Identification and Characterization of Copper-Responsive Proteins in Arabidopsis

A Thesis Submitted to the College of

Graduate Studies and Research

in Partial Fulfillment of the Requirements

for the Degree of Doctor of Philosophy

in the Department of Plant Sciences

University of Saskatchewan

Saskatoon

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ABSTRACT

For the successful development of a hyperaccumulating plant sufficient for use in phytoremediation strategies, a thorough understanding of the mechanism of hyperaccumulation is required. A proteomic survey of the response of plants to metal exposure is a step towards this understanding. The frd3-3 metal accumulating mutant of *Arabidopsis thaliana* and its non-accumulating wildtype parental ecotype, Columbia, were grown hydroponically in growth chamber experiments and exposed to copper in the growth medium. The responses of the global and copper-targeted proteomes were examined both spatially and temporally. Exposure to copper caused a general increase in protein abundance, however, a prolonged exposure to copper that approached toxicity caused a decrease in protein abundance. The protein species differed between the roots of the two genotypes, with more defense- and stress-related proteins, and fewer transport and storage proteins identified in the mutant when compared to the wildtype. Proteomic evidence suggests that in the mutant the uptake and transport of copper ions to the aerial tissues is regulated. The protein expression patterns over time demonstrate a constitutive expression of defense- and stress-related proteins in the mutant, whereas the wildtype expression was one of induction. The constitutive expression of key defense proteins suggests a “state-of-readiness” for metal exposure in the mutant. The plant response to reactive oxygen species, as a consequence of copper exposure, is important in the overall metal accumulation mechanism. A suppression of the oxidative burst produced upon exposure to heavy metals is suggested by the proteomic evidence.

ACKNOWLEDGEMENTS

I would like to express thanks to my co-supervisors, Drs. Andrew Ross and Graham Scoles. I would also like to thank the members of my Graduate Committee; Drs. Rosalind Bueckert, Rich Farrell and Michele Loewen for their assistance, advice and encouragement throughout the entire process of this degree.

A great deal of thanks must go to the members of the Mass Spectrometry Facility at the National Research Council – Plant Biotechnology Institute; Dr. Richard Hughes, Dr. Lianglu Wan, Jenny Yang and Dr. Uma Aryal and especially to Steve Ambrose and Doug Olson for years of help, teaching, guidance and commiseration. I would also like to thank my summer student, Angela Chiang for her immense help, dedication and tireless cheerful attitude. Special thanks must go to Dr. Wilf Keller who really got the ball rolling in the first place.

I would also like to thank the National Research Council-Plant Biotechnology Institute for providing me with such a stimulating environment in which to conduct this research. Lastly, I would like to acknowledge the National Research Council, the Natural Sciences and Engineering Research Council and the Departments of Plant Sciences and Soil Science of the University of Saskatchewan for funding support.

DEDICATION

To you, Jeff.

Here’s to getting out of limbo!

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LIST OF ABBREVIATIONS

1DE – one dimensional electrophoresis

2DE – two dimensional electrophoresis

ABC – ATP binding cassette

ABRC – Arabidopsis Biological Resource Center

AdoMet - *S*-adenosylmethionine

AOC – allene oxide cyclase

APS – ATP sulfurylase

BSA - bovine serum albumin

Ca - calcium

CAT - catalase

CCH – copper chaperone

Cd - cadmium

CDF – cation diffusion facilitator

cDNA - complimentary deoxyribonucleic acid

CHAPS - 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate

CIMS - cobalamin-independent methionine synthase

Co - cobalt

CRT - calreticulin

Cu - copper

cv - column volume

CYS - cystatin

Cys - cysteine

DM – dry matter

DREPP - developmentally regulated plasma membrane polypeptide

DTPA - diethylenetriaminepentaacetate

DTT - dithiothreitol

EDDHA - ethylenediaminedihydroxyphenylacetate

EDDS - ethylenediaminedissuccinate

EDTA - ethylenediaminetetraacetate

EGTA - ethylene glycol tetraacetate

EMS - ethyl-methane sulfonate

ER – endoplasmic reticulum

Fe - iron

frd – ferric reductase defective

FRX - ferredoxin

GAPC - glyceraldehyde-3-phophate dehydrogenase C

GE – genetic engineering

GLP - germin-like protein

Glu - glutamate

Gly - glycine

GO – Gene Ontology

GR - glutathione reductase

GRX - glutaredoxin

GSH - glutathione

GSSG - glutathione disulfide

GST - glutathione-*S*-transferase

HEDTA - hydroxyethylethylenediaminetriacetate

HEPES - 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid

Hg - mercury

HMA - heavy metal ATPase

HMW - high molecular weight

HR - hypersensitive response

HSP – heat shock protein

IEF - isoelectric focusing

IMAC – immobilized metal affinity chromatography

IPG - immobilized pH gradient

LC – liquid chromatography

LH - lipoxygenase homology

LMW – low molecular weight

LMWOA – low molecular weight organic acid

LP - lipid transfer protein

m/z - mass-to-charge

man – manganese accumulator

MAT - *S*-methionine adenosyltransferase

MATE - multi-drug and toxin efflux

metSO – methionine sulfoxide

Mg - magnesium

MGDA - methylglycinediacetate

ML - MD-2-related lipid recognition domain-containing protein

Mn - manganese

mRNA – messenger ribonucleic acid

MS – mass spectrometry

MSR - methionine sulfate reductase

MT - metallothionein

NA - nicotianimic acid

NCBInr - National Center for Biotechnology Information non redundant

Ni - nickel

NPRI – National Pollutant Release Inventory

NRAMP - natural resistance-associated macrophage protein

OSM - osmotin

PA – protease-associated

PAGE - polyacrylamide gel electrophoresis

PAP – purple acid phosphatase

Pb - lead

PBP - PYK-10 binding protein

PC - phytochelatin

PDF – plant defensin

PDIL - protein disulfide isomerase-like

Pi – inorganic phosphate

pI – isoelectric point

PLAT - polycystin-1/lipoxygenase and alpha toxin

PMSF - phenylmethylsulfonyl flouride

POX - peroxidase

PR – pathogenesis related

PRX - peroxiredoxin

PVPP - polyvinylpolypyrrolodone

QP-C - ubiquinone-binding protein

Q-TOF - quadrupole-time of flight

ROS - reactive oxygen species

RNAi – ribonucleic acid interference

SAR – systemic acquired resistance

SDS - sodium dodecyl sulfate

SOD – superoxide dismutase

SRM – standard reference material

TAIR – The Arabidopsis Information Resource

TBP - tributylphosphine

TCA - trichloroacetic acid

TFA - triflouroacetic acid

TRX - thioredoxin

UCC - uclacyanin

UPLC – ultra performance liquid chromatography

USP – universal stress protein

VSP – vegetative storage protein

VTC4 - L-galactose-1-phosphate phosphatase

WT - wildtype

YSL – yellow stripe-like

ZIP - zinc-regulated transporter, iron-regulated transporter-like protein

Zn – zinc

1.0 INTRODUCTION

Since the industrial revolution, human activities have led to the widespread release of toxic anthropogenic compounds into the environment (Nriago, 1979; Settle & Patterson, 1980). A well-known and important example is the dispersion of heavy metals due to activities such as mining, agriculture and the burning of fossil fuels (Salt *et al.*, 1995; McGrath *et al.,* 2001; Ni *et al.,* 2004; Arthur *et al.,* 2005). The accumulation of heavy metals in the environment threatens the health of plant and animal species, and the environment as a whole (Chaney *et al.,* 1987; Cunningham *et al.,* 1995; McGrath *et al.,* 1995; Garbisu & Alkorta, 2001). Furthermore, the deleterious effects of these inorganic contaminants on ecosystems can, in turn, negatively impact human health.

Methodologies exist for the removal of toxic amounts of heavy metals from soil, sediment and water; however, most conventional strategies involve the mechanical removal of polluted strata, thereby scarring the landscape. While comparatively quick to implement, these traditional methods are also expensive and tend to negatively impact the surrounding ecosystems (McGrath *et al.,* 2001). Phytoremediation is an emerging "green" technology that offers many subsidiary benefits. This approach involves the use of plants to remediate areas contaminated with organic or inorganic compounds. Plants are capable of achieving environmental remediation by a variety of means. In the case of heavy metals, such means include the process of hyperaccumulation (or phytoextraction), whereby plants accumulate heavy metals into their aerial tissues from areas of high metal concentration. Although a much slower process than conventional practices, phytoremediation by use of hyperaccumulators can reclaim an area to levels below the toxicity threshold (Salt *et al.,* 1998; McGrath *et al.,* 2002). In so-doing, the process of hyperaccumulation actually improves the surrounding ecosystems by providing habitat and improving soil quality (Schnoor *et al.*, 1995; Cunningham *et al.*, 1996). Also significant is the state of the landscape following this process. Instead of the scarred, de-vegetated landscape that commonly results from mechanical remediation (McGrath *et al.,* 2001), phytoremediation leaves behind a planted landscape flourishing with life. In addition to its aesthetic value, phytoremediation is generally cost-effective (Salt *et al.,* 1995; Ensley, 2000) as it requires low inputs and is solar-driven.

Hyperaccumulating plants are defined as those capable of concentrating metals to 0.1 – 1% of their dry weight in aerial tissues (Meagher, 2000). Known hyperaccumulators represent less than 0.2% of all angiosperms (Baker & Whiting, 2002). Because some metals are actually required by plants as essential micro-nutrients (Marschner & Romheld, 1995; Salt *et al.*, 1995), plants already possess mechanisms for the uptake, translocation and storage of certain heavy metals. These mechanisms involve the secretion of metal complexing compounds such as organic acids, small proteins, and a variety of transporters and chaperones, as well as proton/metal ion channels and storage bodies (Yang *et al.,* 2005). Hence, the basic framework for metal uptake already exists in most plants, regardless of whether they are non-accumulating, metal-accumulating or hyperaccumulating. Proteins are involved in virtually all biological processes, and are therefore likely to play an important role in the mechanism of hyperaccumulation. However, it has been suggested that, rather than the presence of a few specific proteins accounting for the hyperaccumulation effect, it is more likely that changes in the regulation and expression of existing mechanisms are responsible for this phenomenon (Cobbett, 2003).

Naturally hyperaccumulating plants pose some significant challenges with regard to their widespread use for phytoremediation. Most hyperaccumulators are small, slow-growing plants, making the use of conventional agronomic practices difficult or infeasible (Pollard *et al,.* 2002; Tong *et al.,* 2004). Consequently, the use of genetic engineering has been suggested as a means of either increasing the biomass of hyperaccumulators or altering the accumulation characteristics of an existing high-biomass plant (Brown *et al.,* 1995). In any event, for the successful implementation of either of these strategies, a thorough understanding of the mechanisms governing hyperaccumulation is necessary. Much of the research conducted on hyperaccumulators to date has focused on the description of new species, and the characterization of key proteins such as phytochelatin, metallothionein (Salt *et al.,* 1995), heavy metal ATPase (Yang *et al.,* 2005; Colangelo & Guerinot, 2006) and ABC-type (ATP binding cassette) transporters (Cobbett & Goldsbrough, 2000).

