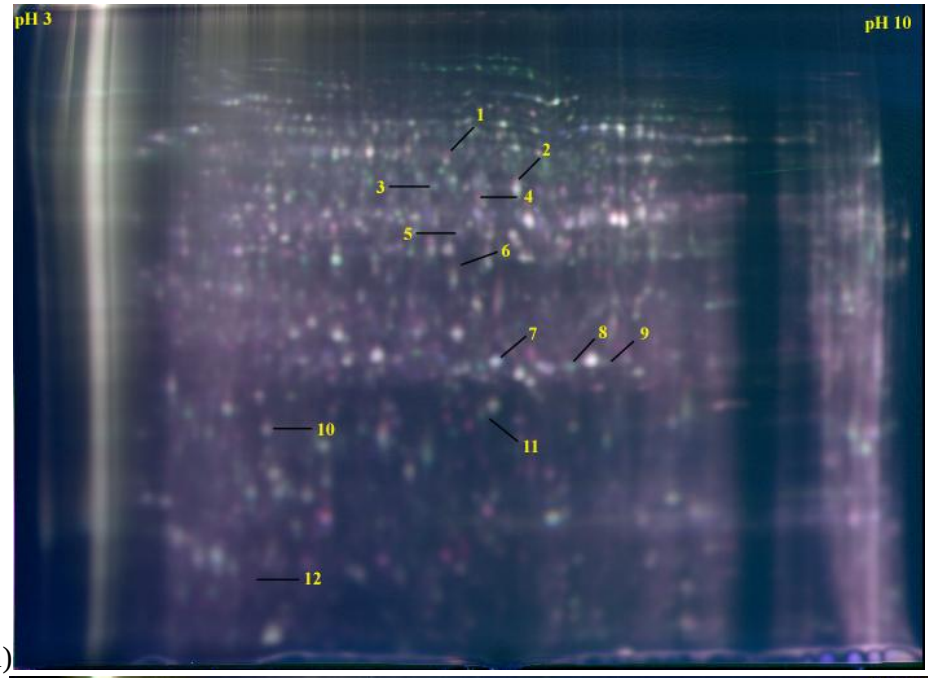


Figure 3.2. Representative DIGE gels of protein samples obtained after 6 h and 48 h of aluminum stress. Proteins were fluorescently labelled, mixed and then separated using 2D gel electrophoresis. a) Twelve successfully identified spots that showed significant differences in abundance after 6 h exposure to aluminum are labelled. b) Seventeen successfully identified spots that showed significant differences in abundance after 48 h exposure to aluminum are labelled. The pH range of the first dimension electrophoresis was from 3-10.

a)



b)

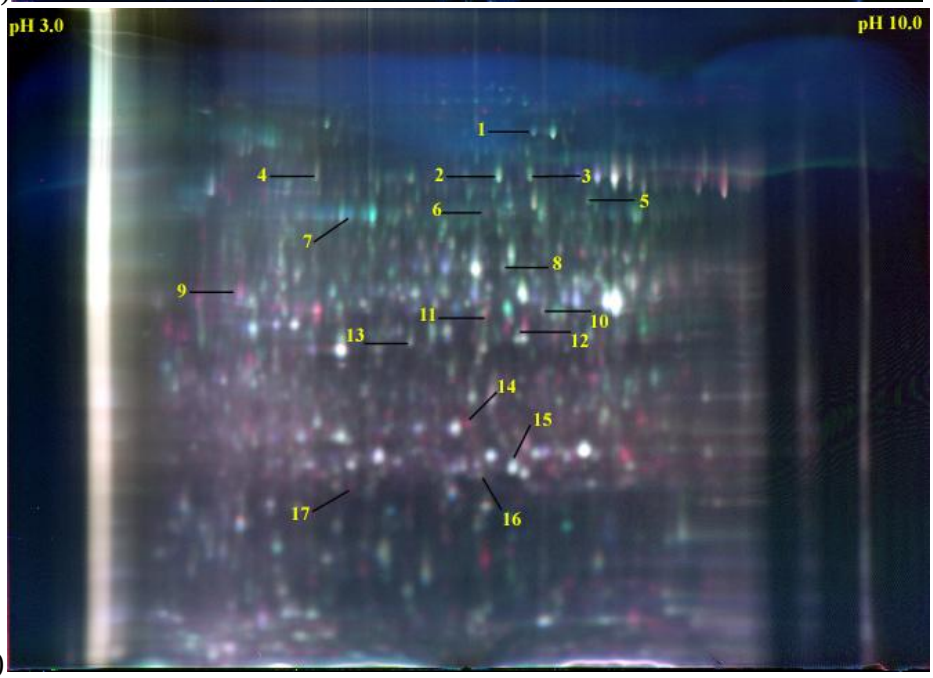


Figure 3.3. Aluminum induced catalytic proteins (shown in red) involved in glycolysis, TCA, and, amino acid biosynthesis pathways.

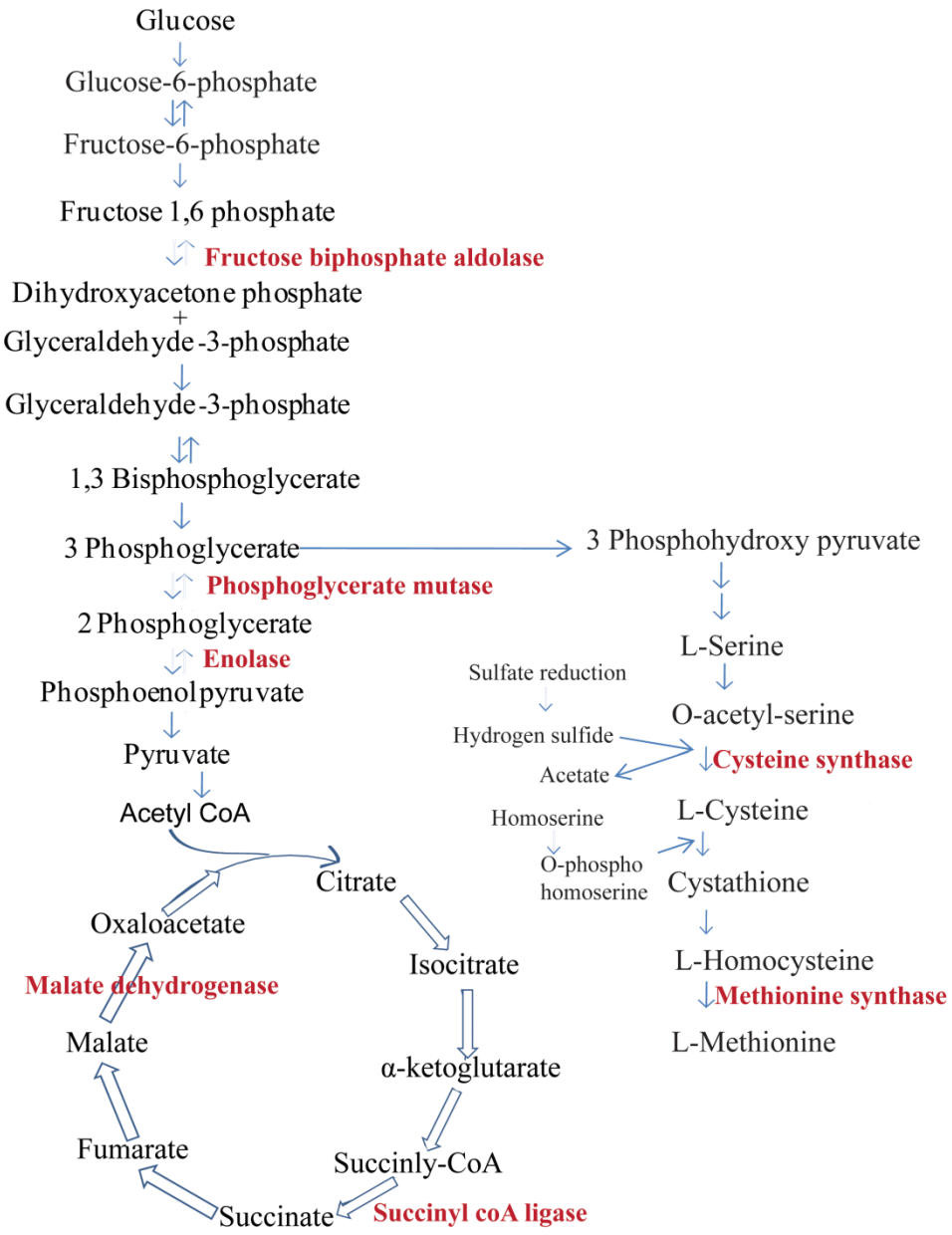


Figure 3.4. Comparison of transcriptomic and proteomic data after 6 h and 48 h of aluminum stress. Correlation coefficients for the relationship between transcript abundance and protein abundance were weak for both 6 h ($r^2 = 0.155$) and 48 h ($r^2 = 0.083$).

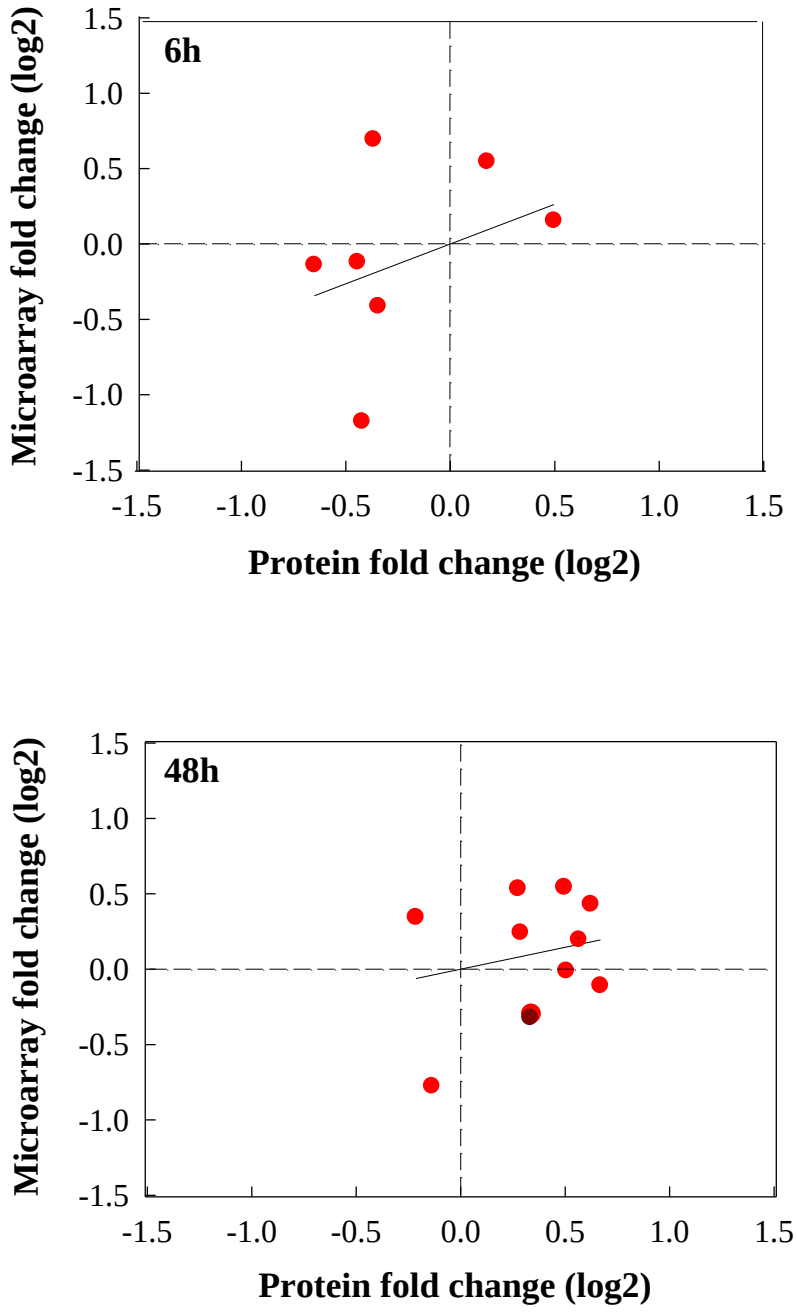


Figure 3.5. Relative transcript abundance of cyt-MDH in roots of *Arabidopsis thaliana* after 1 h, 6 h, 24 h, 48 h, and 78 h of exposure to 25 µM $AlCl_3$.

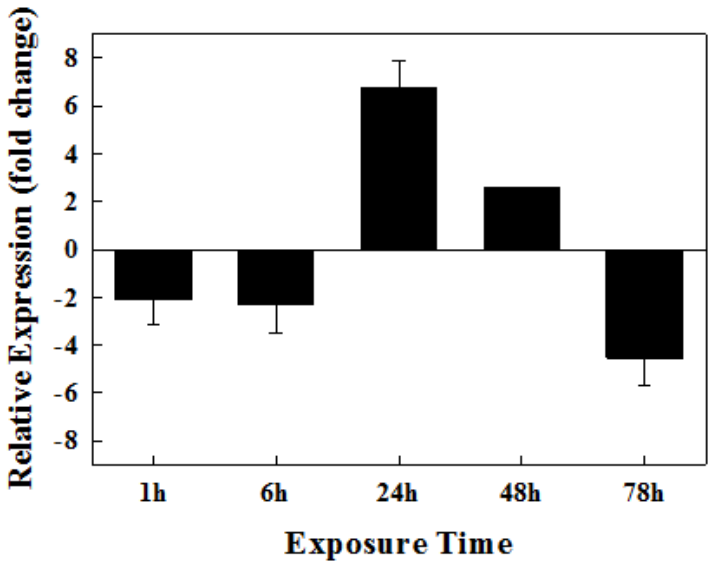


Figure 3.6. Agrose gel image of PCR products to show confirmation of homozygous T-DNA insertions in *mdh-1* and *mdh-2* insertion lines as compared to WT. Both *mdh-1* and *mdh-2* insertion lines generated PCR products when gene-specific and T-DNA specific primer were used. Since WT plants do not harbor a T-DNA, these failed to generate a PCR product. Conversely, when a gene-specific primer pair was used, only WT plants generated a PCR product hence confirming absence of any WT copy of gene in *mdh-1* and *mdh-2* insertion lines, thus confirming homozygous insertion of T-DNA. BP: Border primer (T-DNA-specific), RP: Right primer (gene-specific), LP: Left primer (gene-specific).

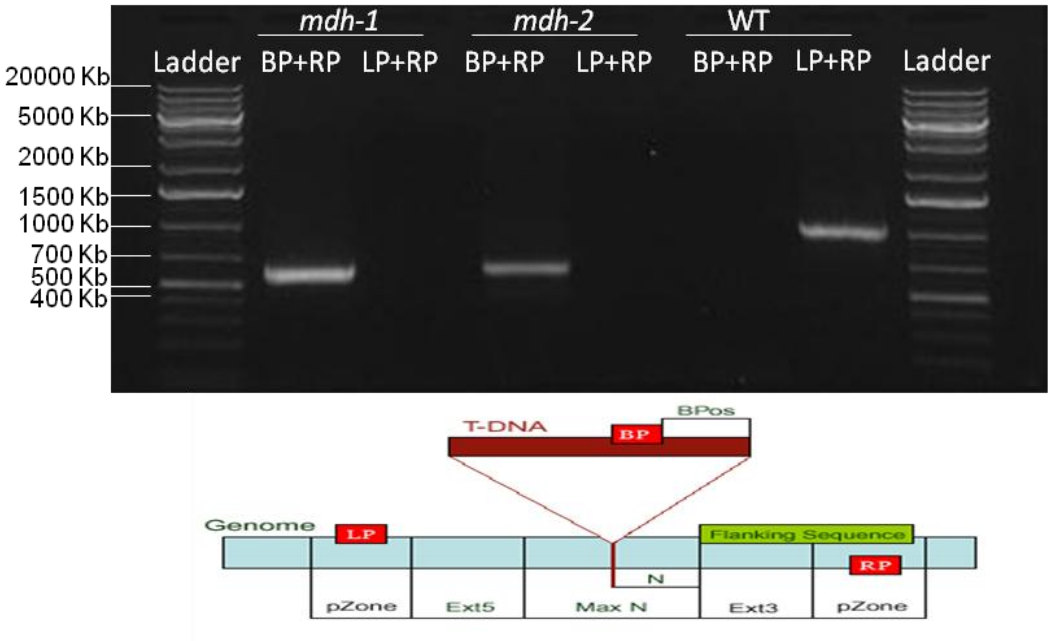


Figure 3.7. Confirmation of decreased relative abundance of cyt-MDH transcripts in homozygous T-DNA insertion lines (*mdh-1* and *mdh-2*) as compared to WT. The abundance of cyt-MDH in mutants was calculated relative to endogenous control (UBC). Insertion lines and WT were both grown under control conditions.

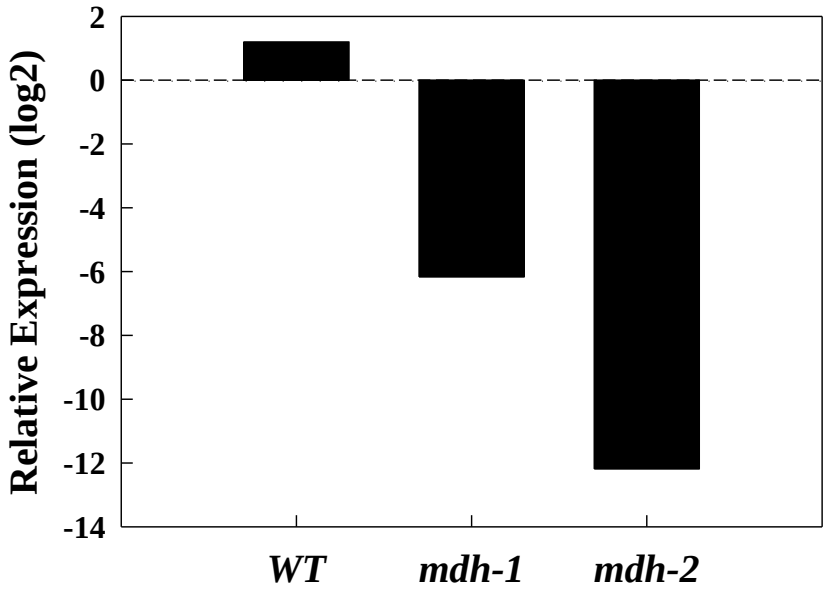


Figure 3.8. Effect of aluminum treatment (0 µM, 25 µM) on growth of WT and two homozygous T-DNA insertion lines (*mdh-1* and *mdh-2*) of *Arabidopsis thaliana*.

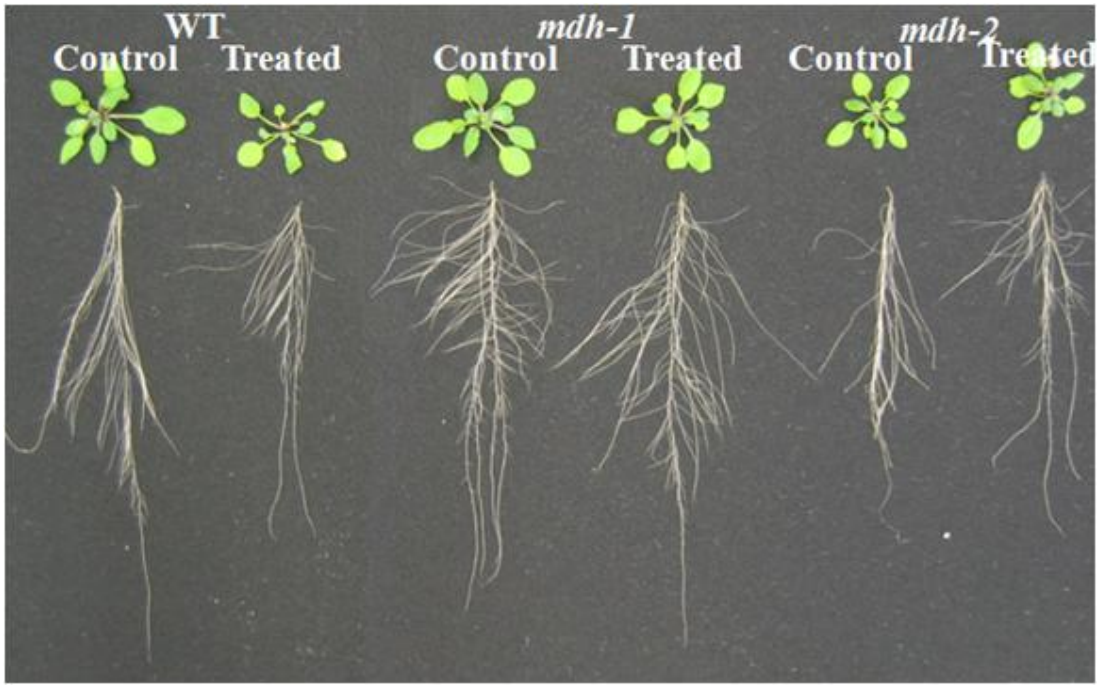


Figure 3.9. Dry weights of roots, shoots, and, root/shoot ratio of two homozygous T-DNA insertion lines (*mdh-1* and *mdh-2*) and WT plants after 48 h of aluminum exposure. An ANOVA indicated no significant affects due to Al (p=0.227), genotype (p=0.098), or the Al x genotype interaction (p=0.390).

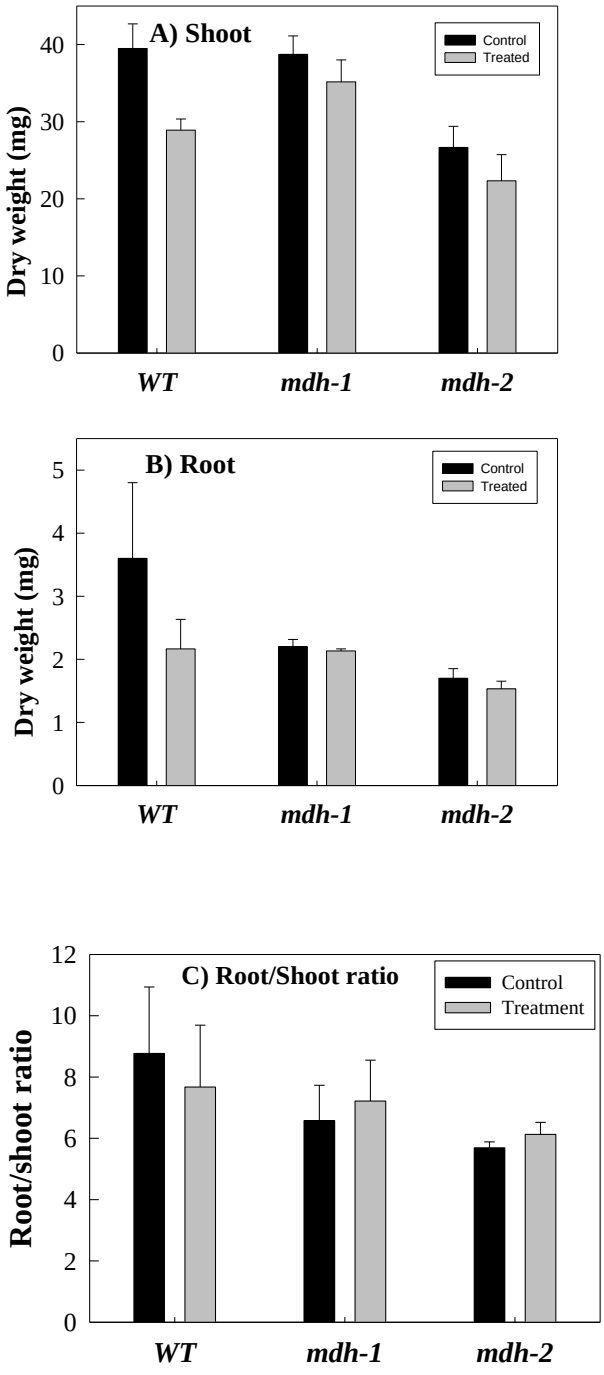


Figure 3.10. Growth of WT and T-DNA insertion lines (*mdh-1* and *mdh-2*) of *Arabidopsis thaliana* to compare aluminum sensitivity. The top layer did not contain aluminum, while the bottom layer contains 150 µM $AlCl_3$ (pH 4.33).

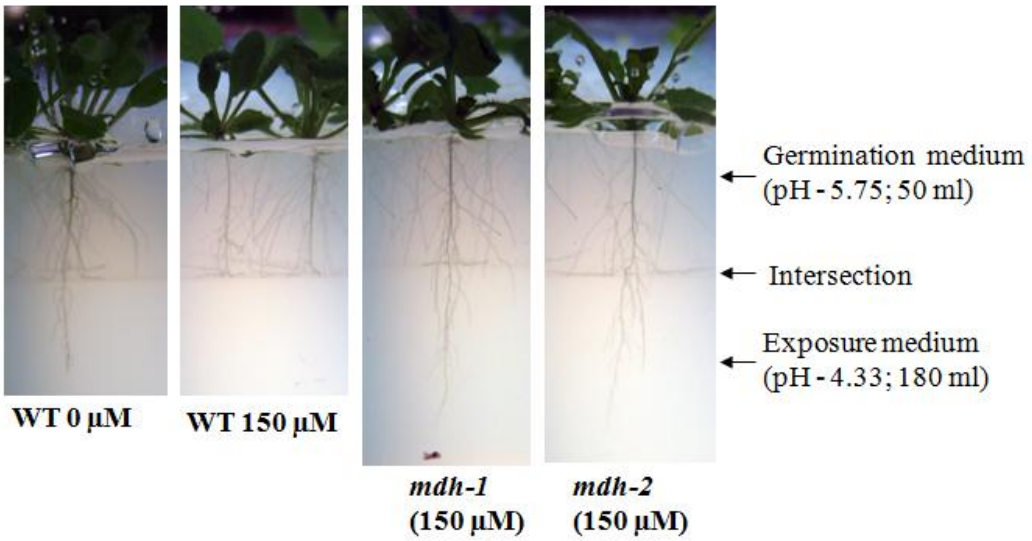


Figure 3.11. Enzyme activities of cyt-MDH in two separate fractions of the cytosol of roots of *Arabidopsis thaliana*. Activities in extracts obtained from control and treated roots are labeled accordingly. a) Enzyme activity of cyt-MDH in total cytosolic fraction after exposure to 1 h, 3 h, 6 h, 12 h, 24 h, and 48 h exposure to Al. b) Enzyme activity of cyt-MDH in cytosol enriched fraction after exposure to 1 h, 3 h, 6 h, 12 h, 24 h, and 48 h exposure to Al.

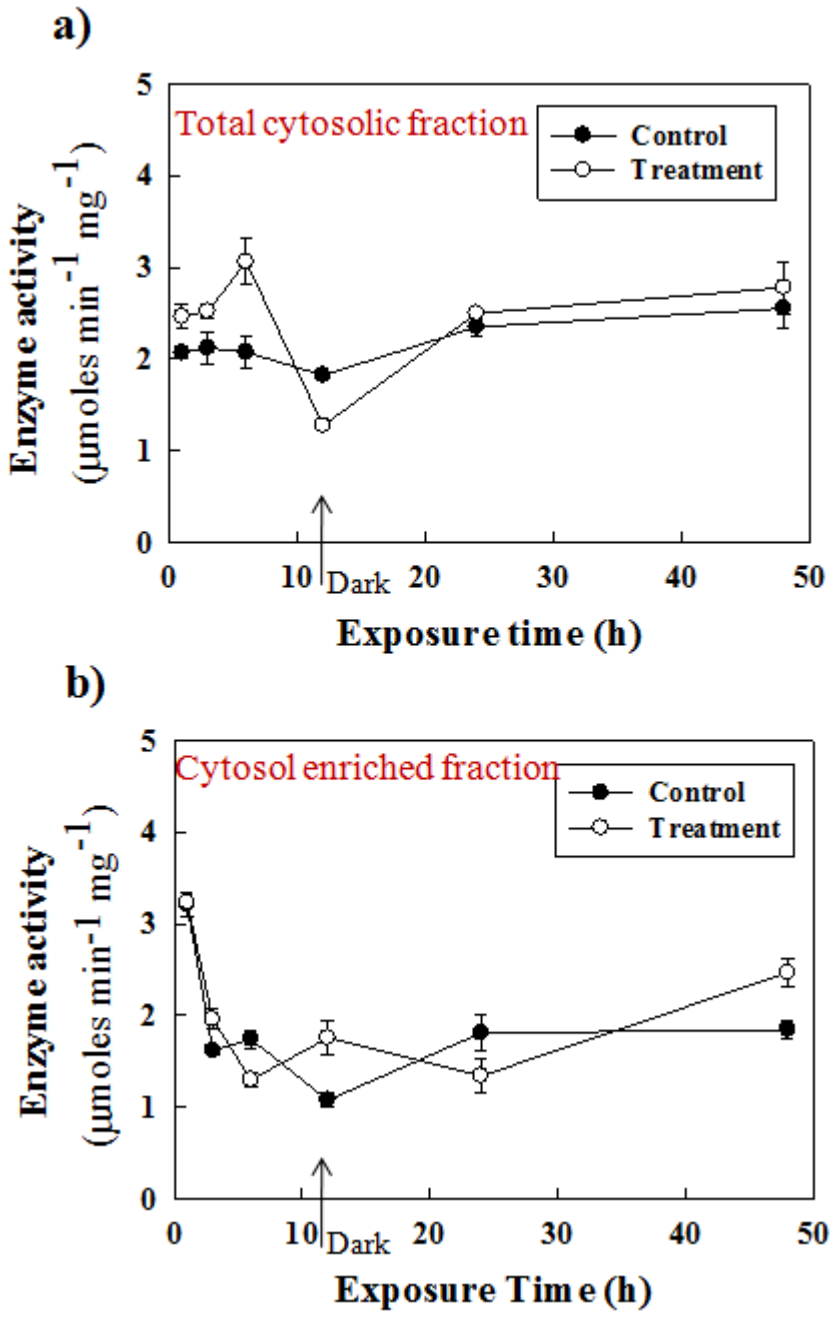


Figure 3.12. Diagrammatic representation of the reactions catalyzed by three differentially abundant proteins (m-MDH, cyt-MDH, and malic enzyme) involved in the malate metabolism and NADPH biosynthesis. Reversibility and reaction intermediates of TCA cycle not shown here.

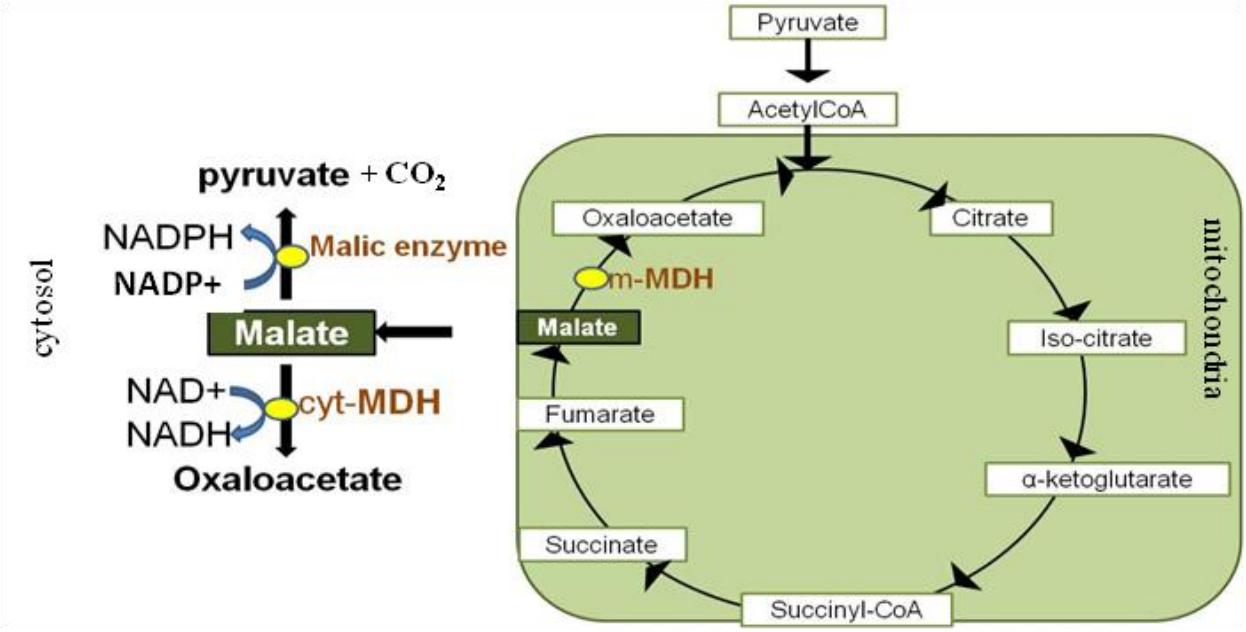


Table 3.1. Total number of protein spots picked and successfully identified in 6 h and 48 h gels.

	6 h exposure			48 h exposure		
	significant		random	significant		random
Picked	55		36	26		37
	Up	Dn		Up	Dn	
Abundance	13	42		22	4	
Identified	3	9	28	15	2	23

.

Table 3.2. List of proteins detected as differentially abundant after 6 h exposure to aluminum. Identity of each protein spot was determined by LC/MS followed by database search in mascot search engine. The mascot scores for identified protein are shown in column 5. Total number of peptides and the sequences of only unique peptides are shown column 6 and 7. The molecular weight (Mr), p*I* and *p*-value of each protein spot is shown in columns labeled accordingly.