Like most complex biological processes, hyperaccumulation is likely to involve the co-ordination of many compounds, networks and/or subsystems within the plant. Proteins have already been implicated in many stages of this process, from initial uptake into the roots to delivery of metals ions to storage bodies. To properly understand the hyperaccumulation

mechanism, an examination of the entire protein network of a plant in response to metal exposure, and subsequent accumulation, is therefore required. This assertion forms the basis for the research described hereafter, the principal objectives of which were:

1. To characterize the global proteomes of two closely related (*Arabidopsis*) plant genotypes that show markedly different responses to copper exposure, one being an accumulator and the other a non-accumulator of metals.
2. To characterize the copper-binding sub-proteomes of the same genotypes in order to identify proteins that may be directly involved in metal uptake.
3. To better understand the mechanism(s) of hyperaccumulation in plants by comparing the differential responses of these two genotypes at the proteome level.

The following thesis is presented in seven chapters. Chapter 1 (Introduction) provides a brief overview of the question being explored and outlines the intended objectives of the thesis. Chapter 2 (Literature Review), summarizes the existing, relevant knowledge in the field and provides justification for the importance of the research detailed herein. In Chapter 3 (Copper-Induced Proteomic Response of *Arabidopsis thaliana* Genotypes), tissue-specific changes in the proteomes of two *A. thaliana* genotypes (one metal-accumulating and one non-accumulating) in response to copper exposure are examined. Proteins in root and shoot tissues that show differential expression during copper exposure are identified, enumerated, and grouped into functional categories. Changes in the percent contribution of each functional category to the total number of differentially expressed proteins at specific time points during copper exposure are then determined. Chapter 4 (Isolation and Identification of Copper-Binding Proteins in Copper-Treated *Arabidopsis thaliana* Genotypes) describes a more targeted approach to exploring the responses of *A. thaliana* genotypes to copper exposure by examining the spatial and temporal expression of copper-binding proteins in the two genotypes. Comparisons of differential expression between the copper-binding sub-proteomes of the two genotypes are made, and conclusions regarding the metal accumulation mechanism are drawn. In Chapter 5 (Changes in the Global and Copper Proteomes of two *Arabidopsis thaliana* Genotypes in Response to Copper Exposure), the expression profiles of individual protein species belonging to key categories, as identified in Chapter 3, are examined. In Chapter 6 (General Discussion), insights regarding the metal accumulation mechanism that have been learned from Chapters 3-5 are synthesized and the broader implications of this knowledge are explored. Finally, Chapter 7 (Concluding

Remarks) provides a summary of the conclusions drawn throughout the thesis and outlines future research.

2.0 LITERATURE REVIEW

2.1 Definition of the Problem – Heavy Metal Pollution

Since the industrial revolution, the global environment has become increasingly contaminated with heavy metals (Nriago, 1979; Settle & Patterson, 1980). The term heavy metal refers to a naturally occurring metallic element that has a specific gravity above 5 g/cm^3 and readily forms sulphides (Adriano, 1986). Metals are immutable and cannot be either biologically or chemically degraded leading to their accumulation over time in the environment (Salt *et al.*, 1995; Garbisu & Alkorta, 2001; Mejáre & Bülow, 2001; Schwedt, 2001). Heavy metals are naturally occurring entities in the earth's crust originating from metalliferous ore bodies. Over time however, natural weathering effects such as geologic erosion and saline seeps have contributed to the accumulation of heavy metals in the biosphere (Arthur *et al.,* 2005). Deposition also occurs through a variety of anthropogenic means: emissions from industrial activities and power generation, combustion of fossil fuels, agricultural activities including use of fertilizers and pesticides, disposal of wastes including municipal, industrial and agricultural, and residues from mining and smelting activities (Salt *et al.*, 1995; McGrath *et al.,* 2001; Ni *et al.,* 2004; Arthur *et al.,* 2005). Deposition can occur directly to soil and surface waters as well as atmospherically. Ultimately this atmospheric release ends up either on soil or surface water.

Many elements are strongly retained at the surface of the soil and do not leach through the soil layers to the groundwater (McGrath *et al.,* 2002). This phenomenon leads to potentially hazardous accumulations of heavy metals. The concentration of heavy metals has been measured as high as 10,000 mg/kg in some soils (Yang *et al.,* 2005). The danger from heavy metals results partially from their persistence in the environment. Residence times in soil for some heavy metals have been estimated to be on the order of thousands of years (McGrath, 1987).

Many heavy metals function as essential trace elements in plants, the primary producers of the trophic system. While heavy metals such as iron (Fe), zinc (Zn), copper (Cu), manganese (Mn), nickel (Ni), cobalt (Co) and magnesium (Mg) are all required in micronutrient levels by plants for normal growth and metabolism (Marschner & Romheld, 1995; Salt *et al.*, 1995), exposure to high concentrations of these elements can also have toxic effects on plants (Cunningham *et al.,* 1995; McGrath *et al.,* 1995; Garbisu & Alkorta, 2001) . Physiological and

biochemical processes are directly affected by toxic levels of heavy metals *in planta* and can manifest as growth reduction, inhibition of photosynthesis and respiration, and degeneration of cellular organelles (Garbisu & Alkorta, 2001). Heavy metal soil concentrations exceeding background levels and reaching contaminant status, can negatively impact the entire pedosphere by adversely affecting the soil micro- and macro-biota (McGrath *et al.,* 2002). Phytotoxic effects can lead to decreased plant growth, decreased ground cover (Cunningham *et al.,* 1995; McGrath *et al.,* 1995; Schwedt, 2001) and poisoning of the food chain due to bio-concentration effects (Chaney *et al.,* 1987).

2.1.1 Copper Contamination

While heavy metals with little or no known biological function such as, mercury (Hg), lead (Pb) and cadmium (Cd) (Gaur & Adholeya, 2004), are thought to be of greatest environmental importance, the toxicity of the remaining biologically relevant heavy metals cannot be ignored. Copper is a widespread environmental contaminant that poses a serious threat to environment health and quality. In Canada, more than 40,000 tonnes of copper and its compounds were released to the environment in 2005 (Environment Canada, 2005); of which 700 were categorized as *on-site releases*, meaning the element is released directly to the environment within the confines of the facility. The remainder was targeted for *disposal* either on- or off-site. Disposal refers to the long-term storage of the chemical by means of underground injection, land treatment by land farming or landfill, or storage containment (Environment Canada, 2002 & 2005). According to the 2002 NPRI (National Pollutant Release Inventory) data, 55% of the on-site copper releases were atmospheric (Environment Canada, 2002). Due to gravitational settling, dry deposition and rain washout (Dameron & Howe, 1998), that 55% is ultimately destined for distribution across surface land and water. It is also important to note that the NPRI is not an exhaustive representation of total chemical releases to the Canadian environment.

Canadian environmental quality guidelines have been set regarding the maximum allowable concentration of copper. In freshwater systems, copper concentrations should not exceed 2-4 μg/L, whereas in residential areas, parklands and agricultural lands, copper should not exceed 63 mg/kg (CCME, 2002). Localized accumulation of copper occurs through natural

phenomena such as volcanic activity, windblown dust, forest fires, decaying vegetation and sea spray (Dameron & Howe, 1998). As metallic copper and copper compounds have many varied uses, anthropogenic activities also contribute to the concentration of copper in the environment. Smelting, combustion sources, anti-microbial uses and agricultural practices all contribute to copper accumulation. The largest releases of copper to the environment from anthropogenic activities however originate from the spreading of sewage sludge and from mining activities (Dameron & Howe, 1998).

2.1.2 Copper in Plants

Copper can exist in its metallic form (Cu^0), or in three oxidation states (Cu^+, Cu^{+2} and Cu^{+3}) (Clarkson & Hanson, 1980; Dameron & Howe, 1998) and is found naturally as a metal and as a variety of mineral salts and organic compounds. Environmentally, the first two oxidation states are the most common, with Cu^{+2} being the form usually found in soils (Clarkson & Hanson, 1980). Copper readily changes oxidation states and thus has a high redox potential (Clarkson & Hanson, 1980; Dameron & Howe, 1998; Pilon *et al.,* 2006). This contributes both to copper's biologically essential nature and to its deleterious effects on biological systems.

As mentioned previously, many heavy metals function as essential trace elements in biological systems. Copper plays a very important biological role. Because copper can exist intracellularly as Cu^+ and Cu^{+2} it therefore is involved in many redox reactions (Ochiai, 1977; Clarkson & Hanson, 1980; Pilon *et al.,* 2006). Copper is a "soft" metal that preferentially binds with "soft" ligands; Cu^+ preferentially interacts with oxygen or sulfide ions found in cysteines and methionines, while Cu^{+2} interacts readily with nitrogen atoms in histidine molecules (Clarkson & Hanson, 1980; Lippard & Berg, 2004; Pilon *et al.,* 2006). Additionally, copper can be used in energy transfer reactions when it is protein bound (Pilon *et al.,* 2006). In plants, copper is an essential co-factor in many proteins (plastocyanin, superoxide dismutase (SOD), cytochrome *c* oxidase and apoplastic oxidases) and as such, is involved in photosynthesis, respiration, antioxidant activity, cell wall metabolism and hormone perception (Clarkson & Hanson, 1980; Marschner, 1995; Pilon *et al.,* 2006;).

As copper is involved in a myriad of functions, it is almost ubiquitous throughout the plant cell, and is required locally in the cytosol, the endoplasmic reticulum, the chloroplast

stroma, the thylakoid lumen, the apoplast, and the inner membrane of the mitochondria (Pilon *et al.,* 2006). Plants normally contain approximately 1-3 ppm of copper on a dry weight basis (Loneragan, 1975). Because of copper's high redox activity, excess copper in the plant can easily become toxic and so its intracellular delivery must be tightly regulated (Dameron & Howe, 1998; Pilon *et al.,* 2006).

2.1.3 Biological Effects

Copper deposited on the soil surface persists in the upper surface layers (Alloway & Jackson, 1991; McGrath *et al.,* 2001) as it readily forms complexes or adsorbs to soil particles. The bioavailability of copper in soil and water can vary greatly depending on the particular situation and available ligands and adsorbents. Many factors affect the bioavailabilty of copper. In soil, for example, pH, type and distribution of organic matter, soil redox potential, cation exchange capacity, rate of decomposition and proportion of clay:silt:sand particles all influence copper availability (Dameron & Howe, 1998). Due to these factors and the high redox potential of copper itself, the bioavailability of copper in the environment can be in constant flux.

Copper is a required trace element for all biota and, as such, all forms of life have evolved mechanisms for copper metabolism. However, when natural homeostatic levels of copper are exceeded, toxicity can result and induce adverse effects on structure and function of biomolecules, membranes and proteins, either directly or through oxygen radical mechanisms (Dameron & Howe, 1998). Some organisms possess a wide tolerance to copper; others' can be quite narrow. Additionally, some organisms are capable of bio-accumulating levels of copper in excess of their requirements. Accumulation may lead to exceptionally high body burdens in certain animals and terrestrial plants which can, in turn, lead to bio-concentration issues for the food chain (Chaney *et al.,* 1987; Dameron & Howe, 1998; Schwedt, 2001).