Category / Spot number	Protein identity	Accession number	Score	No. of peptides	Identified peptides (MS/MS)	Fold change	M_r	p*I*	*p*-value
a) Energy and Metabolism									
1	Ribulose bisphosphate carboxylase	gi|1944432	120	3	LTYYTPEYETK, TFQGPPHGIQVER, DLAVEGNEIIR	2.1	48038	6.12	0.021
2	Alcohol dehydrogenase	gi|469467	106	2	IIGVDFNSK, TDIPGVVEK	1.4	41862	5.83	0.02
4	Fructose bisphosphate aldolase-like protein	gi|7529717	115	3	GILAADESTGTIGK, LASINVENVETNRR, AAQEALYVR	1.13	38882	6.05	0.023
5	Malate dehydrogenase (cytosolic)	gi|15219721	106	3	MELIDAAFPLLK (oxid), VLVVANPANTNALILK, LSVPVSDVK	-1.3	35913	6.11	0.015
10	Phosphoglycolate phosphatase	gi|30685622	43	1	MANLTTNAK (oxid)	1.13	25369	5.43	0.069

b) Oxidative stress									
3	ATP sulfurylase	gi|452470	52	2	NADAVFAFQLR	-1.3	51429	6.4	0.007
6	Isoflavone reductase-like protein	gi|7268070	54	2	VLVVGGTGSLGR, TYVSGNDFLADIEDK	-1.4	35586	5.79	0.013
7	Glutathione Stransferase (ATGSTF2)	gi|2262152	71	2	LAFEQIFK, VLDVYEAR	-1.3	24128	5.92	0.054
11	Glutathione S transferase (ATGSTF10)	gi|15224582	98	3	AVVTLVEK, SQGPDLLGK, YSLPV	-1.3	24230	5.49	0.032
12	Thioredoxin	gi|992966	53	3	FTNVVFFK, FIAPVFAEMAK (oxid), VEAMPTFVFMK (oxid)	-1.3	13293	5.19	0.034
c) Others									
8	Proteasome subunit	gi|577531	135	3	MSTGYSILSR (oxid), SPSPLLLPK, QDSNTPLSEAEAVDLVK	-1.6	24872	6.95	0.005
9	Unknown protein	gi|18401381	42	1	EAAKEVPK	-1.3	38084	8.28	0.087

Table 3.3. List of proteins detected as differentially abundant after 48 h exposure to aluminum. Identity of each protein spot was determined by LC/MS followed by database search in mascot search engine. The mascot scores for identified protein are shown in column 5. Total number of peptides and the sequences of only unique peptides are shown column 6 and 7. The molecular weight (Mr), p*I* and *p*-value of each protein spot is shown in columns labeled accordingly.

Category / Spot number	Protein identity	Accession number	Score	No. of peptides	Identified peptides (MS/MS)	Fold change	M_r	p*I*	*p*-value
a) Energy and Metabolism									
2	Succinyl-CoA-ligase beta subunit	gi|3660469	181	5	YGVNVPK, SAGPLIIACK, LYELFR, LNFDDNAAFR, LITADDLDDAAEK	1.5	45630	6.3	0.1
3	Malic enzyme-like protein	T48526	160	5	GIQVIVVTDGER, YMALMDLQER, TYDLGLASNLPR, FAESSMYSPVYR, AIFGSGSPFDPVVYDGK	1.2	64771	6.01	0.013
4	2,3-bisphosphoglycerate-independent phosphoglycerate mutase	gi|18391066	182	7	LCDQALASGK, IFEGEGFK, VHILTDGR, ENGVDAQIASGGGR, YENDWEVVK	1.2	60807	5.32	0.1

5	Dihydrolipoamide dehydrogenase 1	gi|8778521	78	3	HIIVATGSDVK, VVSVDSSSDGVK, AIDNAEGLVK	1.5	54070	6.96	0.018
7	Phosphopyruvate hydratase/Enolase	gi|15227987	129	4	AGAVVSGIPLYK, TYDLNFK, SCNALLLK, YNQLLR	1.5	48004	5.54	0.074
12	Malate dehydrogenase-mitochondrial	gi|3929649	362	7	NLCTAIAK, LFGVTTLDVVR, EGLEALKPELK, TQDGGTEVVEAK, DDLFNINAGIVK, SEVVGYMGDDNLAK, ALEGADLVIIPAGVPR	1.2	36032	8.54	0.016
b) Oxidative stress related									
14	L-Ascorbate peroxidase	gi|16173	153	5	NYPTVSEDYKK, LLDPIR, EGLLQLVSDK, LSELGFADA	1.3	27846	5.72	0.062
15	Glutathione S-transferase (ATGSTF8)	gi|20197312	198	6	LISQDCK, VLATLYEK, VLFDSRPK, VLDVYEAR, DLQFELIPVDMR, AITQYLAEEYSEKGEK	1.2	24134	6.09	0.03
16	Glutathione S-transferase (ATGSTF8)	gi|20197312	59	2	VLATLYEK, VLDVYEAR	-1.13	23761	6.08	0.009
c) Protein and amino acid synthesis and degradation									
1	Cobalamin-independent	gi|15238686	194	6	EVITELK,	1.4	84698	6.09	0.1

	methionine synthase (ATCIMS)				YLFAGVVDGR, VVEVNALAK, VSEEDYVK, LLSVFR, IPSSEEIADR				
10	19S Proteosome subunit 9 : signalosome complex	gi|3450889	41	3	TAKIVRR, AVADAHSK	1.4	47515	6.14	0.044
11	Branched-chain amino acid aminotransferase-like protein	gi|26391664	81	3	TDYMYVAK, AFPSGTGGVK, LYETLSDIQTGR	-1.2	39418	5.9	0.1
13	Cysteine synthase	gi|804950	291	6	IGFSMISDAEK, LIITMPASMSTER, IDGFVSGIGTGGTITGAG, LFVAIFPSFGER, YLSTVLFDATR, KEAEAMTFEA	1.6	33956	5.67	0.091
d) Others									
6	Signal transducer	gi|15229647	42	1	NELSKLNR	1.5	60495	6.41	0.055
8	Disease resistance family protein	gi|15230023	45	2	DALLELK, QNCSSFK	1.4	97986	6.28	0.1
9	Endomembrane-associated protein	gi|7268821	98	4	TFDESKETINKEIEEK, VVETYEATSAEVK, YLEELVK, TEGTSGEKEEIVEETK	1.3	24584	4.99	0.049
17	Universal stress protein / Ethylene-responsive protein	gi|18399413 / gi|21594903	156	3	QPETTTEAEAPSLTTK, AQQETSAALLSR, TETLVLEGEAK	-1.7	21686	5.53	0.066

Supplemental Table 3.1. List of proteins that were excluded for comparison of transcriptomic and proteomic data after 6 h and 48 h of aluminum exposure.

a. Identified protein spots whose corresponding AGIs failed to pass above background cut off in microarrays for 6 h data.

Protein ID	**Fold change**	**Corresponding AGIs**	**Printed on arrays**
Ribulosebisphosphate carboxylase	2.1	AtCg00490	no
Unknown protein	-1.3	At2g27285	no
Thioredoxin	-1.3	At1g45145	no
Catalytic/ hydrolase/ phosphoglycolate phosphatase	1.13	At2g33255	y
ATP sulfurylase	- 1.2	At3g22890	y

b. Identified protein spots whose corresponding AGIs failed to pass above background cut off in microarrays for 48 h data.

Protein ID	**Fold change**	**Corresponding AGIs**	**Printed on arrays**
NADP dependent malic enzyme-like protein	1.2	At5g11670	y
Dihydrolipoamide dehydrogenase 1	1.5	At1g48030	y
Signal transducer	1.5	At3g49970	y
Kinase/ protein binding	1.4	At3g05650	y
Unknown protein /universal stress protein	-1.7	At3g11930	y

Supplemental Table 3.2. Comparison between root proteomic and transcriptomic data obtained after 6 h and 48 h exposures to aluminum.

a. List of proteins and corresponding genes that were compared after 6 h treatment.

Protein fold change (log_2)	Microarray fold change (log_2)	AGI	Protein ID
-0.34	-0.41	At3g22890	ATP sulfurylase
0.50	0.15	At1g77120	Alcohol dehydrogenase
0.18	0.55	At3g52930	Fructose bisphosphate aldolase-like
-0.37	0.69	At1g04410	Malate dehydrogenase
-0.44	-0.12	At4g13660	Isoflavone reductase-like protein
-0.42	-1.18	At4g02520	Glutathione S transferase
-0.65	-0.14	At3g60820	Proteasome subunit

b. List of proteins and corresponding genes that were compared after 48 h treatment.

Protein fold change (log_2)	Microarray fold change (log_2)	AGI	Protein ID
0.50	0.54	At5g17920	Cobalamine-independent methionine synthase
0.27	0.53	At1g09780	2,3-bisphosphoglycerate-Independent phosphoglycerate mutase
0.57	0.20	At2g20420	Succinyl-coa-ligase beta subunit
0.62	0.43	At2g36530	Enolase
0.51	-0.01	At1g29150	19S proteosome subunit 9
-0.21	0.34	At3g19710	Branched-chain amino acid aminotransferase-like protein
0.33	-0.31	At4g20260	Endomembrane-associated protein
0.29	0.24	At1g53240	Mitochondrial malate dehydrogenase
0.67	-0.11	At4g14880	Cysteine synthase
0.33	-0.32	At1g07890	L-ascorbate peroxidase
0.21	-0.78	At2g47730	Glutathione S-transferase (GST6)

Note: The protein corresponding to At2g47730 was represented by two distict spots.

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4. Identifying the role of Class III plant peroxidases under aluminum stress using a reverse genetics approach

4.1. Introduction

Peroxidases (EC 1.11.1) catalyze oxidation-reduction reactions in which a peroxide is reduced and a substrate is oxidized. Peroxidases are found in animals, plants, and microorganisms, and are divided into three super-families based on their structural and catalytic properties (Hiraga *et al.* 2001). These super-families are: animal peroxidases, catalases, and plant peroxidases (Table 4.1). Despite its name, the plant peroxidase super-family includes enzymes from bacteria, fungi, and plants. This super-family is further divided into Class I, II, and III (Table 4.1). Within these three classes, five distinctly positioned amino acids (important for catalysis, structure, and the helical folding of the polypeptide) are strictly conserved. Therefore all members share a similar three-dimensional structure (Schuller *et al.* 1996, Gajhede *et al.* 1997) despite less than 20% amino acid similarity between the most divergent sequences.

Class I plant peroxidases are intracellular enzymes present in all organisms except animals, whereas Class II plant peroxidases are extracellular peroxidases found exclusively in fungi. Structurally, Class II plant peroxidases are unique in having an additional 40 to 60 amino acid residues in their C-termini as compared to other plant peroxidases (Welinder *et al.* 2002). Class III plant peroxidases (EC 1.11.1.7) are unique among this superfamily, in that they are found exclusively in plants and differ structurally by having three structural helices instead of just one as reported for Classes I and II (Schuller *et al.* 1996, Gajhede *et al.* 1997). Class III peroxidases, which I will refer to by the abbreviation PER, have also been known by the abbreviations POX, PRX, or POD. PER genes are the focus of this chapter.

PERs are heme-containing enzymes that are secreted either outside cells or transported into vacuoles. Functionally, Class III plant peroxidases have been proposed to be regulators of ROS abundance. Depending on whether the peroxidative (catalytic) or hydroxylic cycle of the enzyme is functional, PERs either decrease the abundance of extracellular H_2O_2 or generate highly toxic radicals such as hydroxyl ($OH^{\bullet}$), and hydroperoxyl ($OOH^{\bullet}$; Bolwell *et al.* 2002, Kawano 2003, Passardi *et al.* 2005; Bindschedler *et al.* 2006). During the peroxidative cycle, PERs catalyze the reduction of H_2O_2 by oxidizing various substrates such as NAD(P)H, indole acetic acid, and saturated fatty acids (Hiranga *et al.* 2001). In the hydroxylic cycle, PERs use the superoxide anion (O_2^{-}) to generate $OH^{\bullet}$. By operating these two cycles, PERs participate in different plant development processes from germination to senescence (e.g. auxin metabolism, cell wall elongation, and stiffening) or help protect against abiotic and biotic stresses (Kawano 2003, Liu *et al.* 2005, Cosio *et al.* 2009).

Sequencing of the Arabidopsis genome has helped to identify 73 sequences encoding putative PER genes (*PER1-PER73*) (Tognolli *et al.* 2002, Welinder *et al.* 2002). The phylogenetic relationship, based on complete amino acid sequences and upstream sequences (Fig. 4.1, Fig. 4.2), suggests that a succession of genomic rearrangements resulted in extensive duplication and diversification of the peroxidase gene family. The extent to which sequence diversification of PERs has any relevance to their possible functional diversification remains to be seen.

Several types of abiotic stress affect expression of PERs. These include Al (Richards *et al.* 1998, Kumari *et al.* 2008, Maron *et al.* 2008), NaCl (Jiang and Deyholos 2006), Cd/As (Weber *et al.* 2006, Abercrombie *et al.* 2008), cold (Llorente *et al.* 2002), ozone (Ludwikow *et al.* 2004), anoxia (Klok *et al.* 2002), and nutrient deficiencies such as sulphur (Nikiforova *et al.* 2003), potassium (Kang *et al.* 2004), and phosphate

(Hammond *et al.* 2003). I focused my attention on the potential role of peroxidases in protecting plants against Al-stress based on my previous microarray-based observation of plants exposed to Al (Kumari *et al.* 2008). My microarray data showed that compared to almost any other gene family, a higher proportion of PER genes was responsive to Al compared to most other classes of genes. Of the 73 PER genes predicted in the Arabidopsis genome, 71 were represented by probes printed on my microarrays. I detected transcripts of 36 and 41 genes above background for 6 and 48 h respectively; of these transcripts of 15 different PERs changed in abundance following exposure to Al (Table 4.2). After 6 h transcripts for ten Class III peroxidase genes increased and three others decreased, whereas after 48 h, transcripts for one gene decreased and one other increased (Fig. 4.3). Among these 15 genes, transcripts for two genes (*PER27* and *PER62*) were found common to have a similar transcript abundance pattern to each other at both 6 h and 48 h data (Fig. 4.3).

Inhibition of elongation of the primary root is a major symptom of Al toxicity and is correlated with modifications in cell wall properties, including reduced extensibility (Tabuchi and Matsumoto 2001), and changes in the distribution and content of polysaccharides such as pectin (Yang *et al.* 2008, Li *et al.* 2009). Peroxidases have been suggested to promote formation of phenolic linkages in the cell wall, thus restricting cell growth (Cosio and Dunand 2009). Also, some PERs reportedly have indole-3-acetic acid oxidase activity (Gazaryan *et al.* 1996), and hence may control auxin activity in the elongation zone through the catabolism of auxin in roots (Kawano 2003, Cosio *et al.* 2009).

Among the 15 distinct PERs identified as Al-responsive, I selected seven for study of transcript profiles at additional Al exposure time points besides the two time points (6 h and 48 h) previously studied (Kumari *et al.* 2008, Chapter 2). I also further

explored the role of PERs in Al-treated root elongation using a reverse genetics approach. In this chapter, I present the hypothesis that the activity of specific peroxidases, regulated in part by their transcript abundance, influences the Al tolerance of roots of Arabidopsis.

4.2. Materials and methods

Seven PER genes (*PER2, PER24, PER27, PER49, PER56, PER62,* and *PER69*) were selected to represent different patterns of transcript abundance previously detected in my microarrays (Kumari *et al.* 2008). I used two separate techniques to determine whether knocking out these PERs has any effect on root elongation or on the dry weight of mutant plants. For root elongation studies, plate assays were used and for dry weight measurements, a hydroponic system was used.

4.2.1. Plant material and growth conditions

Seeds of WT *Arabidopsis thaliana* (Col-0) were obtained from Lehle Seeds. The following T-DNA insertion mutants were obtained from either the Arabidopsis Biological Resource Center or the Nottingham Arabidopsis Stock Centre: *PER2* (Salk_008488, SALK_101287), *PER21* (SALK _021989, SALK_024315), *PER69* (Salk_137991, Al752351). The mutants were named as *per2-a, per2-b, per21-a, per21-b, per69-a,* and *per69-b*.

Seeds were surface sterilized (2.25% NaClO, 0.05% Tween-20) and were sown on 1/2 X MS medium (0.3% Phytagel). Aluminum (Al) treatments were conducted by transferring rafts to exposure solutions containing $AlCl_3$ in 200 µM $CaCl_2$. Throughout their life cycle, plants were exposed to 16 h light/8 h dark cycle in a growth chamber with approximately160 µmol m^{-2} s^{-1} photosynthetic photon flux density and 65% relative humidity.

4.2.2. Aluminum treatment

For plate assays, exposure medium (Snowden *et al.* 1995) containing 0.15 mM NH_4NO_3, 0.3 mM KNO_3, 0.2 mM NaCl, 0.1 mM $MgSO_4$, 5 µM $(NH_4)H_2PO_4$, 5 µM H_3BO_4, 1 µM $MnSO_4$, 0.2 µM $CuSO_4$, 1 µM $ZnSO_4$, 0.2 µM $CoCl_2$, 5 µM $FeCl_3$ and 500 µM $CaSO_4$ was prepared. Prior to autoclaving, pH was adjusted to 4.3 and 14 g Phytagar was added. Aliquots of $AlCl_3$ stock solution (filter sterilized) were added after autoclaving. To obtain 0 µM, 100 µM, 150 µM and 200 µM $AlCl_3$, 0 ml, 4 ml, 6 ml or 8 ml of 25 mM stock of $AlCl_3$ was added to media (respectively) followed by plating in 15 cm square plates.
For hydroponics, exposure medium containing $CaCl_2$ (200 µM, pH 4.33) was prepared to avoid precipitation of Al. To obtain 25 µM $AlCl_3$ exposure medium, 1ml of $AlCl_3$ stock solution was added to a liter of 200 µM $CaCl_2$, and pH was adjusted to 4.3. The use of alkali was avoided while adjusting the pH of exposure solutions to prevent the formation of non-toxic Al species (Kinraide and Parker 1987). Aluminum (Al) treatments were conducted by transferring rafts (containing 14 day old plants) to Al exposure solutions (Chapter 2; Kumari *et al.* 2008). Stock solutions of Al (25 mM) were prepared by adding $AlCl_3$ to water that had been adjusted to pH 3.0 with HCl.

4.2.3. Root elongation, shoot area, and dry weight measurements

Seeds of WT and *per* mutants were grown on 1/2 X MS medium (pH 5.75; 0.65% Phyagar, Sigma) for 7 d in vertically positioned plates to prevent penetration of roots into the medium. Seedlings with uniform root lengths were transferred onto the surface of Al-containing exposure medium and control medium in petri plates. Control medium contained no Al (0 µM) whereas exposure medium contained 150 µM $AlCl_3$. Based on a preliminary experiment, differences in root elongation were detectable after exposure to 150 µM $AlCl_3$ only. Therefore, exposure media containing 100 µM and 200 µM $AlCl_3$ were included only for the purpose of photographs. Each plate contained 10 seedlings. To

compare seven genotypes (including WT) under control and treatment condition (0 µM and 150 µM) a total of 56 plates were used (7 genotypes x 2 concentrations x 4 replicates = 56 plates). I marked the position of root tip after every 24 h for 72 h and photographed the plates after 7 days of exposure. Differences in root lengths were calculated for WT and all six *per* mutants for three time points (0-24 h, 24-48 h, 48-72 h) under both control and treatment conditions. For statistical analysis, root lengths were averaged for each plate. A two-way ANOVA was used to test for differences between genotypes, concentration of Al, and the genotype x Al interaction, and a Tukey pairwise multiple comparison was used to identify differences in means using Sigma Stat ver. 3.5. For plants grown for 7 days, root lengths of plants grown under control conditions could not be measured as roots elongated and accumulated at the bottom of plates. Root lengths for plants grown under Al stress were analyzed by one-way ANOVA to test for significant effects due to genotype. All plates were photographed and images were processed in Photoshop to remove square-patterned background of petri-plates. Precise measurements of root elongations were calculated using ImageJ software.

To measure leaf areas of primary leaves, rosette leaves were cut from one representative plant from each plate and spread between two glass slides. The slides were scanned using Cannon Scan LiDE90 and resulting images were processed in Photoshop to measure the leaf area using ImageJ software.

For dry weight analyses, plants were grown in hydroponics (as described previously; Kumari *et al.* 2008) for 14 d and were exposed to Al treatment for 7 d. Exposure solutions contained 0 or 25 µM $AlCl_3$ at pH 4.33. Two control and two treatment tanks used to collect root tissue. Therefore, a total of 28 tanks were used (2 treatment x 2 control x 7 genotypes = 28 tanks). After 7 d of exposure, the two longest roots were selected from 100 plants and pooled for dry weight measurement from each of

control (0 µM) and treatment (25 µM) for WT and all *per* mutants. Dry weights were estimated by drying whole plants for 3 days in a hot air oven set at 65° C.

4.2.4. Isolation of RNA and QRT-PCR

To detect the transcript abundance pattern of selected PER genes at time points between 1 h and 48 h following Al exposures, I used QRT-PCR. Root tissues from more than 120 plants from three independently maintained hydroponic tanks were pooled together to comprise one replicate. In total, three replicates were used. For a single replicate, 54 tanks were used (3 control x 3 treated x 6 time point = 54 tanks). For three replicates a total of 162 tanks (54 tanks x 3 replicate = 162 tanks) were used for six time points (1 h, 3 h, 6 h, 12 h, 24 h and 48 h).

Total RNA was isolated using a silica membrane-based RNA isolation kit (Qiagen). QRT-PCR primers were designed using PrimerExpress 2.0 software (Applied Biosystems). The specificity of each primer pair was checked against the Arabidopsis genome using WU- BLAST 2.0 (http://www.Arabidopsis.org/wublast/index2.jsp). The primer sequences were as follows:

PER2 (At1g05250) F'GATGCCGTGGCAGTGAT,

R'CTTTATGTCGGCGAAAGGAG;

PER27 (At3g01190) F: ACTCGGAGTACGCCGCTAAG, R:

TTGAAACTCCCCGGATCCA;

PER34 (At3g49120) F: CTAGCCCCAATGCCACTGA, R:

GCCTCCACAAATGCATTGAA;

PER62 (At5g39580) F: TTTCCTTGGCATCGAACGTT, R:

AGGCGAAAAGCGGAGAATTT;

PER69 (At5g64100) F: CTCTTGTTGGCGGACACA, R:

GTCGATTGATGGGTCAGGTT;

UBC (At5g25760) F: CTTAACTGCGACTCAGGGAAT, R: GGCGAGGCGTGTATACATTT.

All RNA samples were treated with DNA*free* (Ambion). I quantified cDNAs in an Applied Biosystems 7500 Real-Time PCR System, with a SYBR green dye detection assay, and ROX passive reference, in 10 µl reactions as follows: 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 1 min; Stage 3, 95.0° C for 0.15 min; Stage 4, 60.0° C for 1 min; and Stage 5, 95.0° C for 0.15 min. C_t values generated for each primer pair set over a range of dilutions were used to calculate the ΔC_t (C_t target – C_t reference). ΔC_t values were then plotted against log input amount. If the slope of the ΔC_t vs. log input was < 0.1 then the relative abundance of each transcript was estimated using the $\Delta\Delta C_t$ (Livak and Schmittgen 2001).

4.3. Results

To validate and extend the results of my previous microarray analysis, I selected seven PER genes for analysis by QRT-PCR: *PER2, PER24, PER27, PER34*, *PER56*, *PER62*, and *PER69*. These were selected to represent different patterns of transcript abundance (Chapter 2; Kumari *et al.* 2008; Fig. 4.4). Due to high sequence similarities among closely related *PER* genes, I could not amplify a homogenous product using primers for either *PER24* or *PER56* (data not shown). Therefore, only five genes (*PER2, PER27, PER34*, *PER62*, *and PER69*) were suitable for QRT-PCR. In addition, I selected ubiquitin conjugating enzyme (*UBC*; At5g25760) as a reference gene (Czechowski *et al.* 2005) and confirmed its stable transcript abundance pattern in root tissue from eight independent samples collected after 6 and 48 h exposures.

To detect dynamic changes in transcriptome with greater temporal resolution than the two time points (6 and 48 h) selected for microarrays, I exposed roots of Arabidopsis to 1, 3, 6, 12, 24, and 48 h of Al treatment in hydroponic culture. I measured transcript abundance in three biologically independent samples for each time point using QRT-PCR. I detected changes in the transcript abundance for all five selected peroxidases, with changes detected as early as 1 h (Fig. 4.4). Furthermore, *PER2*, *PER27*, and *PER69* showed similar patterns, as transcripts for each of these genes increased in abundance after 1 h, 3 h, and 6 h, but decreased after 24 h, and 48 h. In general, transcript abundance of all selected PERs increased after 1 h and then decreased after 48 h, except for *PER62*.

I compared the protein sequences and transcript expression patterns of each of the five PER genes and found that sequence identity was not correlated with similarity in expression pattern. For example, despite 78% amino acid identity, the transcript expression pattern of *PER62* and *PER69* were less (Fig. 4.1, Fig. 4.4).

I further compared the identities in the upstream sequence and amino acid sequence of each PER gene with the transcript abundance pattern (Chapter 2; Kumari *et al.* 2008) using the published phylogenetic data (Tognolli *et al.* 2002, Cosio and Dunand 2009). For example, *PER27* and *PER56* genes have a close phylogenetic relationship based on upstream sequence (Fig. 4.2; Cosio and Dunand 2009) as well as amino acid sequence (Fig. 4.1; Tognolli *et al.* 2002). Both genes also showed increased transcript abundance after 6 h and 48 h of Al stress; respectively (Table 4.2). This may suggest that both genes arose due to a recent gene duplication event (Tognolli *et al.* 2002) as minimal diversification of expression pattern and amino acid was observed. On the other hand, *PER*50 and *PER*56 also have a close phylogenetic relationship in their upstream sequences but displayed opposite transcript abundance patterns (*PER50* decreased whereas *PER56* increased in transcript abundance after Al exposure) and diverged widely

at the level of amino acid sequence (Fig. 4.1). This may suggest functional diversification of *PER50* and *PER56* and also supports a previous analysis that conservation of upstream sequences is independent of coding sequences (Chiba *et al.* 2008). A population genetic theory suggests that most duplicate gene pairs revert to a single copy on a short evolutionary timescale, however, pairs that are retained are likely to have at least partially diverged in function (Lynch and Conery 2000). Also, Haberer *et al.* (2004) estimated that twothirds of duplicate gene pairs had divergent expression in Arabidopsis. Thus, it is not surprising to observe functional diversification within large gene family of Class III peroxidase (Passardi *et al.* 2005) and the diversity of expression patterns within these closely related members (Kumari *et al.* 2008).

4.3.1. Comparison of root elongation of WT and *per* mutants after aluminum treatment

To identify the role of peroxidases in root elongation, I used a reverse genetics approach, focusing my attention on *PER2*, *PER21*, and *PER69*, because two different T-DNA insertion mutants were available for each these genes. According to previous microarray based gene expression map of Arabidopsis development (Birnbaum *et al.* 2003) transcripts of two of these (*PER2* and *PER69*) are expressed primarily in roots and the other (*PER21*) is expressed in both roots and shoots (Fig. 4.5; Winter *et al.* 2007). Specifically, *PER2* is expressed in root epidermis and the stele whereas *PER69* is strongly expressed in both epidermal atrichoblasts and the stele, but weekly expressed in epidermis, endodermis and cortex tissue (Fig. 4.5)

I obtained T-DNA insertion lines for *per* mutants and named them *per2-a, per2-b, per21-a, per21-b, per69-a*, and *per69-b*. I examined sequences flanking the T-DNA in each line (Fig. 4.6). For *per2-a,* the insertion was located in an intron whereas for *per2-b* the insertion was located in the exon. For both *per21-a* and *per21-b* the insertions were

located in separate exons. For *per 69-a* mutant the insertion was in exon and for *per69-b* the insertion was in upstream sequence.

I grew plants to the T_6 generation and confirmed the inheritance of a homozygous insertion (data not shown) by PCR on genomic DNA using primers specific for T-DNA and each respective gene (as described in chapter 3, Fig. 3.7).

Seedlings were grown on vertical agar plates to detect differences in root elongation between WT and six *per* mutants after exposure to Al (0 µM, 100 µM, 150 µM, and 200 µM $AlCl_3$). Detectable differences in root length increments were observed after exposure to 150 µM $AlCl_3$. Exposure to this concentration resulted in complete inhibition of elongation of primary roots after 3 days of exposure. A two way ANOVA indicated a significant effect due to genotype ($p< 0.001$), concentration of Al ($p< 0.001$), and the genotype x Al interaction ($p< 0.001$). Mean root lengths of WT plants grown on 150 µM $AlCl_3$ were 37% (± 1.2 standard error) of those grown under control conditions (Fig. 4.7). Comparison between the genotypes showed that only two *per* mutants, namely *per21-a,* and *per69-b* were significantly different from WT. These mutants showed greater primary root length than WT (44% ± 1.3 and 47% ± 1.7 respectively; Fig. 4.7, Fig. 4.9a).

Although primary root elongation stopped after 3 d of exposure to Al, lateral roots continued to elongate in some circumstances. Considering primary and lateral roots together, differences in total root lengths were more apparent when *per* mutants were compared with WT after 7 d of exposure to 150 µM $AlCl_3$ (Fig. 4.8, 4.9b). Under these conditions, a one way ANOVA indicated that all *per* mutants except for *per2-a* showed significant increases in total root lengths (Fig. 4.9b). The differences between WT and *per* mutants were observed only after exposure to 150 µM $AlCl_3$ and not after exposure to 100 or 200 µM $AlCl_3$ (Fig. 4.8).

To determine whether any of the *per* mutations affected biomass accumulation under Al stress, I measured dry weights of roots from WT and mutant plants exposed to 25 µM $AlCl_3$ for 7 days in hydroponic culture. No differences in dry weight were observed when *per* mutants were compared to WT plants after exposure to Al (data not shown).

4.3.2. Inhibition of leaf area expansion

Shoot growth of WT and *per* plants was significantly inhibited by treatment with 150 µM $AlCl_3$ (Fig. 4.10, Fig. 4.11). I calculated percent decrease in leaf area in treated plants as compared to untreated controls, but observed no significant ($p=0.760$) differences in leaf area for any of the mutants when compared to WT.

4.4. Discussion

The large PER gene family in Arabidopsis is proposed to have arisen through gene duplication (Tognolli 2002, Cosio and Dunand 2009). *PERs* have been implicated in a number of functions in plants and extensive duplication may have allowed for functional diversification. Recently, in maize, Maron *et al.* (2008) also observed differential abundance of transcripts encoding peroxidases after exposure to Al. Alignment of Al-induced maize probe sequences with the Al-induced Arabidopsis transcripts I reported earlier, identified a single PER (*PER27*) as one of the several genes found common to both datasets (Kumari *et al.* 2008, Maron *et al.* 2008).

Although it is evident that PER genes do play an important role in abiotic stresses (Klok *et al.* 2002, Llorente *et al.* 2002, Hammond *et al.* 2003, Kang *et al.* 2004), their specific cellular functions remain unknown (Cosio and Dunanad 2009). Root elongation for most *per* mutants after 3 d exposure to Al was not significantly different from WT,

except for *per21-a,* and *per69-b*. Although root elongation in both *per21-a,* and *per69-b* was less affected by Al than WT, other alleles of these genes (*per21-b* and *per69-a*) did not show any difference as compared to WT. I looked closely at the positions of T-DNA insertions to see whether these could explain these observed phenotypic differences. Interestingly, *PER21* gene has two gene models owing to two mRNA splice variants. These two splice variants arise depending on whether first exon is spliced out or not. In *per21-b,* the T-DNA insertion is in the first exon, whereas in *per21-a* insertion is in the second exon (Fig. 4.6) which is retained irrespective of alternative splicing. Since the insertional effect of mutation in *per21-a* mutant line was independent of splicing, it is possible that this could be a reason that *per21-a* showed increased root elongation after 3 d of exposure to Al as compared to *per21-b*. Reverse transcriptase -PCR and sequencing would help to determine whether under Al stress *PER21* actually undergoes alternate splicing. Also, in the case of *per69-a* the T-DNA insertion was in an exon and in *per69-b* the insertion was in sequence upstream of 5'UTR. Thus differences observed between *per69-a* and *per69-b* could be due to a positional effect of T-DNA insertion.

All *per* mutants (except *per2-a*) had greater total root lengths compared to WT after 7 d of exposure. Why most mutants exhibited a significant response to long-term stress (attributed to growth of lateral roots), but not after short- term stress (attributed to growth of primary roots) is intriguing. This could indicate some benefit of reduced abundance of peroxidases after a long period of stress. For instance, *PER2* and *PER69* had reduced abundance of transcripts after 48 h exposure as compared to shorter exposure periods 1 h, 3 h, and 6 h (Fig. 4.4). Although I did not measure transcript abundance of *PER2*, *PER21* and *PER69* genes beyond 48 h exposure, it appears that reduced transcript abundance of these PERs in respective *per* mutants is favourable under prolonged

exposure to Al stress because *per* mutants showed increased root elongation as compared to WT.

Also, it was interesting that although *per2-a,* and *per2-b* have insertion mutations in the same gene, each allele had a different response to long term exposure to Al. I examined sequences flanking the T-DNA in each line (Fig. 4.6). For *per2-a*, the T-DNA insertion was located in an intron; whereas for *per2-b*, the insertion was in an exon (assuming that the predicted insertion sites are accurate; Fig. 4.6). While T-DNA insertion in introns might not be expected to impact function of genes, the impact on gene expression is complicated to predict. For instance, aberrant transcriptional termination and/or inappropriate intron processing due to insertion in an intron can affect function of a gene (Bennetzen *et al.* 1984). On the other hand, if insertion does not disrupt *cis* elements involved in intron processing, then they can be spliced out of the transcript together with the introns (Miesak and Coruzzi 2002, Gusmaroli *et al.* 2007, and Xu *et al.* 2007). Splicing out of T-DNA contained in introns has been suggested be a widely occurring phenomenon (Ulker *et al.* 2008). Nonetheless, excision of the T-DNA sequences does not occur with equal efficiency in various mutant lines and efficiency has been reported to be always less compared to non-inserted introns of WT plants. This may lead to expression level of these mutants ranging from null mutants to WT (Ulker *et al.* 2008). Although I did not measure the transcript abundance of *PER2* gene in *per2-a* and *per2-b* mutant lines, it is possible that the T-DNA insertion in the intron was spliced out (with undertermined efficiency) resulting in WT like phenotype of *per2-a* mutant plants under Al stress. Thus, the differences observed between *per2-a* and *per2-b* could be due to the position of the T-DNA insertion.