Excessive levels of copper in either terrestrial or aquatic systems have been shown to have adverse effects on reproduction, biochemistry, physiology and behaviour of a variety of organisms including; phytoplankton communities, freshwater and marine invertebrates and fish, plants and soil micro- and macro-organisms such as earthworms, bacteria and fungi (Dameron & Howe, 1998; McGrath *et al.,* 2001).

2.1.4 Health Effects

Instances of copper poisoning among a normal, healthy, human population are rare and the chances of copper accumulating in the body to toxic levels are small outside of occupational exposures (Dameron & Howe, 1998). In non-occupational settings, the major route of exposure is oral. According to a report sanctioned by the World Health Organization, gastro-intestinal problems have been attributed to repeated ingestion of copper and liver failure and intravascular haemolysis can result from chronic ingestion. Dermal exposure may illicit an allergic response in sensitive individuals. In occupational settings, metal fever, a condition that results in flu-like symptoms, can result from inhalation of high concentrations of copper (Dameron & Howe, 1998). Long term exposure to copper has also been attributed to mouth, nose and eye irritations, headaches, nausea, dizziness, diarrhea and in cases of extreme exposure, kidney damage (Lenntech, 2005).

2.2 A Proposed Solution - Remediation Technologies

When concentrations of toxic substances reach hazardous levels in soils, traditionally, those land areas have been either cordoned off and essentially abandoned or conventionally remediated using largely mechanical and/or chemical techniques. Likewise, contaminated surface and groundwaters have largely been managed using conventional remediation strategies. Both *in situ* and *ex situ* methods exist for the remediation of soils or sediments. *In situ* methods include volatilization of chemicals by air venting, leaching of chemicals by the addition of acids or other leachants, vitrification and isolation, and containment using physical barriers (Arthur *et al.,* 2005). *Ex situ* techniques involve first the excavation of contaminated soils to depth, followed by thermal or chemical treatment, extraction or solidification prior to disposal in a landfill (Arthur *et al.,* 2005). Surface and groundwater treatments include ion exchange, reverse osmosis, microfiltration and precipitation or flocculation followed by sedimentation and disposal of the resultant sludge by landfilling (Salt *et al.,* 1995).

Conventional remediation techniques require extensive energy inputs and most require sophisticated equipment, making the remediation of contaminated areas very costly. It is currently estimated that the remediation of a full-scale commercial site would cost US$200,000 +

$40-$70 per cubic yard (Business Publishers, 2004). In the United States alone, 2% of the Gross National Product is estimated to be spent annually on remediation and pollution control activities (Sparks, 1995). Compounding these high costs are the additional drawbacks that follow the remediation of a site using *ex situ* methods; the soil's structure is destroyed and left biologically inactive (McGrath *et al.,* 2001) leaving a visible scar on the land as well as the inaesthetics of the operations during treatment. It is therefore vital that an alternative method for the remediation of contaminated sites is sought.

2.2.1 Phytoremediation

Phytoremediation, the use of plants to remediate soils, sediments and waters contaminated with organic and/or inorganic compounds, is an alternative to conventional remediation that has received growing attention over the past three decades. Phytoremediation has many advantages over traditional methods. While applicable to different toxic substances, it simultaneously provides ground cover (which helps to eliminate erosion by air and water,) is aesthetically pleasing (which helps lead to a high degree of public acceptance) and minimizes the disturbance to the environment. Additionally, in the case of metals, it can generate a metal-rich plant residue from which the metals can be recycled (Kumar *et al.,* 1995). Perhaps its greatest advantage however, is the low cost of the technology in comparison to traditional methods. It has been estimated that the use of phytoremediation can cost 10-25% of conventional techniques (Salt *et al.,* 1995; Ensley, 2000).

Several methods of phytoremediation exist. Their details are highlighted in Table 2.1. The archetypal plant for phytoremediation is fast-growing, has high biomass production with an extensive root system, is easy to harvest, and can both tolerate and remediate a range of toxic compounds (Yang *et al.,* 2005). While plants can directly remediate an area, they also provide indirect advantages. Through their roots, plants improve soil structure, add organic matter to the soil and improve the water holding capacity (Schnoor *et al.*, 1995; Cunningham *et al.*, 1996). Plants also provide carbon and other nutrients in the form of root exudates to the rhizosphere microbial community (Cherian & Oliveira, 2005; LeDuc & Terry, 2005). These fungi and bacteria can significantly affect the remediation process by contributing to the detoxification of the medium.

Table 2.1: Types of phytoremediation techniques.

Technique	Process	Medium
Hyperaccumulation	Accumulation of contaminants in shoots and subsequent shoot harvest.	Soil
Rhizofiltration	Absorption/adsorption of contaminants in/on roots.	Surface water
Phytostabilization	Root and root exudates reduce bioavailability of contaminant.	Soil, Groundwater
Phytovolatilization	Evaporation of contaminants through plant transpiration.	Soil, Groundwater
Phytodegradation	Plant-assisted microbial degradation of contaminants in rhizosphere.	Soil, Groundwater
Phytotransformation	Plant uptake and degradation of contaminants.	Soil, Groundwater, Surface water
Removal of Aerial Contaminants	Uptake of volatile contaminants by leaves.	Air

(Sources: Yang *et al.,* 2005; Salt *et al.,* 1995; Arthur *et al.,* 2005)

2.2.2 Hyperaccumulators

As early as the 19th century, plants that accumulated high amounts of metals in their tissues whilst suffering no adverse effects, were identified (Baumann, 1885). In order to cope with increasing concentrations of metals in soils, plants either evolved as excluders or as hyperaccumulators (Yang *et al.,* 2005). Excluders function by restricting either the uptake or the root-to-shoot translocation of metals (Nascimento & Xing, 2006). Hyperaccumulators are generally defined as those plants capable of concentrating heavy metals in their above ground tissues to 0.1-1% of their dry weight (Meagher, 2000) or to levels 50-500 fold greater than typical levels (Lasat, 2000). This translates to threshold levels (w/w) of 1% for Zn and Mn, 0.1% for Ni, Cu, Co, Pb and 0.01% for Cd (Baker *et al.,* 2000). Hyperaccumulation, or phytoextraction as it is sometimes called, was first proposed as a means of reclaiming metals from polluted soils by Chaney (1983).

To date, over 400 species of vascular plants have been identified as hyperaccumulators (Baker *et al.,* 2000; Reeves & Baker, 2000; McGrath & Zhao, 2003; McIntyre, 2003). This represents less than 0.2% of all angiosperms (Baker & Whiting, 2002) making it a fairly rare phenomenon. Hyperaccumulators are found from a wide range of taxonomic groups (45 different families) (Baker *et al.,* 2000) and geographic areas and possess a wide variety of morphologies, physiologies and ecological characteristics (Pollard, 2002). The first hyperaccumulators identified were from the Brassicaceae and Fabaceae families (Yang *et al.,* 2005). The majority of hyperaccumulators are endemic to highly metalliferous soils and are regarded as strict metallophytes. A few species are found on both metal and non-metal sites and are described as facultative metallophytes (Pollard, 2002). The majority of hyperaccumulators accumulate only one metal (Pollard, 2002) although a significant number show the ability to accumulate more than one (He *et al.,* 2002; Yang *et al.,* 2002, 2004; McIntyre, 2003). This is of great significance as many sites requiring remediation are polluted with more than one metal. In fact, 70% of all Superfund sites in the United States are contaminated with at least two metals (Forstner, 1995).

Several hypotheses regarding the evolution of the hyperaccumulation phenomenon exist and it is important to recognize that the function of a trait as it is regarded today may not be that

for which it originally evolved. Boyd (2004) proposed four hypotheses for the evolution of hyperaccumulation:

i. The *tolerance/disposal hypothesis* suggests that hyperaccumulation evolved as a method of sequestration of metals into tissues and the subsequent shedding of those tissues.
ii. The *interference* or *elemental allelopathy hypothesis* suggests that hyperaccumulating plants enrich the soil directly under their canopies with the decomposition of high-metal leaf litter thereby excluding the establishment of non-hyperaccumulating competitor species.
iii. The *drought-resistance hypothesis* suggests that high levels of metals *in planta* can help plants avoid drought-stress.
iv. The *defense hypothesis* suggests that hyperaccumulation evolved as a means of pathogen resistance and/or deterring herbivory.

The goal of using hyperaccumulators in phytoremediation strategies is to decrease the metal concentration of the stratum to below regulatory levels within a suitable timeframe (Nascimento & Xing, 2006). The accumulation ability of a plant depends on both the plant biomass as well as the bio-concentration efficiency (the harvestable plant tissues:soil ratio of metal concentration (Cherian & Oliveira, 2005)) (Yang *et al.,* 2005). Most plants have a bioconcentration factor <1 (McGrath & Zhao, 2003) whereas hyperaccumulators are characterized by bioconcentration factors >1, and in some cases, ranging from 50-100 (Ma *et al.,* 2001; Zhao *et al.,* 2003). The hyperaccumulation of metals involves several steps. Firstly, hyperaccumulators must by definition be hypertolerant of metals (McGrath *et al.,* 2001). Secondly, mechanisms allowing the transport of metals across the root cell plasma membrane must exist. Xylem loading and translocation of metals to the aerial tissues then follows. Finally, mechanisms to detoxify and/or sequester the accumulating metals at both the whole plant and cellular levels must be present (Yang *et al.,* 2005). Hyperaccumulation is therefore a complicated interplay requiring co-ordination of several systems within the plant.

2.2.2.1 The Mechanics of Hyperaccumulation

2.2.2.1.1 Root Uptake

To increase the bioavailability of soil-bound metal ions it is generally believed that plants secrete exudates from their root systems into the surrounding rhizosphere, either to acidify the soil and mobilize metal ions, or to form metal-chelate complexes. Exudates may be in the form of protons, low molecular weight organic acids (LMWOA), phenolics, amino acids such as histidine or nicotianimic acid (NA) or phytosiderophores (Romheld, 1991; Ross, 1994; Salt *et al.,* 1995; Lasat, 2002; Yang *et al.,* 2005). It has also been suggested that specific metal-chelating compounds such as enzymes or phytochelatins (PC) may also be secreted (Yang *et al.,* 2005). Plasma membrane-bound root reductases may also function to decrease soil pH (Salt *et al.,* 1995). Of the implicated root exudates, LMWOAs (acetic, oxalic, fumaric, citric, tartaric) are believed to be the most important (Nascimento & Xing, 2006), and that complex formation is likewise more important than the reduction of soil pH (Bernal *et al*., 1994; McGrath *et al.,* 1997; Gupta *et al.,* 2000; Quartucci *et al.,* 2005). Solubilized metal ions must then enter the root cells by symplastic or apoplastic pathways. Most likely, entrance is via metal ion carriers or channels (Clarkson & Luttge, 1989), however, specialized carriers must also exist for the transport of metal-chelate complexes (Crowley *et al.,* 1991).

2.2.2.1.2 Root-to-Shoot Translocation

Hyperaccumulators are at least partially defined by the efficient root-to-shoot transport of metals which likely occurs via xylem sap (Stephan & Scholz, 1993). Enhanced root-to-shoot transport can be accomplished either by reducing the sequestration of metals in root vacuoles (Lasat *et al.,* 1998) or by enhancing xylem loading (McGrath & Zhao, 2003). Metal transport likely occurs in the form of metal-organic acid complexes (Baker & Brooks, 1989) although PCs have also been implicated as a possible complexing agent in xylem sap (Przemeck & Haase, 1991). While evidence suggests a xylem-based root-to-shoot metal transport, it is also believed that metal redistribution in the leaves is performed partially by the phloem (Stephan & Scholz,

1993). In the phloem, metal is thought to be chelated by NA (Stephan & Scholz, 1993), PC, metallothionein (MT) or organic acids (Salt *et al.,* 1995).

2.2.2.1.3 Transporters

Large families of metal transporters are encoded in plant genomes and transporter proteins both within and between families vary greatly in their substrate specificities, expression patterns and cellular localizations (Colangelo & Guerinot, 2006). As metal homeostasis must be tightly controlled in cells, the regulation of metal transporters must consequently be strict. Regulation may occur at the transcriptional level (initiation sites, mRNA stability and splicing) or post-translationally (targeting, protein stability) (Yang *et al.,* 2005).

Transporters can be broadly divided into two groups; efflux and influx transporters. Efflux transporters are responsible for the movement of metals from the cytoplasm either across the plasma membrane or into organelles (vacuole, endoplasmic reticulum (ER), golgi body, chloroplast). Efflux transporters include members from the heavy metal (or CPx-like) ATPases (HMAs), the cation diffusion facilitators (CDFs) (Yang *et al.,* 2005; Colangelo & Guerinot, 2006) and ABC-type transporters (Cobbett & Goldsbrough, 2000). Influx transporters, like the natural resistance-associated macrophage proteins (NRAMPs), the yellow-stripe like proteins (YSL) and the zinc-regulated transporter, iron-regulated transporter-like proteins (ZIP), function to move metals from the apoplast or remobilize metals from organelles into the cytoplasm (Yang *et al.,* 2005; Colangelo & Guerinot, 2006).

2.2.2.1.4 Chaperones

Metallochaperones provide the plant with a mechanism to avoid high levels of free metals in the cytoplasm and their function is two-fold. The main function of chaperones is to control the specific delivery pathways in response to metal ion supply and in doing so they also prevent inappropriate metal ion interactions (Pilon *et al.*, 2006). Two examples of metal chaperones in plants are CCH (copper chaperone) and cytosolic Cu/ZnSOD (superoxide dismutase) (Pilon *et al.*, 2006). The two best characterized chaperones to date however, are

metallothionein and phytochelatin. They are both widely distributed throughout the plant and form stable metal-complexes in the cytosol (Zenk, 1996; Goldsbrough, 2000).

Metallothioneins (MTs) are gene-encoded, low molecular weight (6-7 kDa), cysteine (Cys)-rich proteins (Hamer, 1986; Yang *et al.,* 1995) that are induced by Cu, Zn and Cd (Zhou & Goldsbrough, 1994, 1995; Guo *et al.,* 2003) and are transcriptionally regulated (Mejáre & Bülow, 2001). MTs occur in all taxa and are well-characterized in the animal kingdom especially in humans, however, the isolation and subsequent characterization of plant MTs has been difficult.

Plant MTs belong to the Type II class of MTs as defined by Cobbett and Goldsbrough (2002). MTs characteristically possess no aromatic or histidine amino acid residues (Mejáre & Bülow, 2001) and bind metals via thiol groups of the Cys residues (Hamer, 1986). Plant MTs consist of two Cys-rich domains, one each at the carboxyl and amino ends of the protein and are separated by a Cys-devoid spacer region which gives MTs characteristic dumbbell shape (Cobbett & Goldsbrough, 2002). Plant MTs are further classified into four subtypes based on the Cys arrangement in the 2 domains (Rauser, 1999; Cobbett & Goldsbrough, 2002). Based on limited data, Subtype 1 MT expression has been localized to roots whereas Subtype II MTs are expressed in shoots (Mejáre & Bülow, 2001).

Phytochelatins (PCs) are post-translationally, enzymatically-synthesized peptides (Yang *et al.,* 2005) that function to reduce free metal concentration in plant cells by chelation (Vatamaniuk *et al.,* 1999). PCs are synthesized from the tripeptide glutathione (GSH) and as such contain only three amino acids; glutamine (Glu), glycine (Gly) and Cys. Primary PC structure consists of increasing repetitions of the Glu-Cys dipeptide with a terminal Gly residue where typically the number of dipeptide repeats ranges from 2-5 (Yang *et al.,* 2005) but up to eleven can occur (Cobbett, 2000). The enzyme phytochelatin synthase, which controls the synthesis of PCs, is rapidly induced and only active in the presence of Cd, Cu, Zn, Ag, Hg and Pb (Yang *et al.,* 2005).

2.2.2.2 Naturally Enhanced Hyperaccumulation

A variety of strategies to enhance the metal uptake and subsequent accumulation capacities of natural hyperaccumulators have been attempted with varying degrees of success.

The most well-researched strategy is the amendment of metal-rich soil with chelators for the purposes of increasing metal bioavailabilty. It was generally believed that by increasing the bioavailable concentration of metals ions in the soil, a hyperaccumulator would thereby be capable of increased total metal accumulation. While the addition of both synthetic and natural chelators has significantly increased the soluble metal fraction, that increase has not been mirrored to the same degree in plants. Ethylenediaminetetraacetate (EDTA), the most studied synthetic chelator in phytoremediation research can markedly increase the mobility of Pb in soils and has been shown to cause an increase in phytoextracted Pb and other metals (Cunningham & Ow, 1996; Blaylock *et al.,* 1997; Chen *et al.,* 2004). However, EDTA is not biodegradable by soil microorganisms and poses a dual threat, by leaching into groundwater itself (Sun *et al.,* 2001; Wenzel *et al.,* 2003; Chen *et al.,* 2004) and also by causing newly mobile metal ions to leach out. Moreover, EDTA has been shown to adversely affect the soil microbiota (Welper & Brummer, 1997) as well as some plant species (Nasciamento *et al.,* 2006). Other synthetic chelators that have been studied include HEDTA (hydroxyethylethylenediaminetriacetate), EGTA (ethylene glycol tetraacetate), EDDHA (ethylenediaminedihydroxyphenylacetate) and DTPA (diethylenetriaminepentaacetate). All have been shown to increase the bioavailability of metals in soil, although to a lesser extent than EDTA (Huang *et al.,* 1997). Two biodegradable synthetic chelators, MGDA (methylglycinediacetate) and EDDS (ethylenediaminedissuccinate), have been tested as environmentally-friendly alternatives to EDTA and although not yet extensively studied, show promise for enhancing hyperaccumulation, especially of Pb (Groman *et al.,* 2003; Tamura *et al.,* 2005). Synthetic chelators generally seem to make metal available too quickly for the existing hyperaccumulation mechanism to handle.

Natural chelators such as citric, acetic, fumaric, tartaric and gallic acids have been tested as possible biodegradable soil amendments (Mench & Martin, 1991; Robert & Berthelin, 1994; Stevenson & Fitch, 1994; Nasciamento *et al.,* 2006). In general, organic acids do not have the same chelating power that synthetic molecules do although they have been shown to improve metal bioavailability and influence a moderate increase in accumulation. Their lower chelating capacity can be attributed to the fact that small amounts of organic acids have been shown to be rapidly mineralized by soil microorganisms (Römkens *et al.,* 2002; Meers *et al.,* 2004). It has been suggested that due to the rapid degradation of these amendments, several applications could be applied throughout the growing season as an alternative (Nascimento & Xing, 2006).

An alternate strategy examined by several groups is the establishment of symbiotic plant-microbial associations, usually mycorrhizal, to improve hyperaccumulation. A general view that most plants in natural conditions form mycorrhizal associations exists (Smith & Reed, 1997) and these relationships have been reported for some hyperaccumulators (Shetty *et al.,* 1995; Weissenhorn & Leyval, 1995; Chaudhry *et al.,* 1998 and 1999). Mycorrhizae (arbuscular or ecto-) functionally increase root surface area and allow access to a greater soil volume for the plant host. Mycorrhizae are also known to produce growth promoting substances and aid in nutrient uptake (Khan *et al.,* 2000) and so could be expected to enhance metal uptake. However, the evidence for this is conflicting as mycorrhizae have been shown to both enhance and reduce metal uptake by plants (Marschner, 1995). Ectomycorrhizal fungi seem to protect plant roots from excess metal as tolerance levels are increased by this association but metal accumulation decreases (Marschner, 1995; Leyval & Joner, 2001). Compounding this is the fact that the Brassicaceae, the family to which a large number of hyperaccumulators belong, do not form mycorrhizal associations (McGrath *et al.,* 2001).

The final strategy to naturally enhance hyperaccumulation that will be discussed is that of "co-culture engineering" as coined by Ni *et al.* (2004). In controlled experiments, co-cropping a hyperaccumulator species with a leguminous non-accumulator caused an increased mobility of metal ions in soil and subsequent accumulation of metal ions in the hyperaccumulator that mimicked levels achieved when soil was amended with an organic acid chelator.

2.2.2.3 Genetic Engineering of Hyperaccumulators

Naturally-occurring hyperaccumulators tend to be small, low-biomass plants that are slow growing (Pollard *et al.* 2002; Tong *et al.,* 2004), and while capable of accumulating, in some cases, high relative amounts of metals in their tissues on a per plant basis, do not in general accumulate high absolute amounts of metal. In addition to the problem of low biomass, the small size of the plants generally precludes the use of standard agronomic practices for their cultivation (Tong *et al.,* 2004). To overcome these limitations it has been suggested that the hyperaccumulation traits be transferred to plants with high biomass (Brown *et al.,* 1995). Traditional plant breeding is limited largely to genetic transfer within a species or between

closely related species thereby limiting the degree of improvement to the genetic variability of the species and its close relatives.

To date, two successful somatic hybridizations of *Thlaspi caerulescens*, a small hyperaccumulator, and the high-biomass *Brassica napus*, have produced hybrids of an intermediate size capable of accumulating high levels of zinc (Brewer *et al.,* 1999) and Pb (Gleba *et al.,* 1999), indicating that the transfer of hyperaccumulation traits is possible. Somatic hybridization however is a technique with a very low success rate.

Genetic engineering (GE) of plants provides an alternative strategy for the transfer of traits that does not rely on sexual compatibility as does traditional plant breeding. GE also allows for the transfer of genes from different kingdoms into plants. For example, the diversity and adaptability of microorganisms has allowed for their colonization of toxic environments where higher plants are incapable of growing. These microbial genomes could provide genes that have the potential to enhance hyperaccumulation (LeDuc & Terry, 2005). Obvious targets for enhancing hyperaccumulation would be (1) to increase the number of uptake sites in the roots, (2) to increase the rate of root-to-shoot translocation, (3) to enhance the sequestration capacity, (4) to enhance metal uptake and, (5) to alter substrate specificity.