In summary, comparisons between WT and *per* roots showed that after a short period of treatment (3 d), only *per21-a,* and *per69-b* were significantly more resistant to

Al than WT; whereas after long term exposure (7 d) most *per* mutants (except *per2-a*) were more resistant. Two alternative theories may explain why a *per* mutant could have longer roots as compared to WT after exposure to Al. First, reduced peroxidase activity could result in decreased cross-linking of cell wall components (such as linking arabino-xylans by diferulic acid bridges) or reduced lignification, thus enhancing the cell wall extensibility. Secondly, perhaps reduced peroxidase activity may decrease peroxidase-mediated catabolism of growth promoting hormones (such as auxin; Kawano 2003), thus affecting root elongation (Cosio *et al*. 2009). Also, since lateral root meristem activation and elongation are dependent on auxin (Peret *et al*. 2009) it is possible that reduced activities of peroxidases perhaps result in decreased degradation of auxin and hence increased elongation of lateral roots. The role of peroxidase in modification of cell walls and elongation of primary or lateral roots correlate with the observation that most PERs are secreted proteins, have an affinity for pectin *in vivo* (Shah *et al.* 2004) which facilitates the distribution of these proteins within the cell wall network.

Analysis of root elongation data and QRT-PCR data suggests that decreased abundance of PERs (*PER2*, *PER21,* and *PER69*) is favorable in roots exposed to Al stress. Although roots of WT plants showed decreased abundance of PERs (detected by QRT-PCR) after 48 h exposure Al, the decreases are expected to be many fold more in *per* mutants, thus resulting in a resistant phenotype. Notably, stronger expression of both the root specific genes (*PER2* and *PER69)* has been reported in stele tissue (Fig. 4.5; Birnbaum *et al*. 2003). Recently, higher accumulation of H_2O_2 in stele and decreased activity of peroxidase has been shown in roots exposed to NaCl (Hernandez *et al.* 2009). This study proposed that higher accumulation of H_2O_2 in treated plants could be due in part to decreased activity of peroxidases. Previously, PERs have been proposed to decrease abundance of H_2O_2 (Hiraga *et al.* 2001). It would therefore be interesting to

study H_2O_2 levels in *per* mutants exposed to Al stress. Though H_2O_2 can be a source of oxidative stress, it is also been shown to be beneficial by acting as a signaling molecule (Wood *et al.* 2003). The burst of H_2O_2 used for signaling can then be regulated (to prevent programmed cell death) by antioxidant system involving ascorbate peroxidases and GSTs (Wood *et al.* 2003) both of which were found differentially abundant after Al stress (Chapter 3, Table 3.2 and 3.3).

In conclusion, this study provides the evidence that activity of specific peroxidases, regulated in part by their transcript abundance, influences the resistance to Al in roots of Arabidopsis.

Table 4.1. Classification of peroxidases.

Super-family	**Class**	**Member**	**EC number**	**Origin**	**Molecular weight (kDa)**
Animal peroxidases		Eosinophill peroxidase	EC 1.11.1.7	Animal	50–75
		Lactoperoxidase	EC 1.11.1.7	Animal	78–85
		Myeloperoxidase	EC 1.11.1.7	Animal	79–150
		Thyroid peroxidase	EC 1.11.1.7	Animal	90–110
		Glutathione peroxidase	EC 1.11.1.9	Animal and plant	6–22 75–112
		Prostaglandin endoperoxide synthase	EC 1.14.99.1	Animal	115–140
Catalases		Catalase	EC 1.11.1.6	Animal, plant, fungus and yeast	140–530
Plant peroxidases	I	Cytochrome *c* peroxidase	EC 1.11.1.5	Yeast and bacterium	32–63
		Catalase-peroxidase	EC 1.11.1.6	Bacterium and fungus	150–240
		Ascorbate peroxidase	EC 1.11.1.11	Plant	30–58
	II	Manganese-dependent peroxidase	EC 1.11.1.13	Fungus	43–49
		Ligninase	EC 1.11.1.14	Fungus	40–43
	III	**Peroxidase (PER)**	**EC 1.11.17**	**Plant**	**28–60**

Table 4.2. All genes from the Class III plant peroxidase family that showed increased or decreased abundance at the transcript level after exposure to 6 h and 48 h aluminum treatments. The transcript abundance pattern dissected into 1.5 fold or 2.0 fold increase or decrease is labeled accordingly in the columns. Genes selected for further analysis are shown in bold.

AGI	Gene name	6 h UP 1.5	6 h UP 2.0	6 h DN 1.5	6 h DN 2.0	48 h UP 1.5	48 h UP 2.0	48 h DN 1.5	48 h DN 2.0	Root specificity
AT1G05250	***PER2***	Y	Y	-	-	-	-	-	-	Yes
AT2G37130	*PER21*	Y	Y	-	-	-	-	-	-	Roots, flower, stem, senescent leaves
AT2G39040	*PER24*	-	-	-	-	-	-	Y	Y	Yes
AT3G01190	***PER27***	Y	Y	-	-	-	-	Y	-	Yes
AT3G49120	*PER34*	-	-	Y	-	-	-	-	-	Most stages of development
AT4G21960	*PER42*	Y	-	-	-	-	-	-	-	Most stages of development
AT4G30170	*PER45*	Y	Y	-	-	-	-	-	-	Yes
AT4G36430	***PER49***	-	-	Y	Y	-	-	-	-	Yes
AT4G37520	*PER50*	-	-	Y	-	-	-	-	-	Seeds, siliques, roots
AT5G15180	*PER56*	-	-	-	-	Y	-	-	-	Seeds, siliques, roots
AT5G19890	*PER59*	Y	-	-	-	-	-	-	-	Yes
AT5G39580	***PER62***	Y	Y	-	-	-	-	Y	Y	24h imbibed seed and root
AT5G42180	*PER64*	Y	Y	-	-	-	-	-	-	No (stem)
AT5G64100	***PER69***	Y	Y	-	-	-	-	-	-	Yes
AT5G67400	*PER73*	Y	Y	-	-	-	-	-	-	Yes

Figure 4.1. Phylogenetic tree of the encoded protein sequences of Arabidopsis peroxidases. Scale at the bottom represents ten substitutions per 100 amino acid residues. Boxes highlight duplications events (modified from Tognolli *et al.* 2002).

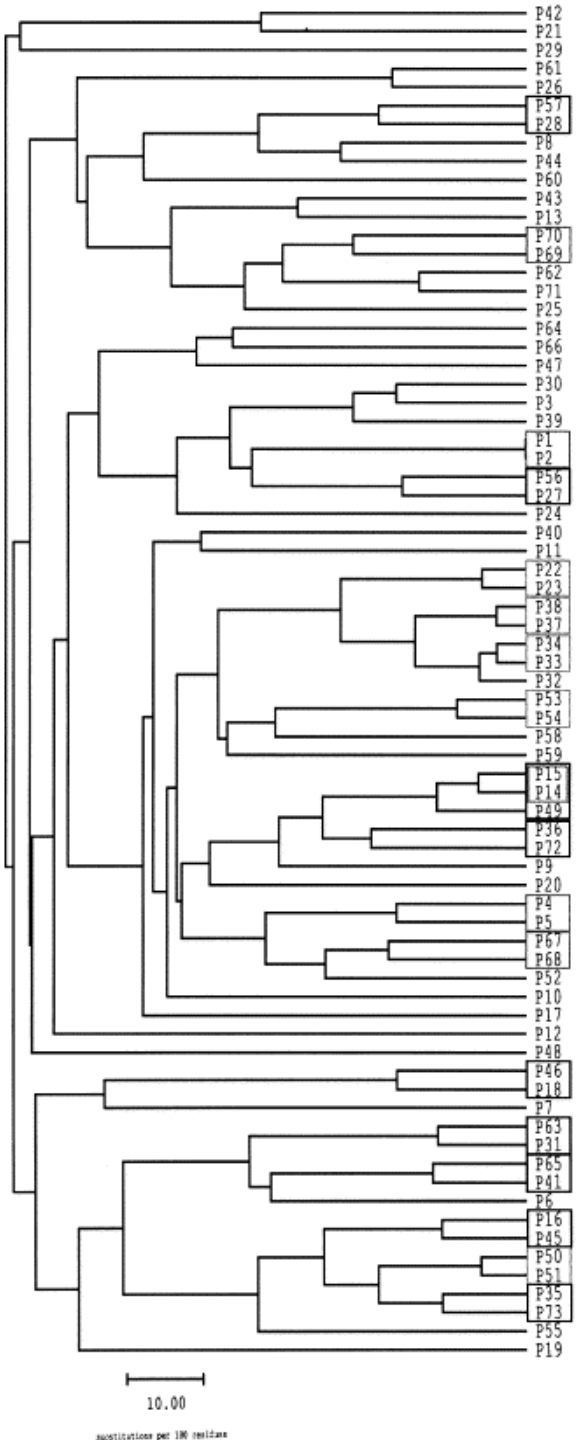


Figure 4.2. Phylogenetic tree of the promoter sequences of Arabidopsis peroxidases (modified from Cosio and Dunand 2009) based on the 1,000 bp regions upstream of the ATG codon. All branches are to the scale and the scale bar represents 0.05 substitutions. Protein sequence homology and identity (%) to the Horse Radish Peroxidase-C amino acid sequence is shown next to the peroxidase name. Circles highlight the 15 PER genes that were found Al-responsive in my microarrays (Kumari *et al*. 2008, chapter 2) and green circles highlight five PER genes whose expression was confirmed using QRT-PCR.

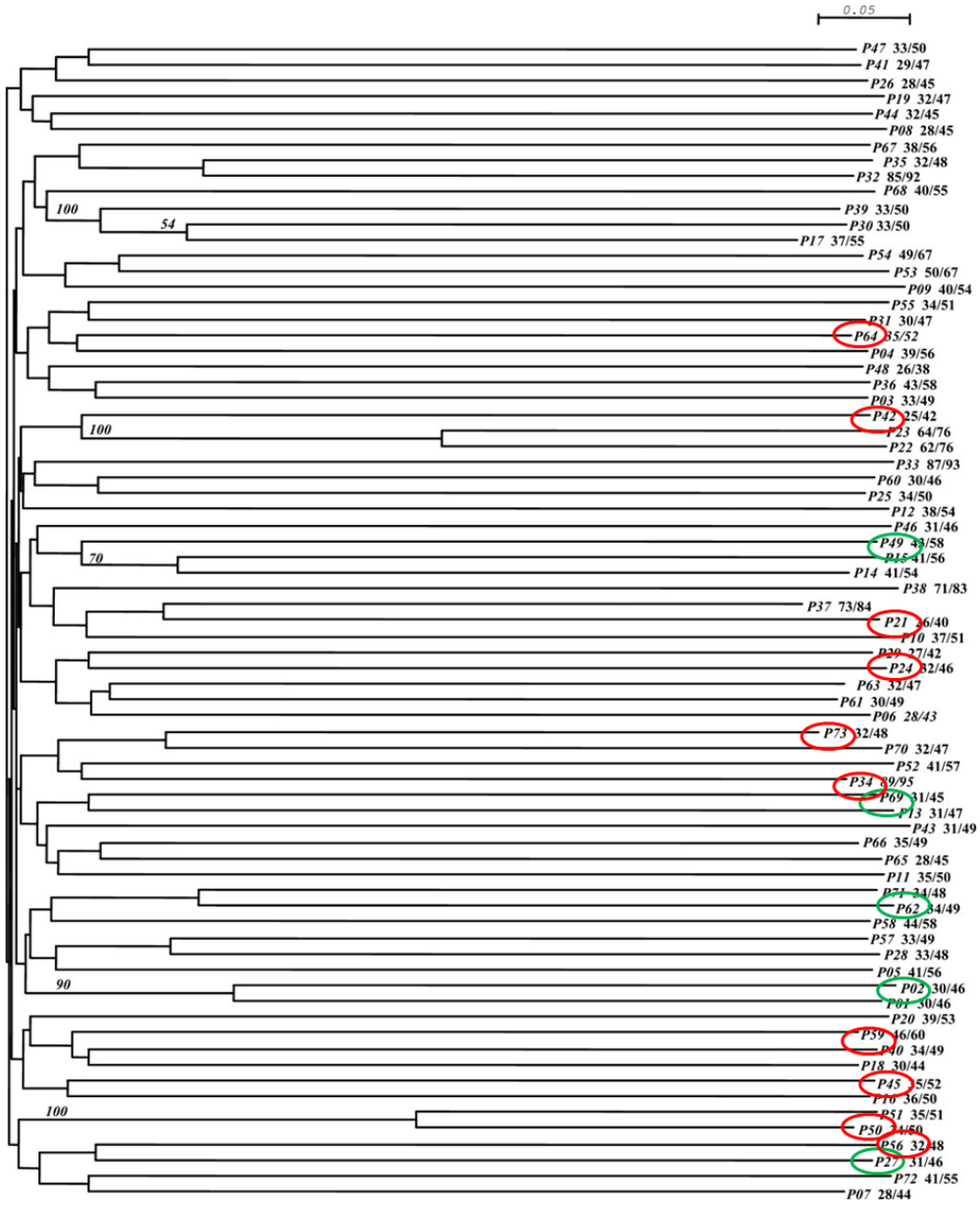


Figure 4.3. Distribution and overlapping pattern of 15 distinct *PER* genes after exposure to aluminum for 6 h and 48 h. Out of a total 73 peroxidase genes, 32 were detected above background and 15 distinct *PERs* were identified as Al responsive. Seven *PER* genes, selected for further analysis of their role in Al stress, are labeled accordingly. These genes were selected to represent diverse transcript abundance patterns observed in my microarrays (Chapter 2; Kumari *et al.* 2008).

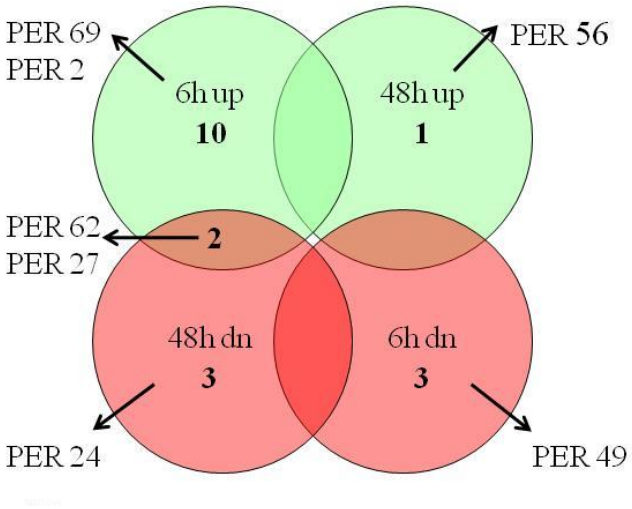


Figure 4.4. The time course of changes in relative transcript abundance of five class III peroxidases in roots of Arabidopsis exposed to 25 µM $AlCl_3$.

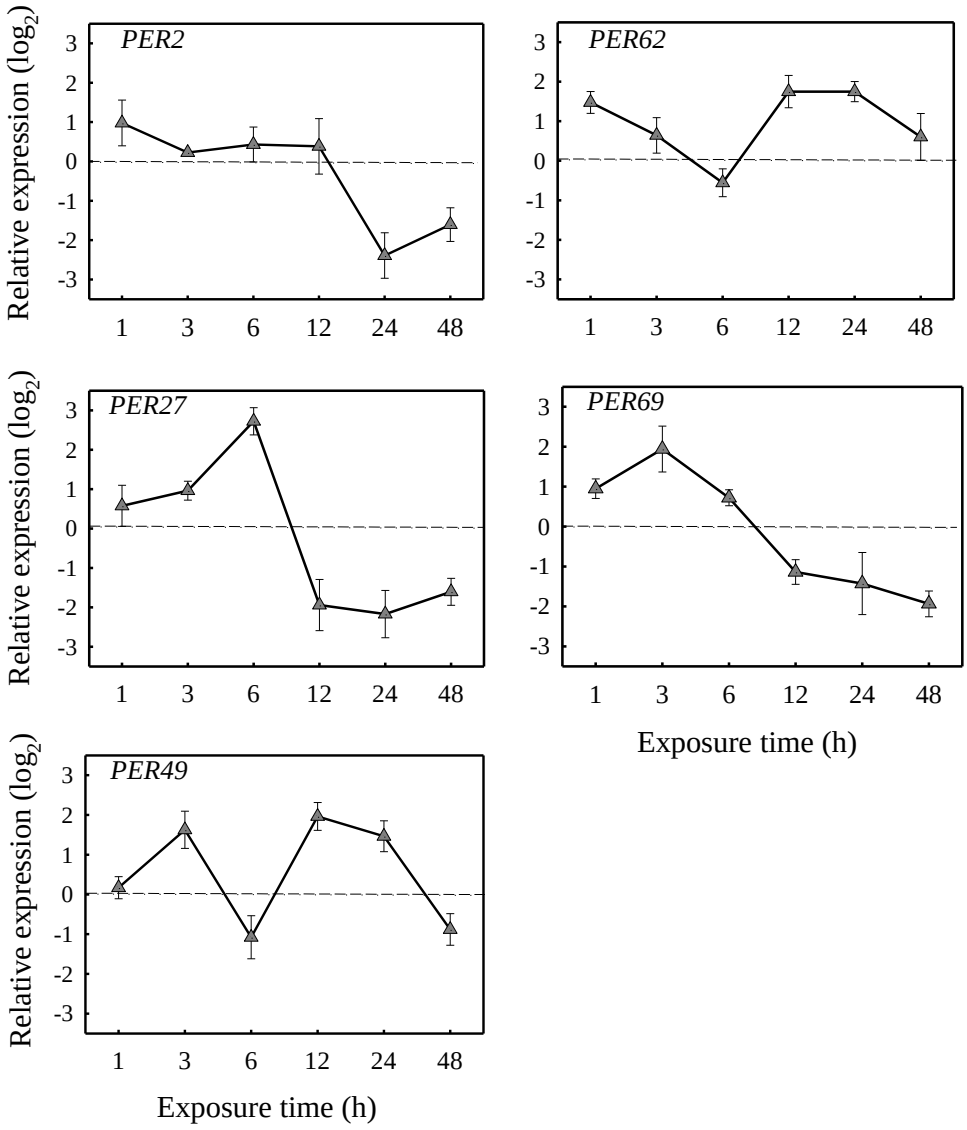


Figure 4.5. Localization of expression for *PER2*, *PER21*, and *PER69* based on data from Birnbaum *et al.* (2003) presented in eFP format (modified from Winter *et al.* 2007). The red color represents the expression in the specific tissue layers. The *PER2* and *PER69* are both root-specific peroxidases, whereas *PER21* is expression in both roots (epidermal atrichoblast cells) and shoots. *PER2* is expressed only in root epidermis and the stele whereas *PER69* is strongly expressed in both epidermal atrichoblasts and the stele, but weekly expressed in epidermis, endodermis and cortex tissue. Stage number is given for the distance from the root tip. Stage 1 is 0.15 mm from root tip, whereas stage 2 and 3 is 0.30 mm, and 0.45 to 2 mm from root tip; respectively.