GE strategies to enhance hyperaccumulation have included the overproduction of intracellular metal chelators such as citrate (De la Fuente *et al.,* 1997), MTs (Evans *et al.,* 1992; Hasegwa *et al.,* 1997) and GSH (Zhu *et al.,* 1999), the precursor to PC, and overexpression of metal-transporters (Samuelsen *et al.,* 1998; Arazi *et al.,* 1999; Van der Zaal *et al.,* 1999; Hirschi *et al.,* 2000). Perhaps two of the best known examples of genetic manipulation for enhanced phytoremediation are (1) the transformation of Indian mustard (*Brassica juncea*) with the *A. thaliana* APS (ATP sulfurylase) gene, resulting in increased accumulation of selenium (Pilon-Smits *et al.,* 1999) and (2) the bioengineering of plants for the removal of methyl-mercury from soil with a bacterial gene (Rugh *et al.,* 1996).

2.3 The Need for a Mechanistic Model

Although great strides in characterizing and understanding the hyperaccumulation phenomenon have been accomplished over the last decade, the science of phytoremediation is still in its infancy. Hyperaccumulation involves a complex and tightly regulated network of

homeostatic mechanisms that will require the collaborative efforts of botany, plant physiology, biochemistry, geochemistry, molecular biology, microbiology and agricultural engineering to fully elucidate (Cherian & Olveira, 2005). The lack of understanding of the network as a whole represents the largest obstacle in the development of efficient hyperaccumulation strategies and it is clear that a more complete understanding is required (Kraemer, 2003; Cherian & Olveira, 2005; Nasciamento & Xing, 2006). Cobbett (2003) has suggested that the underlying mechanisms in metal tolerance and accumulation are the same in both *Thlaspi caerulescens*, a well-known hyperaccumulator, and *Arabidopsis thaliana*, the model genetic plant and a non-accumulator. He postulates that the two species possess the same suite of genes and that it is the differential gene expression and differential protein activities that contribute to the differing physiologies. Nonetheless, it remains clear that an overall model of the hyperaccumulation mechanism is required.

3.0 COPPER-INDUCED PROTEOMIC RESPONSE OF *ARABIDOPSIS THALIANA* GENOTYPES

3.1 Introduction

Phytoremediation is the use of plants to clean-up contaminated waters and soils. It is a cost-effective, emerging technology that requires low inputs and enjoys high public acceptance. It has been suggested that the remediation of metal contaminated sites may be achieved by a subset of plants known as hyperaccumulators (Chaney, 1983). Metal hyperaccumulators have the ability to sequester large amounts of metals (0.1-1% dry weight) in above-ground tissues whilst suffering no toxic effects (Meagher, 2000). While many metals are essential plant micronutrients, these, and other non-essential metals can cause severe toxicity at elevated intracellular levels (Cunningham *et al.,* 1995; Marschner & Romheld, 1995; McGrath *et al.,* 1995; Salt *et al.,* 1995; Garbisu & Alkorta, 2001). Hyperaccumulators however, have evolved mechanisms by which this toxic effect is avoided and include the highly regulated uptake, transport, chelation and sequestration of metals (Yang *et al.,* 2005).

It is likely that the protein network involved in the hyperaccumulation response does not differ largely from that utilized by plants to maintain normal intracellular metal homeostasis. For example, *Arabidopsis halleri*, a hyperaccumulator, is genetically very similar to *Arabidopsis thaliana*, a non-accumulator (Koch *et al.,* 2001). Further, the frd3-3 mutant, described as an accumulator of various metals, is virtually genetically identical to *A. thaliana* (Delhaize, 1996). Therefore, the hyperaccumulation phenomenon likely arises due to the differential expression and regulation of existing mechanisms (Cobbett, 2003). Large families of metal transporters in plants have already been identified as part of the metal homeostatic network; P_{1B}-ATPases (Baxter *et al.,* 2003), CDF (cation diffusion facilitators) (Curie *et al.,* 2001), NRAMP (natural resistance-associated macrophage proteins) and ZIP (zinc-regulated transporter, iron-regulated transporter-like proteins) (Lasswell *et al.,* 2000; Maser *et al.,* 2001) as well as metal chaperones such as phytochelatins and metallothioneins (Zenk, 1996; Goldsbrough, 2000).

Of the more than 400 species of hyperaccumulators characterized to date (Baker *et al,* 2000; Reeves & Baker, 2000; McGrath & Zhao, 2003; McIntyre, 2003), a large proportion are found in the family Brassicaceae and include such species as *A. halleri* (Sarret *et al.,* 2002)*, Brassica juncea* (Bennett *et al.,* 2003)*, Thlaspi caerulescens* (Lombi *et al.,* 2001)*, T. goesingense*

(Reeves & Brooks, 1983) and *Alyssum lesbiacum* (Ingle *et al.,* 2005a ; Prasad, 2005). With its small size, short generation time and completely sequenced genome (Arabidopsis Genome Initiative, 2000), *A. thaliana* is an attractive model plant for studying molecular paradigms especially within the Brassicaceae. Additionally, the sequence identity at the amino acid level based on cDNA sequence information has been shown to be greater than 90% between metal-hyperaccumulating *Brassica* species and *A. thaliana* (Persans *et al.,* 1999, 2001; Lasat *et al.,* 2000; Pence *et al.,* 2000; Ingle *et al.,* 2005b). This high degree of genetic similarity between *A. thaliana* and other hyperaccumulators provides an effective tool for the study and identification of proteins involved in the metal response.

Proteomics offers the ability to study the actively translated portion of the genome. While the expression levels of mRNA can be affected by environmental stimuli, they do not directly correlate with protein expression levels resulting from the same environmental stimuli (Anderson & Seilhamer, 1997; Haynes *et al.,* 1998; Gygi *et al.,* 1999). Protein expression is controlled at many levels including translation rates, targeting and post-translational modification which contribute to the discrepancy between protein and transcript levels (Coleman *et al.,* 2005). Proteomic analysis, which involves the identification and relative quantification of differentially expressed proteins, is a powerful way to study the responses of plants to varying environmental stimuli, and the regulation of that response, at the protein level. Previous plant proteomic studies have focused on the protein responses to different physiological processes such as germination (Gallardo *et al.,* 2001), and senescence (Wilson *et al.,* 2002), whereas others have studied the protein complements of specific tissues (Kamo *et al,* 1995; Giavalisco *et al.,* 2005), organelles (Kruft *et al.,* 2001; Friso *et al.,* 2004) or sub-cellular components (Santoni *et al,* 1999; Ndimba *et al.,* 2003). Other studies have examined the differential proteomic responses of plants to environmental stressors such as drought (Leymarie *et al.,* 1996; Hajheidari *et al.,* 2005; Plomion *et al.,* 2006), heat stress (Majoul *et al.,* 2004; Sule *et al.,* 2004; Ferreira *et al.,* 2006), cold stress (Goulas *et al.,* 2006; Yan *et al.,* 2006), pathogen attack (Colditz *et al.,* 2004), salt (Abbasi & Komatsu, 2004) and heavy metal exposure (Ingle *et al.,* 2005a ; Requejo & Tena, 2005; Roth *et al.,* 2006; Sarry *et al.,* 2006).

An alternative strategy for studying mechanisms of environmental stress response is to compare the proteome of wildtype plant with that of a closely-related mutant grown under the same conditions (Gallardo *et al.,* 2002; Kim *et al.,* 2006; Liu *et al.,* 2006; Sorin *et al.,* 2006).

Likewise, the examination of two closely-related plants with opposing strategies for dealing with a particular environmental stress should also provide useful insights. In this study, the proteomic analysis of two *Arabidopsis thaliana* genotypes grown in the presence of copper is carried out. Non-accumulating wildtype (ecotype Columbia) and metal-accumulating frd3-3 mutant plants were grown hydroponically and exposed to copper. Two-dimensional gel electrophoresis (2DE) was used to separate proteins extracted from roots or shoots of the two genotypes at four timepoints (0, 8, 12 and 48 hours) following addition of copper to the growth medium. After comparing the gels for each genotype/tissue combination to determine which proteins were differentially expressed over time, the protein spots of interest were excised from the gels, digested with trypsin, and identified by nanoLC-MS/MS analysis and database searching. By using genotypes that differ in their abilities to accumulate metals, and challenging them to copper exposure over time, this research aims to elucidate some of the molecular mechanisms that co-operatively control the overall metal homeostatic network in plants.

3.2 Materials and Methods

All reagents unless otherwise noted were obtained from Sigma (St. Louis, MO, USA). All solutions were prepared with Milli-Q water (Millipore, Billerica, MA, USA) unless otherwise stated.

3.2.1 Plant Material and Growth Conditions

The two genotypes of *Arabidopsis thaliana* (L.) used in this study were the wildtype (WT) Columbia ecotype (a metal non-accumulator) and the frd3-3 (ferric reductase defective) mutant (an accumulator of various metals), formerly known as man1-1. The mutant line was derived from Columbia by EMS (ethyl-methane sulfonate) mutagenesis and represents a mutation in a single recessive locus (Delhaize, 1996). The frd3-3 mutant has constitutive ferric chelate reductase activity (Rogers & Guerinot, 2002) causing it to overaccumulate a variety of metals over WT levels including manganese (Mn), iron (Fe), copper (Cu), magnesium (Mg) and zinc (Zn) (Delhaize, 1996; Rogers & Guerinot, 2002). Seeds of frd3-3 were obtained from the

ABRC (Arabidopsis Biological Resource Center, Columbus, OH, USA).

Plants were grown hydroponically in three sequential growth chamber experiments under identical conditions as detailed below. Seeds from a common seed batch were placed on individual 0.5% agar-filled 500-µL microcentrifuge tubes with the bottoms trimmed off and placed in germination boxes (Figure 3.1) supplied with 0.1-strength Hoagland solution (Hoagland & Arnon, 1938) with 10 µM Fe-EDTA in distilled water. Following vernalization (48 hours at 4°C), germination boxes were placed in a growth chamber (16 hour photoperiod, 24°C, 125-150 µE $m^{-2}s^{-1}$) for approximately 20 days until roots protruded from the bottom of the agar plugs. Lids of the germination boxes were left slightly ajar for the following 24 hours to harden off the seedlings. Twenty seedlings with good root and shoot development were transferred to each hydroponic growth vessel (Figure 3.2) containing 0.1-strength Hoagland solution. The solution was well-aerated and changed weekly. Seedlings were grown for 21 days until sufficient root and shoot biomass had developed. Inflorescences were removed to encourage and prolong the vegetative growth phase. The hydroponic solution was inoculated with 30 µM $CuCl_2$ and plants were sampled at 0 (just prior to inoculation), 8, 12 and 48 hours. The unchallenged (0 hour) plants thus serve as the treatment control. The Cu concentration used in this study is in good agreement with other studies examining the effect of Cu on plant genes, transcripts and proteins (Murphy & Taiz, 1997; Xiang & Oliver, 1998; Granger & Cyr, 2001; Maksymiec & Krupa, 2002; Mira *et al.,* 2002; Suzuki *et al.,* 2002). Ten plants, selected randomly from all tubs, were separated into root and shoot at the base of the rosette, pooled, rinsed in distilled water, blotted dry, and flash frozen in liquid nitrogen prior to storage at -80°C. Pooled plants were randomly selected from all hydroponic tubs so as to minimize micro-environment variation. All three sequential growth experiments were conducted with similar results.

Mutant and WT tissues produced in Growth Experiment #1 were used for the determination of biomass and *in situ* Cu concentrations (Figure 3.3A). Like-tissues produced in Growth Experiments #2 and #3 (Figure 3.3B) were combined in the fashion of Richards *et al.* (1998) and Holmes-Davis *et al.* (2005) and used for protein extractions. As in Holmes-Davis *et al.* (2005), the combined tissues represent "averaged samples" and should not be considered biological replicates. The pooling of tissues from Growth Experiments #2 and #3 was performed so as to account for experimental variability arising from the sequential nature of the growth experiments (*i.e.* changes in growth chamber micro-environments, seed ageing, media variation)

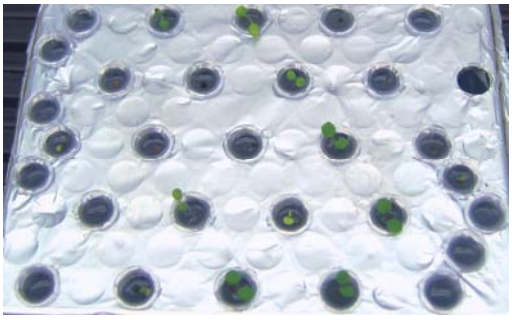

Figure 3.1: Hydroponic seedling germination box.

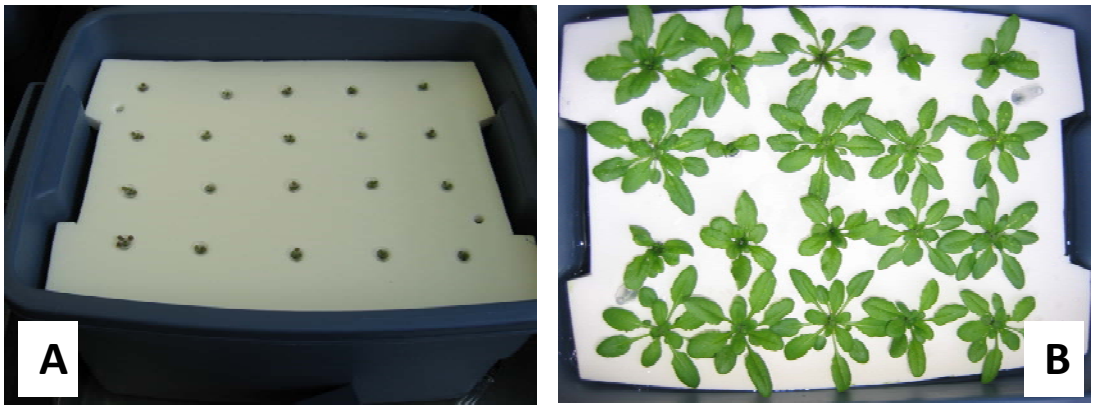

Figure 3.2: Hydroponic growth vessels. **A** – plants at time of transfer from germination boxes; **B** – plants at time of inoculation.

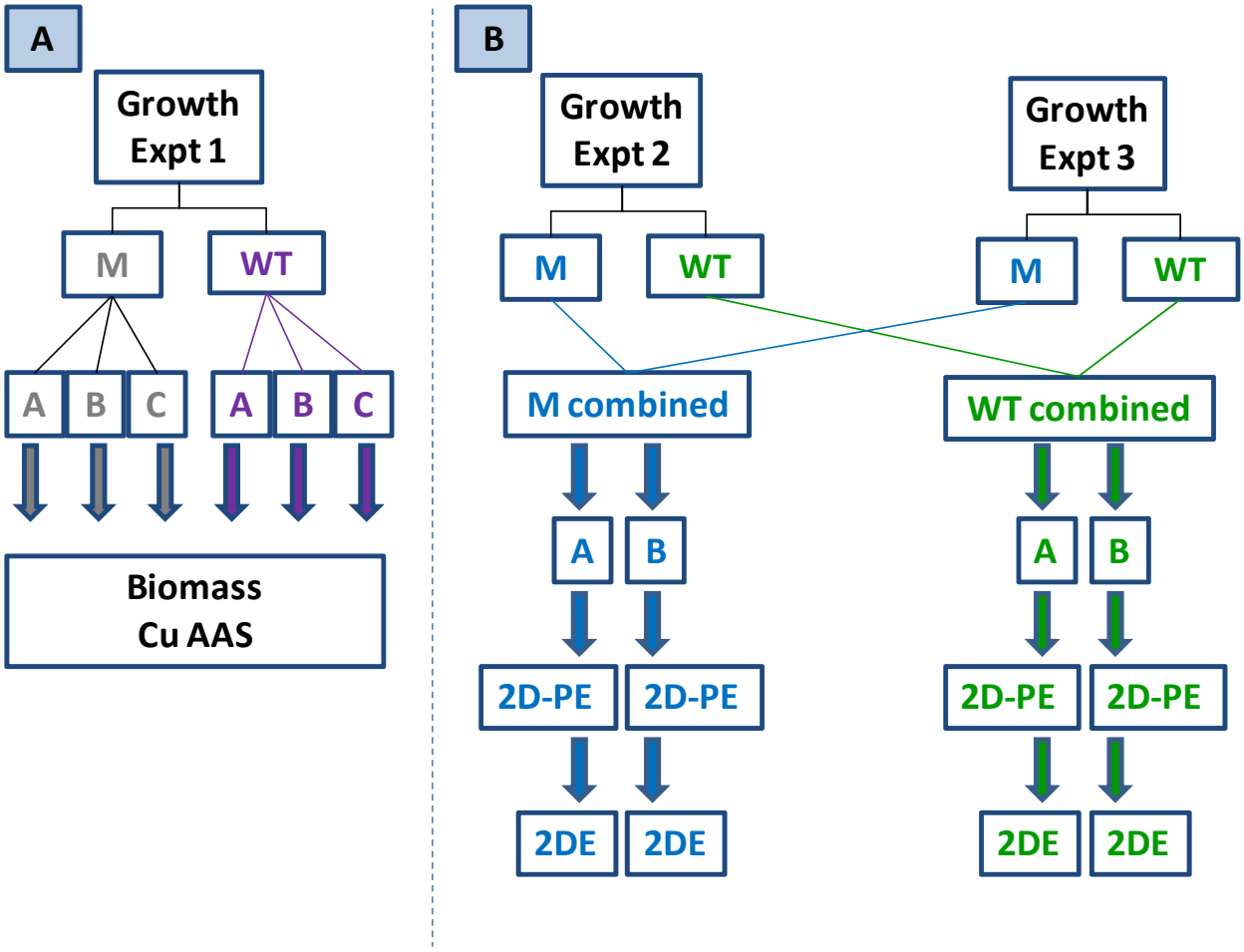

Figure 3.3: Schematic representation of three sequential hydroponic growth experiments and subsequent experimental procedures. M = mutant; WT = wildtype; 2D-PE = protein extraction for 2DE gels; Panel A contains all experiments that used tissues produced in Growth Experiment #1. **A**, **B** & **C** and **A**, **B** & **C** in Panel A represent three technical replicates. Panel B contains experiments that used tissues produced in Growth Experiments #2 and #3. In Panel B, mutant tissues and wildtype tissues produced in Growth Experiment #2 were combined with like-tissues produced in Growth Experiment #3. **A** & **B** and **A** & **B** represent 2 independent subsamples of the combined tissues.

as growth facilities large enough to produce all required tissues in one experiment were unavailable.

3.2.2 Biomass and Copper Analyses

Biomass and Cu concentration analyses were conducted on 3 subsamples from the population of Growth Experiment #1. Each subsample (represented as **A**, **B** & **C** and **A, B** & **C** in Figure 3.3A) is comprised of root or shoot tissues pooled from 10 randomly selected plants and represents 3 technical replicates. Tissues, freeze-dried for biomass determination, were then pulverized using a Mini-Beadbeater (BioSpec Products, Bartlesville, OK, USA) at 2,100 rpm for 2 minutes. Samples were prepared and analyzed as in Lipoth & Schoenau (2007) with modifications. At least 20 mg of tissue was transferred to microwave digestion vessels and 5 mL of concentrated nitric acid was added. Samples were digested at increasing power (up to 90%) for 35 minutes in a MDS-2000 microwave (CEM, Matthews, NC, USA) and subsequently diluted to 25 mL with deionized water. Cu analysis was performed by graphite furnace atomic absorption using a 3100 Atomic Absorption Spectrometer with a HGA-600 Graphite Furnace (Perkin Elmer, Waltham, MA, USA). For every 15 samples, one blank (acid only) and one SRM (Standard Reference Material) (NIST 1573a) were digested as above for quality assurance-quality control analysis. The biomass and Cu concentration results presented represent the means of three technical repetitions. Statistical analyses of the means were performed using the Student's paired t-Test with a significance level of 0.05. Tissue dry weight (DW) pairwise comparisons were made between genotypes for a particular tissue/timepoint combination (*i.e.* WT shoot DW at 8 hours post-inoculation was compared with mutant shoot DW at 8 hours post-inoculation). Cu concentration comparisons were made within a tissue/genotype combination and compared all timepoint pairs. For example, the Cu concentrations determined in mutant roots were compared as follows: 0 hour to 8 hour; 0 hour to 12 hour; 0 hour to 48 hour; 8 hour to 12 hour; 8 hour to 48 hour; 12 hour to 48 hour.

3.2.3 Protein Extraction

Total soluble protein was extracted from roots and shoots of hydroponically-grown *A. thaliana* genotypes harvested at different timepoints following Cu inoculation, essentially following the method of Natera *et al.* (2000). Two independent protein extractions were prepared from two independent samples of tissues pooled from Growth Experiments #2 and #3. The independent tissue samples are schematically represented by **A** & **B** and **A** & **B** in Figure 3.3B. For protein extraction, tissues were ground in liquid nitrogen with a mortar and pestle. Approximately 1-2 g of tissue was suspended in 5 mL of ice-cold acetone containing 10% (w/v) TCA (trichloroacetic acid) and 0.07% (w/v) DTT (dithiothreitol), and sonicated on ice with a Labsonic U Probe Sonicator (B. Braun Biotech, Melsungen, Germany) 6 times for 10 seconds with one-minute interval incubations on ice. Following a 20-minute incubation at -20^{o}C, samples were centrifuged at 35,000 x *g* for 15 minutes at 4^{o}C. The pellets were washed twice by re-suspension in 5 mL of ice-cold acetone, incubation for 30 minutes at -20^{o}C and centrifugation (12,000 x *g*, 15 minutes, 4^{o}C). The pellet was air-dried to remove residual acetone and re-suspended in 2 mL 9 M urea, 4% (w/v) CHAPS, 1% (w/v) DTT, 0.8% (v/v) BioLyte™ 3/10 ampholytes (Bio-Rad, Hercules, CA, USA), 10 mM Tris, 1 mM PMSF (phenylmethylsulfonyl flouride) and 5 mM EDTA (ethylenediaminetetraacetic acid). Re-suspended protein remained at ambient temperature for 2 hours and was vortexed frequently. Finally, the re-suspensions were centrifuged (20,000 x *g*, 5 minutes) and the supernatant retained. Protein concentration was determined by the RC DC Protein Assay (Bio-Rad).