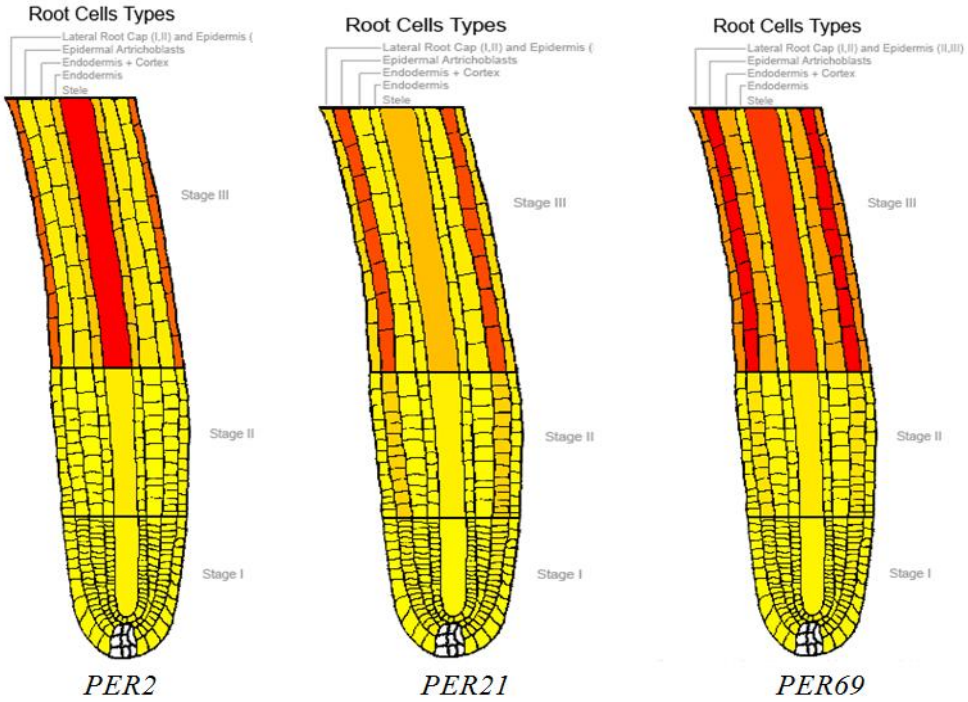


Figure 4.6. Location of T-DNA insertions in six *per* mutants of Arabidopsis. The exact location of T-DNA inserts are not certain and could be a maximum of 300 bp from the predicted site. Position of the PER gene on chromosome is highlighted in yellow. Gene model is shown in blue, where dark blue rectangle represent exon and light blue rectangles represents 5'UTR. The thin linesjoining the exons represent introns. The light blue arrow represents a 3' UTR containing termination sequence or polyA site.

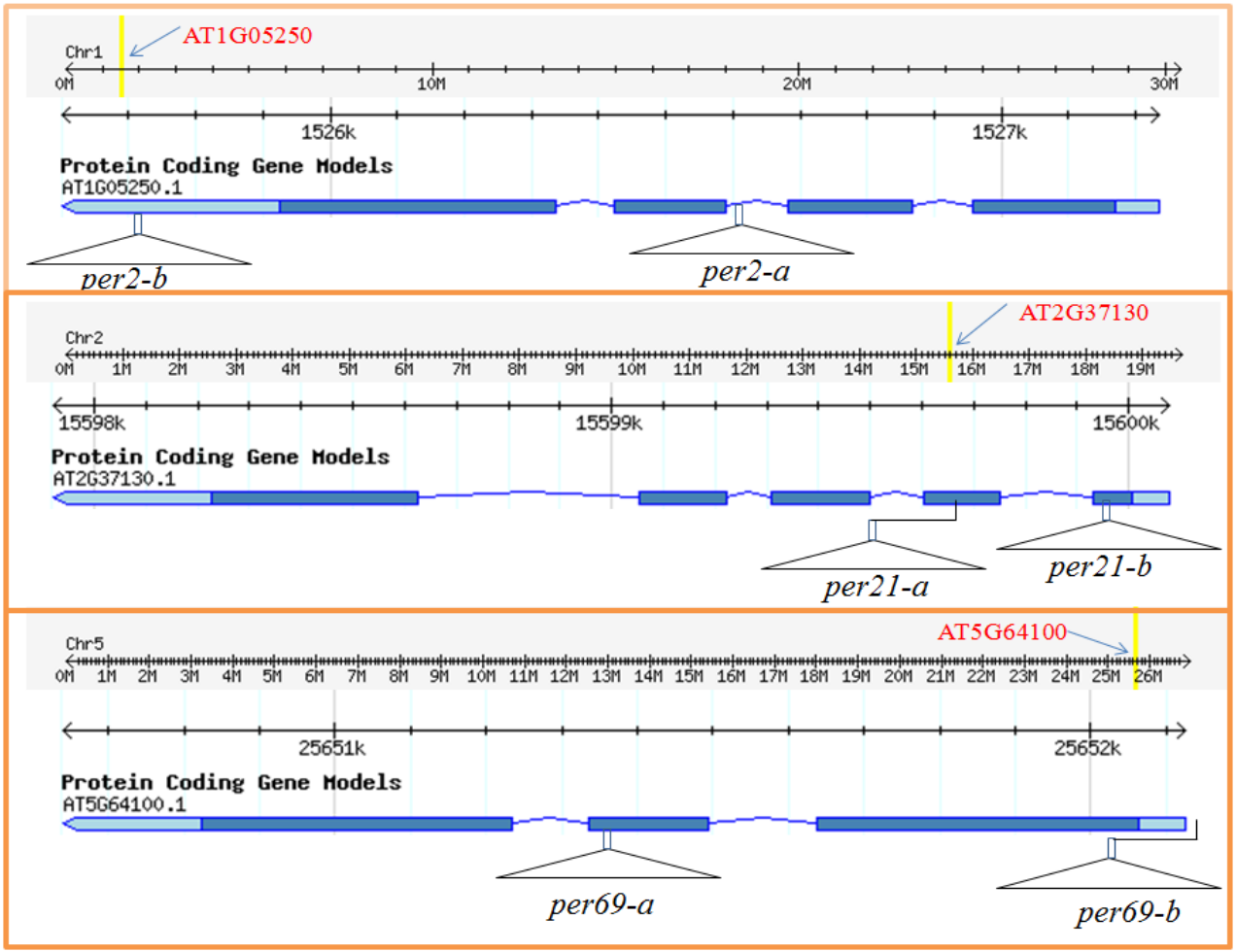


Figure 4.7. Root lengths of *Arabidopsis thaliana* expressed as percent of control for WT and six *per* mutants after exposure to aluminum. The percentage values were calculated as {root length increment after 3 days exposure to 150 µM} / {root length increment after 3 days growth in 0 µM $AlCl_3$} x 100. A two way ANOVA indicated a significant effect due to genotype ($p < 0.001$), concentration of Al ($p < 0.001$), and the genotype x Al interaction ($p < 0.001$). Only two *per* mutants (*per21-a*, and *per69-b*) were significantly different from WT after exposure to Al (*: $p<0.05$).

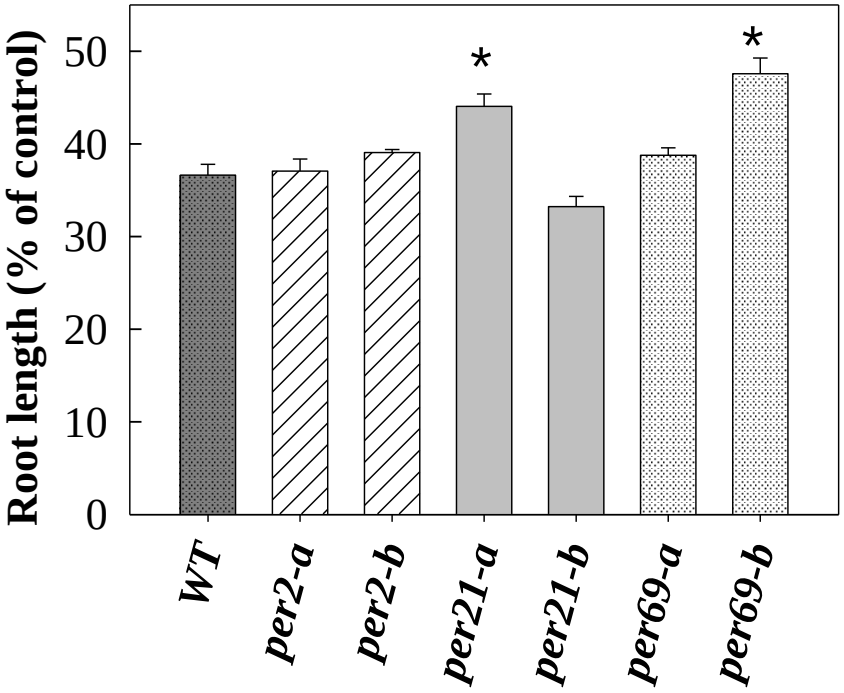


Figure 4.8. Growth of WT and mutant plants of *Arabidopsis thaliana* after exposure to different concentrations of aluminum for seven days. Differences in the root lengths of WT and *per* mutants were scored for 150 µM $AlCl_3$. Most of the *per* mutants (except for *Per2-a*) showed greater increases in root elongation compared to WT after exposure to 150 µM $AlCl_3$.

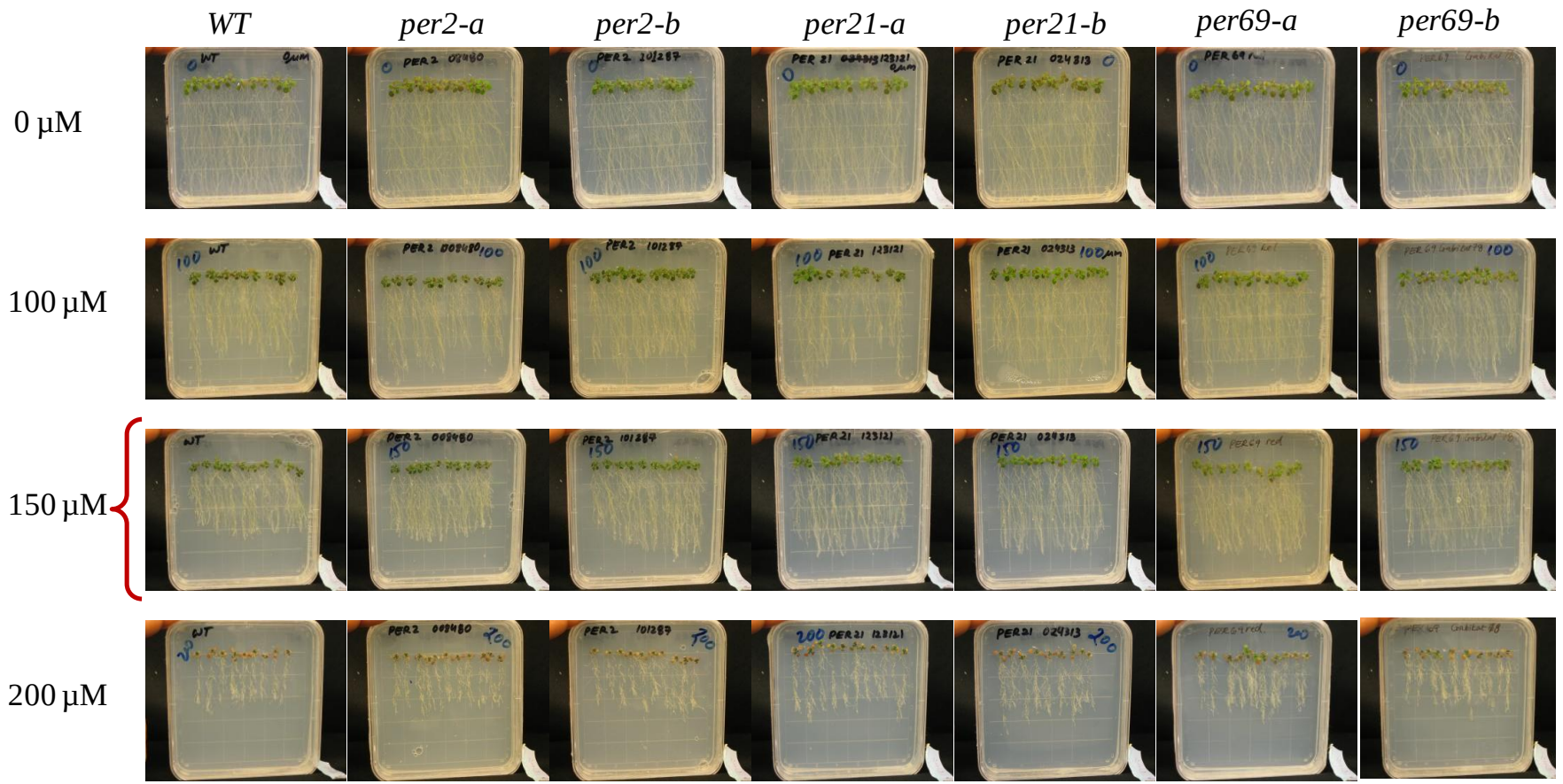


Figure 4.9. Root length increment of WT and six *per* mutants of *Arabidopsis thaliana* after exposure to 150 µM $AlCl_3$. a) Total root length increment of WT and mutants after 3 days of exposure to Al. Root lengths of most of *per* mutants (except for *per21-a* and *per69-b*) were not significantly different from WT. b) Total root length increment of WT and mutants after 7 days of exposure to Al. Root lengths of all *per* mutants except *per2-a* were significantly greater than root lengths of WT Plants. (*: $p<0.05$).

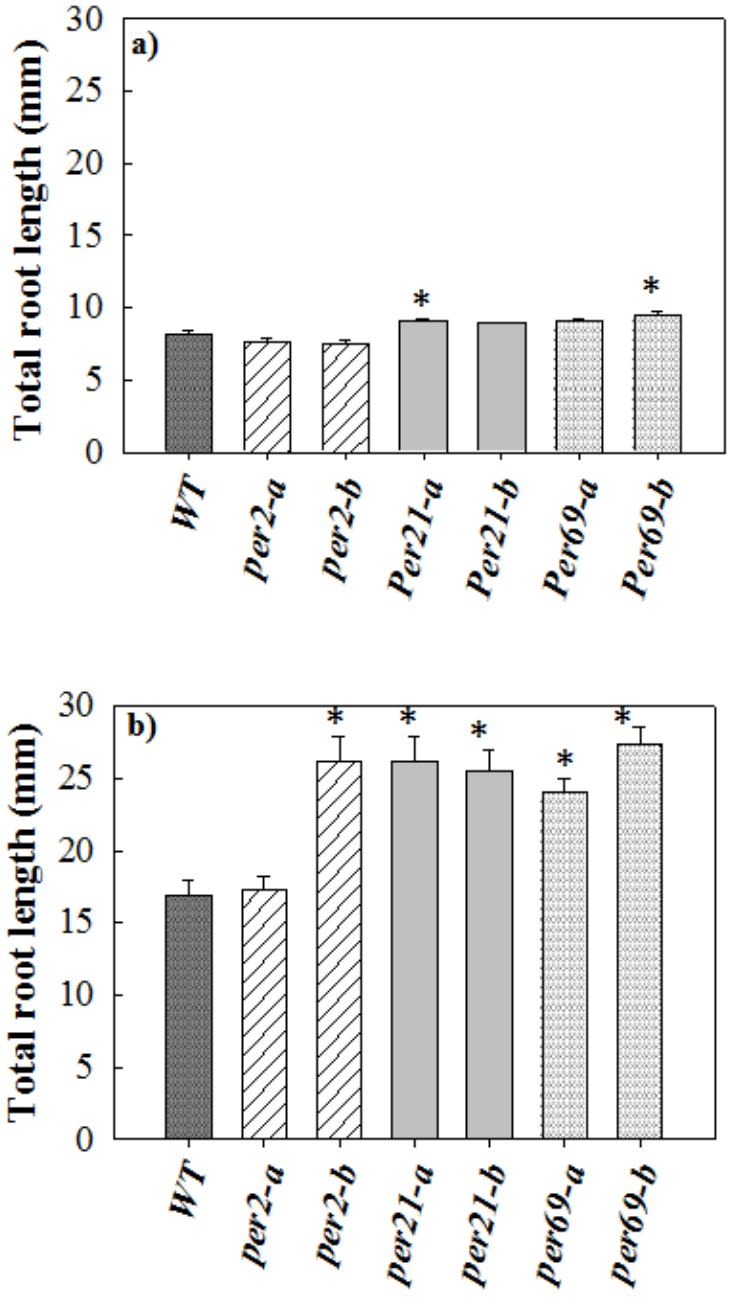


Figure 4.10. Shoot growth of WT and mutant plants of *Arabidopsis thaliana* under control (0 µM $AlCl_3$) and treatment conditions (150 µM $AlCl_3$).