3.2.4 Two-Dimensional Electrophoresis

3.2.4.1 Isoelectric Focusing

Total protein extracts were volume adjusted to 200 µL with 8 M urea, 2% (w/v) CHAPS, 50 mM DTT, 0.05% (v/v) ReadyPrep™ TBP (tributylphosphine) Reducing Agent (Bio-Rad) to a final concentration of 200 µg. Protein extracts were applied to 11 cm, non-linear pH gradient 3-10 ReadyStrip™ IPG (immobilized pH gradient) strips (Bio-Rad) and allowed to rehydrate overnight at room temperature according to manufacturer's instructions. First dimensional

separation by isoelectric focusing (IEF) was performed in a Protean™ IEF Cell (Bio-Rad) with the following program at 20°C: 1 hour conditioning at 500 V; 2 hour exponential voltage ramping to 4,000 V; focusing with exponential voltage ramping to 35,000 volthours with a current maximum of 35 μA/gel.

3.2.4.2 SDS-PAGE

Focussed IPG strips were equilibrated by gentle shaking for 10 minutes with 6 M urea, 0.375 M Tris (pH 8.8), 2% SDS, 20% glycerol, 2% (w/v) DTT and 6 M urea, 0.375 M Tris (pH 8.8), 2% SDS, 20% glycerol, 2.5% (w/v) iodoacetamide successively. Each IPG strip was positioned across the top of a Criterion Tris-Cl ReadyCast™ Gel (Bio-Rad). High molecular weight (HMW) proteins were separated with a 10% gel while low molecular weight (LMW) proteins were separated with a 10-20% gradient gel. Two microlitres of Precision Plus Prestained Protein Kaleidoscope Standards (Bio-Rad) were loaded as molecular weight markers. Second dimensional, polyacrylamide gel electrophoresis (PAGE) separation was carried out using a Criterion™ PreCast Gel System (Bio-Rad). PAGE was performed at 100 volts for 30 minutes followed by 200 volts for 40 minutes starting with a current of 90-120 mA/gel and finishing with a current of 35-55 mA/gel at completion. Gels were fixed in 50% methanol, 12% acetic acid, 0.05% formalin and stored at 4°C. All gels were run in duplicate from two independent protein extractions.

3.2.5 Visualization

Gels were visualized with a mass spectrometry (MS)-compatible silver staining protocol (Shevchenko *et al.,* 1996) using a Hoefer Processor Plus™ unit (GE Healthcare, Piscataway, NJ, USA). Briefly, gels were washed in 35% ethanol and sensitized with 0.02% $Na_2S_2O_3$. Gels were subsequently stained with a 0.2% $AgNO_3$, 0.076% formalin solution and finally developed with 6% Na_2CO_3, 0.05% formalin, 0.0004% $Na_2S_2O_3$. Development was halted by bathing the gels in 50% methanol containing 12% acetic acid.

3.2.6 Image Analysis

Duplicate gels were scanned on an Image Scanner™ (GE Healthcare) and visually examined for consistency. Comparative analysis of 2DE gel images was performed using Phoretix 2D™ Software (UBI, Calgary, AB, Canada). Following background subtraction of all gels within a suite (*i.e.* the 0, 8, 12 and 48 hours HMW gels from WT root tissue), spots were detected and manually edited. Gels were then aligned and corresponding spots were matched between gels. Spot volumes were assessed by *Total Spot Volume Normalisation* and the differential expression ($\Delta \pm 2$) was determined by comparing the 8, 12 and 48 hour treatment gels with the 0 hour control gel for each tissue/genotype combination.

3.2.7 Proteomic Analysis

Gel spots of interest from 8, 12 and 48 hour treatment gels were excised using a ProteomeWorks™ Spot Cutter (Bio-Rad) equipped with a 1.5 mm cutting probe. Excised spots were subjected to automated in-gel tryptic digestion using the MassPREP™ Robotic Digest Station (Waters, Mississauga, ON, Canada). Gel spots were de-stained, reduced with DTT, and alkylated with iodoacetamide prior to digestion with a sequencing grade modified trypsin (Promega, Madison, WI, USA). The resulting peptides were extracted from gel pieces, deposited into 96-well micro-titre plates, dried in a DNA 120 Speed Vac vacuum centrifuge (Thermo Scientific, Waltham, MA, USA) and reconstituted in 1% TFA (triflouroacetic acid).

The peptide digests were analyzed by reverse-phase nanoLC-MS/MS on a Q-Tof™ Ultima™ Global™ quadrupole-time of flight (Q-TOF) instrument (Waters) and CapLC (capillary liquid chromatography) system (Waters), using a 1-hr solvent gradient (8-45% acetonitrile and 0.2% formic acid), an injection volume of 6 µL, and a flowrate of 200 nL/min. Peptides were loaded onto a 350 µm x 0.5 cm, 5 µm Symmetry 300™ C_{18} trapping column (Waters) and washed of salts prior to separation along a 75 µm x 15 cm, 5 µm PepMap™ C_{18} analytical column (Dionex, Sunnyvale, CA, USA).

Data-dependent acquisition was carried out using a mass-to-charge (*m/z*) range of 400-1900 for MS and 50-1990 for tandem MS scans. Data were processed using MASCOT™ Distiller (Matrix Science, Boston, MA, USA) and searched against *Arabidopsis* protein

sequences within the NCBInr (National Center for Biotechnology Information: non-redundant) database using MASCOT™ Daemon (Matrix Science). Searches were performed using carbamidomethylation of cysteines and oxidation of methionines as fixed and variable modifications, respectively; peptide charge states of $^{+}2$ and $^{+}3$; and MS and MS/MS tolerances of 0.8 and 0.6 Da, respectively.

3.3 Results and Discussion

3.3.1 Copper Uptake and Plant Performance

WT and mutant *A. thaliana* plants were exposed to 30 μM Cu in a hydroponic medium. Over the 48 hour sampling period, WT shoot biomass decreased (Figure 3.4), a situation analogous to that reported by Drążkiewicz *et al.* (2004), who observed that the fresh weight of 7 day-old *A. thaliana* shoots decreased with increasing Cu concentration (up to 300 μM) in the growth medium. Likewise, following an initial increase from 0-8 hours, mutant shoot biomass also decreased while the biomass of roots from both genotypes remained relatively constant. Significantly more shoot and root tissue was produced in the mutant than in the WT in all cases except in shoots at 0 hours. Within genotype changes in biomass were not statistically different. No differences in overall plant health could be discerned visually between the two genotypes. However, after 48 hour exposure to Cu, shoot tissues in both genotypes began to show typical signs of Cu toxicity (necrosis, chlorosis and flaccid leaves) (Figure 3.5). The 30 μM Cu concentration is undoubtedly toxic to both genotypes as is evident in Figure 3.5. Exposure of plants to a drastic shock has proven to be an effective means for the study of many molecular mechanisms of plant stress response (Kwon *et al.,* 2007; Larkindale & Vierling, 2008; Yoshida *et al.,* 2008). Furthermore, exposure of plants to toxic levels of metal ions has also been shown to be an effective method for the investigation of plant responses to metal stress (Richards *et al.,* 1998). It follows then, that exposure to toxic metal concentrations can also be an effective means of examining the metal accumulation responses of plants.

Cu concentration in tissues was determined by atomic absorption spectroscopy (Figure 3.6). As expected, root and shoot Cu concentrations increased over the 48 hour sampling period.

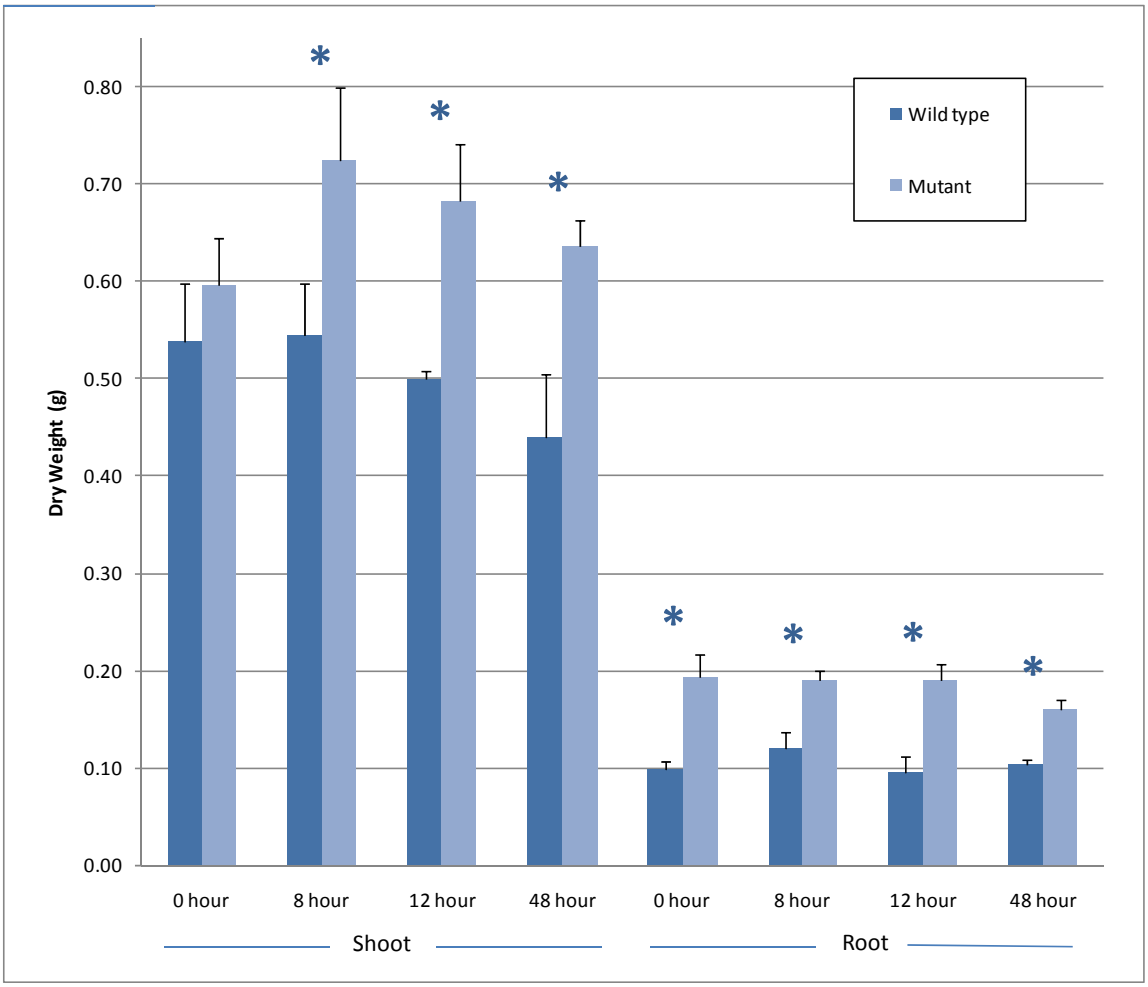

Figure 3.4: Dry weight comparison of two hydroponically-grown *A. thaliana* genotypes exposed to 30 µM Cu over a 48 hour timecourse. Data presented are means of 3 technical replicates. Error bars represent + standard deviation. Pairwise comparisons are made between genotype means of a particular tissue/timepoint combination. Significant differences (p=0.05) are indicated with ***.**