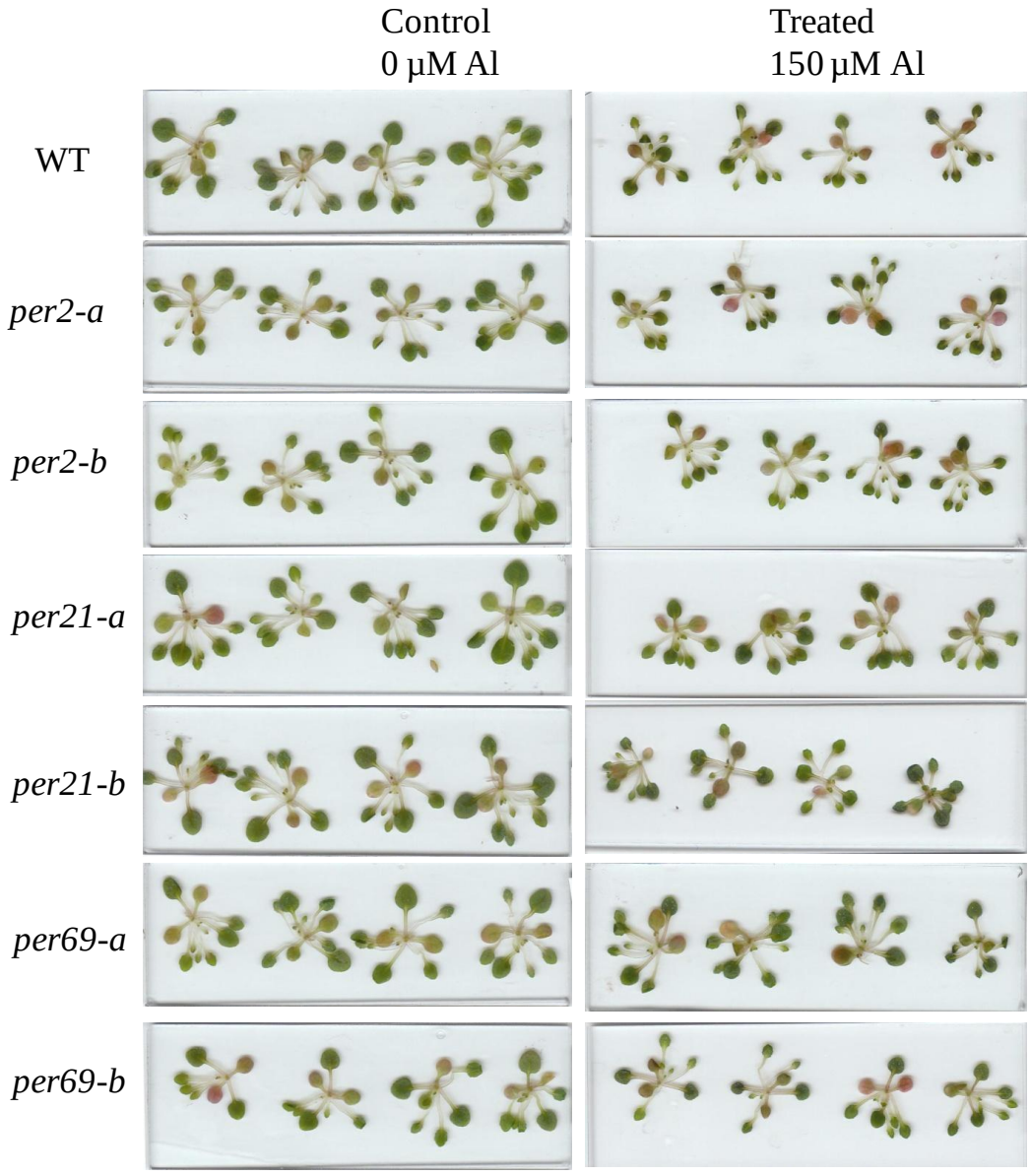
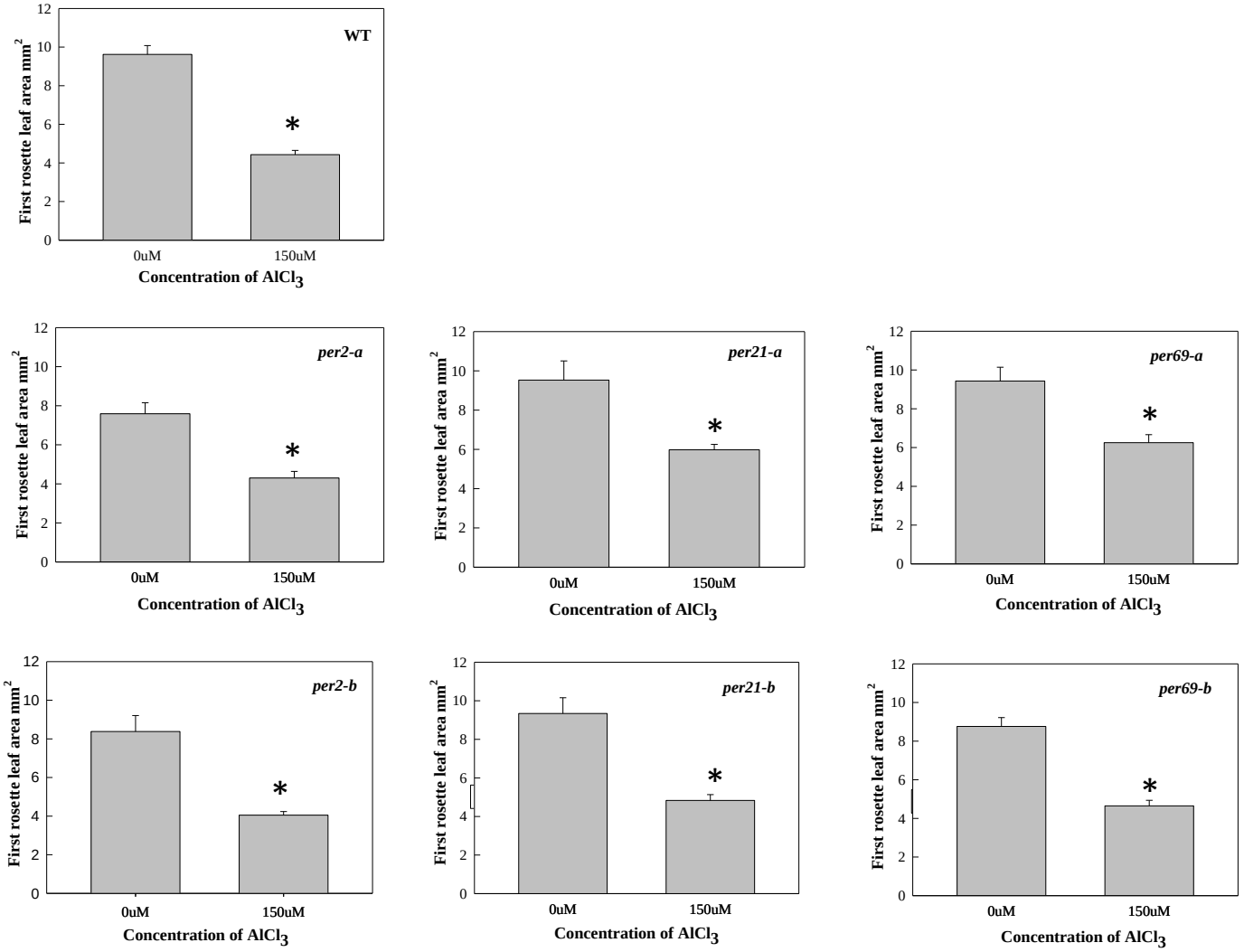


Figure 4.11. Effect of aluminum treatment on the surface area of first rosette leaves of WT and six *per* mutants of *Arabidopsis thaliana*. Exposure to aluminum (150 µM $AlCl_3$) resulted in significant (*:<0.05) decreases in leaf area of WT and *per* mutants.



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5. General discussion and conclusions

Aluminum toxicity is a threat to plant productivity in acidic soils. To investigate molecular responses of plants to Al, I conducted a microarray-based transcriptome survey, and a 2-D electrophoresis-based proteomics survey of roots of Arabidopsis treated with 25 µM $AlCl_3$ for 6 h and 48 h. Based on these results, I selected Class III peroxidases (PER) and malate dehydrogenase (MDH) for further characterization.

5.1. Transcriptomic responses to aluminum stress in roots of Arabidopsis

Although microarrays have emerged as a widely accepted tool for transcript profiling, microarray technology has some inherent disadvantages. These include limited sensitivity, non-specific hybridization, labelling biases, auto-fluorescence, and detector noise. Also, oligo-microarrays can only measure the abundance of transcripts that are represented by a pre-defined number of probe sets printed on slides. Furthermore, oligo-probe sets are available for limited plant species thus limiting the choice of plant species to study microarrays analysis of stress responses.

Despite the comprehensive scale of information obtained, microarrays have made somewhat limited contributions to stress physiology (Deyholos 2010). This is largely due to inherent limits of transcriptomics in physiology because of extensive post-transcriptional regulation, which could account forthe weak correlations that have been reported between transcriptomic and proteomic responses to stress (Feng *et al.* 2009, Fu *et al.* 2009,Lee *et al.* 2009, Minic *et al.* 2009). Thus, results are often generalized and reported to match existing knowledge of stress physiology. Furthermore, the biology behind complex patterns of transcript abundance obtained within gene families is ignored or understated.

Even if microarrays accurately identify transcripts that increase or decrease in abundance, the data may not have direct functional relevance to stress. Decreased

abundance of transcripts may suggest that the gene product is no longer required under stress, however such changes in abundance could perhaps be a secondary effect arising from of changes upstream in a metabolic pathway (Jiang, Yang and Deyholos 2009). It is, therefore, necessary to validate interpretations of microsarry data functional analyses using knock-out mutants or over-expressing transgenic plants.

Despite these limitations, microarrays are still a widely accepted tool for transcript profiling. Its main strength lies in the simplicity of use and power to generate comprehensive gene expression data. Use of this technology has led to association of candidate genes to a biological phenomenon which would otherwise not have been obvious (Kubo *et al.* 2005). Also, microarrays have helped in better understanding of different forms of breast cancer and prognosis of disease (Weigelt *et al.* 2009). The technology is here to stay until the next generation of sequencing technologies (RNA-seq) provide better sensitivity, abolish the requirement for pre-defined probe sets, and eliminate issues associated with cross hybridization (Morozova and Marra 2008). Further improvements that improve spatial resolution will increase the utility of transcriptomic data because relevant changes in gene expression within specific cells are often diluted by transcripts from surrounding cells.

In the current microarray study, the majority of Al-responsive transcripts were found to be associated with seven functional categories: oxidative stress responses, transporters, energy and primary metabolism, polysaccharide and cell wall metabolism, protein metabolism, signaling and hormones, and transcription factors.

I observed a broader range of statistically significant changes in transcript abundance after 48 h (1,114 genes) as compared to 6 h (401genes) of Al treatment. In this regard, my data contrast with most previously published microarray analyses of other abiotic stress responses, in which the bulk of transcriptomic changes were generally

detected at early (e.g. 6 h) rather than later (e.g. 24 h, 48 h) intervals after the imposition of stress (Kreps *et al.* 2002, Wang *et al.* 2003, Jiang and Deyholos 2006). This suggests that treatment with Al imposes some unique challenges to plants that are reflected in the transcriptomic response. Furthermore, there was relatively little overlap in the complement of Al-responsive transcripts detected at each time point (Fig. 2.3). These two trends were conserved in each of the eight biologically independent samples that I analyzed at each time point, and also when comparisons were made for genes where transcript abundance changed by ≥ 1.5-fold or ≥ 2-fold following Al treatment. Because I detected hybridization signal intensities above background for an almost equal number of probes at each time point, the differences between 6 h and 48 h samples were not due to systematic biases in measurement. Thus, distinct sets of transcripts appear to be expressed following 6 h and 48 h of Al treatment. Also, Al stress affects a relatively small proportion of transcripts (e.g. ~3%, Kumari *et al*. 2008 and 1%, Goodwin and Sutter 2009 in Arabidopsis; ~2% in maize, Maron *et al.* 2008; ~4% in alfalfa, Chandran *et al.* 2008; and ~2% in wheat, Houde and Diallo *et al*. 2008) when compared to microarray data from other abiotic stresses (Kreps *et al*. 2002, Jiang and Deyholos 2006).

My data appears to accurately represent the Al-responsive transcriptome, because of the reproducibility over eight independent replicates and good correlation between microarray and QRT-PCR data. However, I found differences between the pattern of gene expression I observed, and those observed in previous studies of Al stress. For example, although exudation of organic anions (OA) is a well known strategy for alleviating Al toxicity, I did not detect a significant increase in transcript abundance for any of the genes of the TCA cycle except for m-MDH. Neither did I observe a response in transcripts for Al-activated malate transporter (*ALMT1*; Sasaki *et al.* 2004). Other microarray-based studies have likewise failed to detect increased transcript abundance of *ALMT1*

(Chandran *et al*. 2008, Goodwin and Sutter 2008, Maron *et al*. 2008). In fact, this transcript has been found to be Al inducible only in wheat microarrays (Houde and Dillo 2008), although *ALMT1* is reported to be constitutively expressed in wheat (Sasaki *et al*. 2004). It is, however, reported to be abundant at both the transcript level (identified by Northern blots) and protein level in Arabidopsis (Hoenkenga *et al.* 2006) after exposure to Al. *In silico* analysis of the Arabidopsis genome indicate that *ALMTs* are encoded by gene families containing 14 members (Hoenkenga *et al.* 2006, Maron *et al*. 2008). Since not all *AtALMT*s are Al responsive (Hoenkenga *et al*. 2006), perhaps cross hybridization may have in part resulted in a failure to detect *ALMT* in microarray experiments. Some recently identified Al-resistance genes e.g. *ALS3* (Larsen *et al.* 2005) and *AtMATE* (Liu *et al.* 2009) are shown to impart resistance to Arabidopsis plants after exposure to Al. Although the respective probe for these genes were printed on my arrays, *ALS3* and *AtMATE* were not identified as Al-responsive due to poor signals intensities measured for these spots, resulting in their elimination for downstream statistical analysis.

Another interesting finding was related to oxidative stress, which is presumed to be a major component of Al stress (Richard *et al.* 1998, Basu *et al*. 2001, Yamamoto *et al*. 2002). My data displayed increased transcript abundance for only a small proportion of ROS network genes. The majority of these oxidative stress-related genes belonged to the multifunctional, Class III family of peroxidases (PERs) which can either generate ROS, or detoxify them (Passardi *et al*. 2005). Production of ROS via Al toxicity was shown to induce cell-death in wheat (Delisle *et al*. 2001) and barley (Simonovicova *et al.* 2004), and this process has been proposed to remove cells that accumulate Al and therefore serve as a mechanism of Al resistance (Delisle *et al.* 2001). Contrary to this conclusion, increasing evidence and critical analysis of previously available data suggest that oxidative stress and the induction of ROS-related transcripts is more likely a marker

for Al sensitivity than resistance (Maron *et al*. 2008). When Al sensitive genotypes were used for transcript profiling, more transcripts related to oxidative stress were found to differentially abundant (Chandran *et al*. 2008, Maron *et al*. 2008) compared to when resistant varieties were used (Kumari *et al.* 2008, Maron *et al*. 2008).

5.2. Proteome analysis of the aluminum stress response in roots of Arabidopsis

A survey of changes in the root proteome using DiGE (Differential in Gel Electrophoresis) was conducted to complement the microarray based transcriptome profile. DiGE has been used to identify proteins associated with stress and development processes in plants (Amme *et al*. 2006, Chivasa *et al*. 2006, Hotte and Deyholos 2008). I detected 55 proteins with statistically significant differences in protein abundance after 6 h exposure, and 26 proteins after 48 h exposure. Identities could only be assigned to 12 (6 h) and 17 (48 h) proteins using MS/MS. Most of the identified Al-responsive proteins were categorized into one of the two functional categories: primary metabolism and oxidative stress as reported previously in rice (Fakuda *et al.* 2007). Most of the identified oxidative stress related genes encoded GSTs. Interestingly, all GSTs identified in my proteomics data belonged to the Phi sub-family of GSTs, which is in contrast to my microarray data (Chapter 2; Kumari *et al*. 2008) where most of the GSTs (identified as Al responsive) belonged to the Tau sub-family. In first glance, data like these arising from microarray and proteomics based studies appear contradictory. But alternatively, it could be argued the benefits of two separate techniques to answer same biological question.

Several of the identified proteins have been previously reported to be Al-responsive at transcript, protein, or phenotypic level (Fukuda *et al*. 2007, Yang *et al.* 2007, Zhen *et al*. 2007, Kumari *et al.* 2008, Zhang *et al*. 2007). My data also identified novel Al responsive proteins, such as cyt-MDH. Only a few proteins detected as Al-responsive at protein level were also detected as Al-responsive at the level of transcript

(Chapter 2; Kumari *et al.* 2008). These included GST, methionine synthase, enolase, succinyl-CoA-ligase, m-MDH, and phosphoglycerate mutase. However, for most of the proteins identified as Al responsive, I did not observe similar changes at transcript level. Thus a poor correlation (6 h, $r^2 = 0.155$; and 48 h, $r^2 = 0.083$) was detected between my microarray data and proteomics data. Though not surprising (Gygi 1999, Tian *et al.* 2004, Jiang and Deyholos 2006), the poor correlation can arise due to several reasons, including post transcriptional regulation of gene expression. Other possible reasons include technological sensitivities such as cross hybridization in microarrays, bias against small or large proteins abundant at low levels in 2-DE, mRNA expression ratios (significant correlation expected when average fold change is greater than 3), and general errors such as sampling effects. By collecting root tissue at the same time for both the proteomics and microarray data, I should have minimized differences due to sampling effects. The weak correlation between transcript abundance and protein abundance illustrates how different techniques provide a useful source of independent information. For example microarrays alone helped me to identify PERs that might play a role in Al stress resistance and proteomics alone helped to me to identify that cyt-MDH may play a role in Al stress resistance.

Although peroxidases were differentially abundant at the transcript level, I failed to detect changes at the protein level using a 2D-gel electrophoresis technique. This could reflect (in part) the fact that some peroxidases are tightly bound to the cell wall (Dunand *et al.* 2002) or plasma membrane (Mika *et al*. 2008), both of which were removed while extracting the soluble protein fraction. Furthermore, several of these proteins have high isoelectric point (e.g. PER27 has p*I* 9.4) resulting in poor resolution on my gels where p*I* range of first dimension was 3-10. Use of narrow ranges for first dimension IEF gels would definitely be helpful. An analysis of half life of these proteins might also explain

why most of the proteomics based studies fail to detect class III peroxidase despite the diverse functional attributes for these proteins.

As mentioned above, technological sensitivities such cross hybridization in microarrays may result in false differences when microarray and proteomics are compared. For instance, in the case of cyt-MDH, a decrease in the abundance of protein was detected after 6 h, which is in contrast to microarrays where an increase in abundance of transcript was detected. However, QRT-PCR (with three replicates) confirmed that cyt-MDH actually decreased as opposed to increases (as detected by microarrays) in transcript abundance after 6 h exposure. These data suggest that cyt-MDH is, regulated in part at the transcript level under conditions of Al stress, however, microarrays failed to detect this. Before drawing major conclusions based upon microarrays, it is important to confirm transcript abundance with more individual gene- sequence-specific techniques such as QRT-PCR.

I was interested in cyt-MDH because the role of this protein in organic acid exudation is unknown. Increased exudation of organic anions (Hayes and Ma 2003) by modulation of TCA cycle enzymes (Tesfye *et al*. 2001, Anoop *et al*. 2003) has been shown to increase Al resistance in plants. Increased abundance of proteins involved in OA synthesis suggests that increased flow of carbon to the TCA cycle is favored under Al stress. This is in contrast to inferences from transcript profiles (Chandran *et al.* 2008, Kumari *et al.* 2008, Maron *et al.* 2008) where transcript abundance for enzymes involved in organic anion (OA) synthesis enzymes remained unaffected (Hayes and Ma 2003). Therefore, it appears that OA synthesis via TCA cycle is generally regulated post transcriptionally.

In this study, the abundance of the cyt-MDH protein (detected by DiGE) and transcript (detected by QRT-PCR) first decreased, and then increased, after 6 h and 48 h

of exposure (respectively). Therefore, it was intriguing to explore role of cyt-MDH in more depth by measuring the activities of this enzyme after exposure to Al. The activities of cyt-MDH in total cytosolic fractions were higher in Al- treated plants compared to untreated controls for most of the time points. However, activities in cytosol enriched fractions decreased after 6 h exposure and then increased after 48 h exposure, a pattern that was consistent with changes at transcript abundance and protein abundance. Differences in the activities in two fractions could imply that total cytosolic fractions might contained membrane-bound proteins that had different activities compared to proteins in soluble fraction. Efraín Ramírez-Benítez *et al*. (2008) also reported decreased activity of cyt-MDH after exposure to Al.

My two DNA insertion lines for cyt-MDH were healthier than WT under Al stress. This observation was short-lived. Prolonged exposures (>2 weeks) to Al made *mdh-1* and *mdh-2* lines flower early and appeared stressed compare to WT. I confirmed the short-term data using a double layer agar assay, in which roots of WT plants did not penetrate the Al containing exposure medium while the roots of almost all *mdh* mutants grew into the exposure medium. This may suggest that mutants are more resistant to Al toxicity, or perhaps, in an alternative explanation, *mdh* mutants were slower to sense Al around the roots. Interestingly, cyt-MDH (At1g04410) maps close to a major Al-responsive quantitative trait loci (QTL; confirmed by personal communication from Dr. Hiroyuki Koyama) in Arabidopsis. This major Al resistant-QTL was found common in two independent studies (Kobayashi and Koyama 2002, and Hoekenga *et al*. 2003) using recombinant inbred lines (L.*er* x Col) by utilizing physiological mechanisms of resistance such as malate exudation and relative root growth.

5.3. Reverse genetics approach to identify role of Class III plant peroxidases under aluminum stress

I focused in the final part of my research on peroxidases because a large number of genes from Class III peroxidase family were differentially abundant after Al exposure and because of the interesting diversity in transcript abundance patterns that were detected within members of this family in my microarray data. Intriguingly, although a larger number of distinct transcripts were detected later (e.g. 48 h) than earlier (e.g. 6 h) in Al exposure, I observed a contrary pattern for peroxidases because more transcripts were differentially abundant after 6 h than 48 h exposure to Al.

QRT-PCR was used to validate the diverse transcript abundances of five peroxidases (*PER2, PER27, PER34, PER62, and PER69*) in a time course analysis after 1 h, 3 h, 6 h, 12 h, 24 h, and 48 h exposure to Al. For most of the PERs, dynamic changes in transcript abundance were detected as early as 1 h with a decreased abundance after 48 h exposure. Although analysis of all published microarray data sets identified a limited number of genes as Al-responsive (as mentioned earlier), PERs were always represented in this small pool irrespective of the species and exposure conditions. This suggests that peroxidases may play an important in Al stress resistance. For comparison at the individual gene level, probe sequences printed on alfalfa, wheat, and maize microarrays were aligned (WU-BLAST) to identify putative orthologs in Arabidopsis. In maize, *PER27* was one of 51genes (data not shown) found to be common between two studies. Also, *PER27* was found to be differentially abundant when sensitive and resistant varieties of maize were compared (Maron *et al*. 2008). Comparison to maize was particularly interesting because of a common time point (6 h) and the use of an Al-resistant variety to detect transcriptome changes in control and treatment conditions for 50% of their microarray hybridizations. In alfalfa, *PER2* (common with my data)

decreased in abundance whereas *PER55* and *PER11* increased in abundance after exposure to Al (Chandran *et al*. 2008). In wheat, a gene with 59% identity to *PER15* increased in transcript abundance (Houde and Diallo 2008) after exposure to Al. When I blasted the given sequence (TC247326) with Arabidopsis TAIR sequences, it resulted in 59% identity to *PER12* rather than *PER15*. In any case, both *PER12* and *15* were not detected in my microarrays. In Arabidopsis, both *PER58* and *PER71* (mistakenly reported as *AtPOX 7* by authors) were reported to be decreased after Al exposure (Goodwin and Sutter 2009). In summary, two PERs namely *PER2* (Arabidopsis and alfalfa) and *PER27* (Arabidopsis and maize) were found common in at least two studies. Notably, both are root specific Class III peroxidases.

Interestingly, inhibition of root elongation correlates well with modifications in cell wall properties including reduced extensibility of cell walls (Tabuchi and Matsumoto 2001) and changes in distribution and content of cell wall polysaccharides such as pectin (Yang *et al*. 2008, Li *et al*. 2009). Peroxidases could form phenolic linkages in the wall consequently restricting cell growth (Cosio and Dunand 2009). Peroxidases reportedly have an indole-3-acetic acid oxidase activity as well (Gazaryan *et al*. 1996), and hence may control endogenous levels of auxin in the elongation zone (Cosio *et al*. 2009), resulting in regulation of root elongation through the catabolism of auxin in roots (Kawano 2003).

Encouraged by QRT-PCR data and a probable role of peroxidases is cell wall extensibility, I used a reverse genetics approach to determine whether knocking out PERs would have any effect on root elongation after exposure to Al. To test this hypothesis I selected *PER2*, *PER21*, and *PER69*, because two independent T-DNA insertions lines were available for each of these genes that I had previously found to be Al-responsive at the transcript level. Most *per* mutants studied exhibited a significantly greater root

elongation after long-term stress (differences attributed to growth of lateral roots) but not after short-term (differences attributed to primary roots) as compared to WT. Detailed studies are required to determine if increased growth of lateral roots is a truly due to Al exposure (as opposed to technical problem of using surface for exposure in plates) because these results were not reproducible when primary rosette leaf area and dry weight measurements of *per* mutants were compared to WT. It would also be interesting to determine whether any differences exist in the accumulation of Al in lateral roots as compared to primary roots in these mutants. Two alternative theories may explain why most *per* mutants had longer roots as compared to WT after exposure to Al. First, reduced peroxidase activity could result in decreased cross-linking of cell wall components (such as linking arabino-xylans by diferulic acid bridges) or reduced lignification, thus enhancing the cell wall extensibility. Secondly, perhaps reduced peroxidase activity may decrease peroxidase-mediated catabolism of growth promoting hormones (such as auxin; Kawano 2003), thus affecting root elongation (Cosio *et al.* 2009). Most PERs are secretory proteins and some of the Arabidopsis peroxidases have an affinity for pectin *in vivo* (Shah *et al.* 2004). This attribute facilitates the distribution of these proteins within the cell wall network and could play a role in mediating changes in roots after exposure to stress.

Several different experimental designs can be applied to identify Al-responsive genes or proteins. For instance, one can compare sensitive and resistant genotypes, or stressed and unstressed tissues of the same genotype. I used an Al resistant genotype (Col- 0) to determine changes in transcript abundance. Maron *et al.* (2008) however, compared both sensitive and resistant genotypes of maize and also compared transcript abundance between stressed and control conditions in individual genotypes. Their data showed that substantially larger numbers of genes are differentially abundant at the

transcript level when sensitive and resistant genotypes were looked at individually as compared to when these responses were compared between the two. This indicates that different sets of genes are affected by Al in each genotype. For instance, more oxidative stress related genes were detected in the sensitive genotype than in a resistant genotype, suggesting that oxidative stress is probably a marker of toxicity as opposed to resistance. It appears that both strategies (i.e. comparisons between two genotypes and comparison within single genotypes) have their own limitations.

Irrespective of the genotypes used, one major problem with microarray-based or proteomics-based approaches is that both produce snapshots of changes at a particular time point. Since limited numbers of genes or proteins are expressed at a given time in a development stage, the use of several time points tightly spread over different developmental stages would help to better understand how plants cope with Al toxicity and resistance. Perhaps next generation sequencing (Ledford 2008) will make these experiments easier and more cost effective and would also sidestep some of the technical limitations of microarrays, such as their relatively low sensitivity.

In summary, this study: 1) provided a comprehensive dataset of Al-responsive genes and proteins in Arabidopsis; 2) suggested that oxidative stress, commonly associated with abiotic stresses, appears to be a marker of Al toxicity or injury; 3) showed that more genes and proteins are differentially regulated after long-term stress, indicating that Al injury could be more progressive over time; 4) provided evidence that the principle enzymes involved in organic acid synthesis in TCA cycle are not regulated at the level of transcription; 5) showed that protein abundance of cyt-MDH in response to Al treatment is regulated, in part, at the transcriptional level, and knocking out of the cyt-MDH gene results in resistance compared to WT after exposure to Al; and 6) showed that

knocking out class III peroxidases result in less inhibition of root elongation after exposure to Al.

5.4. Future prospects

My proteomics and QRT-PCR data suggested that cyt-MDH may be regulated in part at the level of transcription. To test this hypothesis I would determine the time course of changes in protein abundance in response to Al stress using western blotting with cyt-MDH specific antibodies. This study would confirm abundance of cyt-MDH (independent of 2-D/ mass-spectrometry approach) and would help to clarify whether abundance of protein correlates with abundance of transcript (previously determined using QRT-PCR).

Furthermore, I proposed that one possible of role of cyt-MDH in response to Al is to fine tune malate exudation from roots by maintaining a pool of malate in cytosol through the action of cyt-MDH and malic enzyme. To test this hypothesis I would determine cytosolic and extracytosolic metabolite levels of malate in control and treated plants. The results would be analysed with the pattern of abundance of cyt-MDH protein obtained from time course western blotting. This experiment would help to understand if the differential abundance of cyt-MDH correlated with cytosolic and extra-cytosolic levels of malate. This would be an interesting experiment because although malate exudation is an accepted bonafide mechanism of Al stress resistance, the finer details of how cell regulate the pool of malate in cytosol has not been studied. There is a possibility that activities of cyt-MDH and/or the protein abundance may play a role in maintaining cytosolic pool of malate. Therefore, it would also be meaningful to measure activities of NAD dependent cyt-MDH. My enzyme activity data obtained from cytosol enriched fractions did not show much difference in the activity of cyt-MDH in response

to Al. I recognize that plant cells contain multiple iso-forms of MDH that differ in co-enzyme specificity, sub-cellular localization and biological function. To differentiate the specific activities of Al responsive isoforms of cyt-MDH, a biochemical and genetic approach could be used. Zymograms have been used previously to differentiate different isoforms of malate dehydrogenase. Using a reverse genetics approach, measurement of cyt-MDH activities in T-DNA insertion mutants could help, however the redundancy within cyt-MDH isoforms might mean that observation of differences in activity is optimistic. Conceivably, use of double mutants could help to determine which isoform(s) is/are Al responsive. Personnel communications from Dr. Steven Smith (University of Western Australia) report that double mutants for NAD-dependent cyt-MDH are unhealthy compared to WT.

I also proposed that concomitant activities of cyt-MDH and cyt-ME together might help to generate NADPH by directing oxidation of malate either towards pyruvate synthesis. To test whether NADPH levels increase in response to Al, it would be interesting to determine NADPH levels after exposure to Al. This could be complicated due to the operation of the pentose phosphate pathway in roots, which generates NADPH (non-photosynthetically) in roots. The activity of cyt-MDH has been shown to be redox-regulated by formation of inactive homodimer which is activated by action of thioredoxin.

Decreased activities of peroxidases have recently been correlated with decreased cellular levels of H_2O_2 and are postulated as a resistance mechanism in roots of Al resistant Melaleuca trees. This is in agreement with my Class III peroxidase (PER) mutant phenotype data. However, I did not measure the H_2O_2 levels and peroxidase activity in roots. I could use Al resistant and sensitive ecotypes (*L.er*) of Arabidopsis and would measure H_2O_2 levels and peroxidase activity in roots exposed to control and

treatment conditions. The diverse pattern of transcript abundance obtained for class III peroxidase was interesting, as was the fact that I could see phenotypes in single mutants. Previously, PER expression has been shown to be tissue-specific, however these data were from microarray analysis of protoplasts obtained from root tissue. To study the spatial and temporal expression pattern of peroxidases and to determine the upstream regulatory elements in selected Al-responsive peroxidases, I decided to fuse the PER promoter to reporter genes in the pCAMBIA 1303 vector. Due to time limitations, and my QRT data that suggested that PERs are down regulated after exposure to Al, this project was not completed. It would still be interesting to study the root-specific spatial expression for selected PERs. This can be done by fusion of 1kb upstream sequence to reporter gene or by using laser-capture-microdissection of specific root layer cells and performing gene specific QRT-PCR.

5.5. Literature cited

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