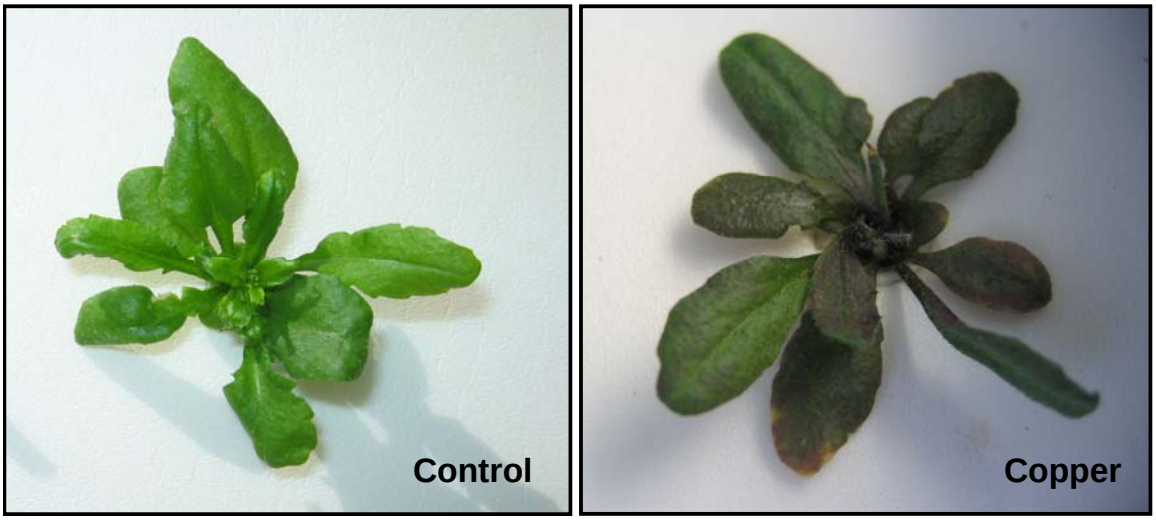

Figure 3.5: Example control and treated hydroponically-grown *A. thaliana* plants 48 hours post-inoculation of the growth medium with 30 μM Cu.

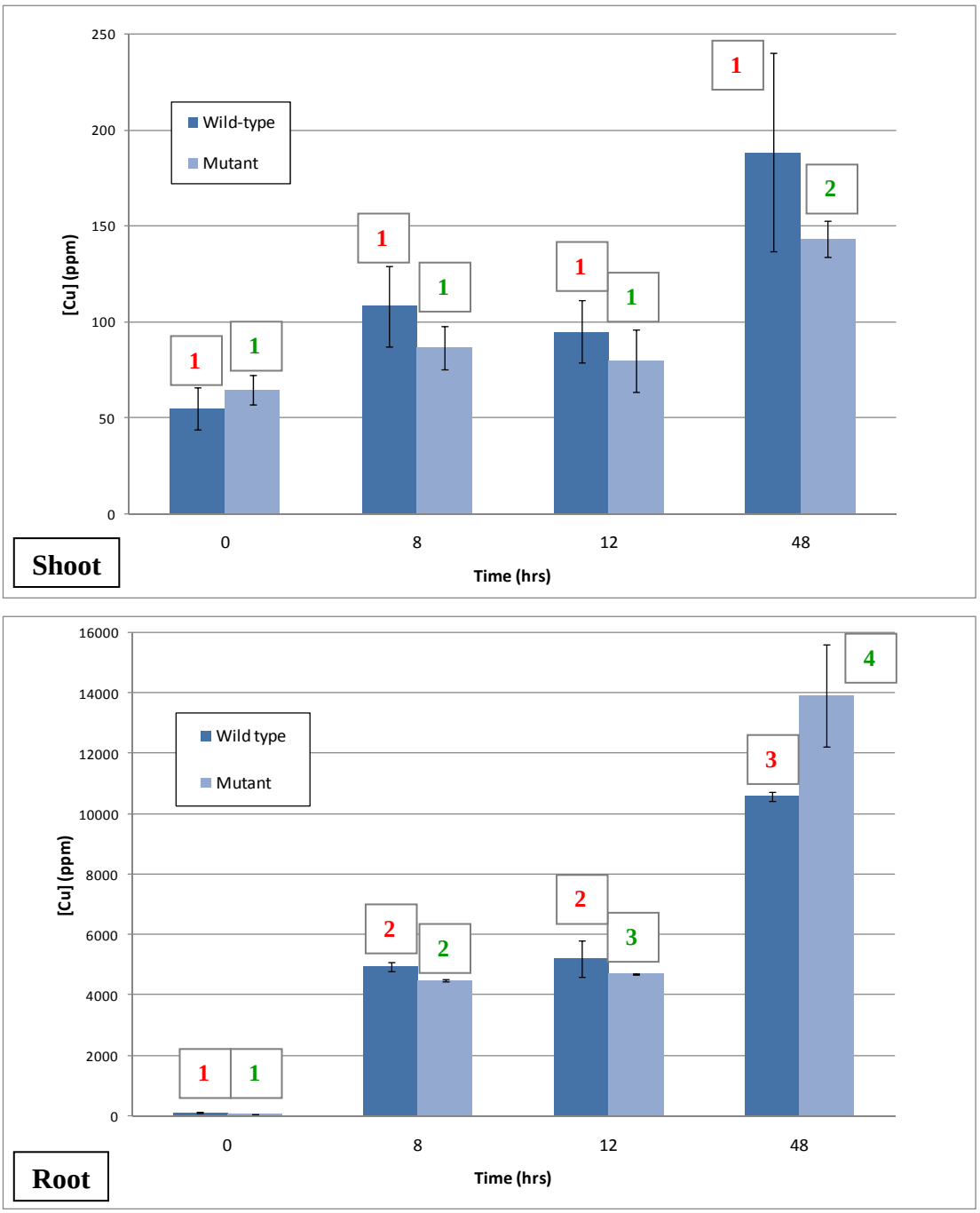

Figure 3.6: *In situ* Cu concentrations over time in roots and shoots of two hydroponically-grown *A. thaliana* genotypes exposed to 30 µM Cu over a 48 hour timecourse. Data presented are the means of three technical replicates and error bars represent ± standard deviation. Comparisons were made between all timepoints within a genotype/tissue series and significant differences (p=0.05) in the Cu concentrations over time are represented by different numbers above bars within a genotype/tissue series. Upper panel = shoot tissue, Lower panel = root tissue, Red denotes WT data series, Green denotes mutant data series.

In both genotypes, root Cu increased significantly from 0 – 8 hours and then remained steady over the next 4 hours. Concentrations again rose significantly from 12 – 48 hours. Cu concentrations in the shoots were numerically lower than those in the roots. Delhaize (1996) noted that when grown in soil, the frd3-3 mutant plants were smaller than the WT individuals grown alongside. The results presented above however, demonstrate that the mutant produced more biomass than the WT, despite the fact that both genotypes were grown side-by-side under identical conditions. This is in agreement however, with the results of Tsang *et al.* (1996) who noted that Cu toxicity manifested itself in non-tolerant plants by amongst other symptoms, reduced plant growth. Perhaps the greater biomass in the mutant is an indication that the mutant is more tolerant than the WT of Cu at high concentrations in the growth medium. From the *in situ* Cu concentrations (Figure 3.6), it appears that the WT, a non-accumulator, had higher Cu concentrations than the mutant. This is somewhat surprising as the mutant, regardless of growth medium (soil, hydroponics) is reported to accumulate more metal than WT (Delhaize, 1996). However, upon examining the per plant Cu concentration (Table 3.1), it is evident that the mutant accumulated more Cu than the WT and is therefore in agreement with previous reports (Delhaize, 1996). The shoot-to-root ratios of Cu concentration (Table 3.2) indicate that there may be less translocation of Cu to the shoots by the mutant. These results suggest that the mutant may inhibit translocation of metals from roots to shoots. This is re-enforced by the overall Cu concentration in which the only significant difference in Cu concentration between the two genotypes occurs after 48 hour exposure, at which point the mutant roots contain more Cu than WT roots and the WT shoots more Cu than the mutant roots, a result consistent with inhibited translocation of Cu in the mutant. If the mutant is in fact more tolerant than the WT of high concentrations of Cu, as is suggested by the greater biomass (Figure 3.4), an effective mechanism for this may be the inhibition or mediation of translocation of metals ions to the shoots. This hypothesis provides a useful framework for interpreting the results of the proteomic experiments to identify proteins that are differentially expressed upon exposure of WT and mutant plants to Cu.

Table 3.1: Average per plant Cu concentration (μg) in two genotypes of hydroponically-grown *A. thaliana* exposed to 30 μM Cu over a 48 hour timecourse.

	0 Hour	8 Hour	12 Hour	48 Hour
Wildtype	4.20	65.45	56.79	114.24
Mutant	5.20	92.05	95.01	232.09

Table 3.2: Shoot-to-root ratios of Cu concentration in two genotypes of hydroponically-grown *A. thaliana* exposed to 30 μM Cu over a 48 hour timecourse.

	0 Hour	8 Hour	12 Hour	48 Hour
Wildtype	2.42	0.10	0.09	0.08
Mutant	2.94	0.07	0.06	0.04

3.3.2 Proteomic Analysis

3.3.2.1 2D Gel Analysis

Global proteome surveys for each genotype/tissue/timepoint combination were performed using equal amounts of total protein to facilitate normalization and comparison of results. The resulting gels (Figures 3.7 to 3.14) show clearly the dynamic proteomic responses of the two *A. thaliana* genotypes to Cu exposure over time. Despite repeated washing of the protein pellets by acetone precipitation to remove unwanted contaminants such as excess salts, polysaccharides, lipids and nucleic acids, some gels suffered from problems with protein streaking. However, protein spot boundaries were still discernible and subsequent protein identification was still achieved with adequate confidence. Protein extracts from each combination of genotype/tissue/timepoint were separated using two polyacrylamide gels of differing compositions to maximize the resolution of low and high molecular weight proteins. Duplicate gels made from the independent protein extracts of independent pooled tissue samples showed similar spot patterns (Figure 3.15) and were reproducible for a given sample and gel composition. Select corresponding spots from duplicate gels were excised, digested and analyzed as for those gels in Figures 3.7-3.14. Comparison of the MS data from duplicate gels shows very good agreement with respect to proteins identified including like peptide sequences matched and MOWSE scores (Appendix 1) demonstrating clearly the reproducibility of the global proteomic analysis undertaken here. The probablility-based MOWSE algorithm (Pappin *et al.,* 1993) used by MASCOT™ Daemon, reports protein scores based on peptide matches where scores above a calculated significance threshold are considered to be non-random events (Matrix Science, 2007). Additionally, the lists of proteins identified from co-localized spots between gels from different timepoints but within a genotype/tissue suite (Figure 3.16) were similar (Table 3.3) further re-enforcing the confidence in the reproducibility of the gels.

Following Cu inoculation, the total number of resolved protein spots increased between 0 and 8 hours in all tissues except WT roots (Table 3.4). The number of spots then decreased between 8 and 48 hours in all tissues again with the exception of WT roots. An initial induction of overall protein expression in response to Cu exposure was expected and, with the exception of WT roots, the observed increase in the number of protein spots mirrors the increase of *in situ* Cu

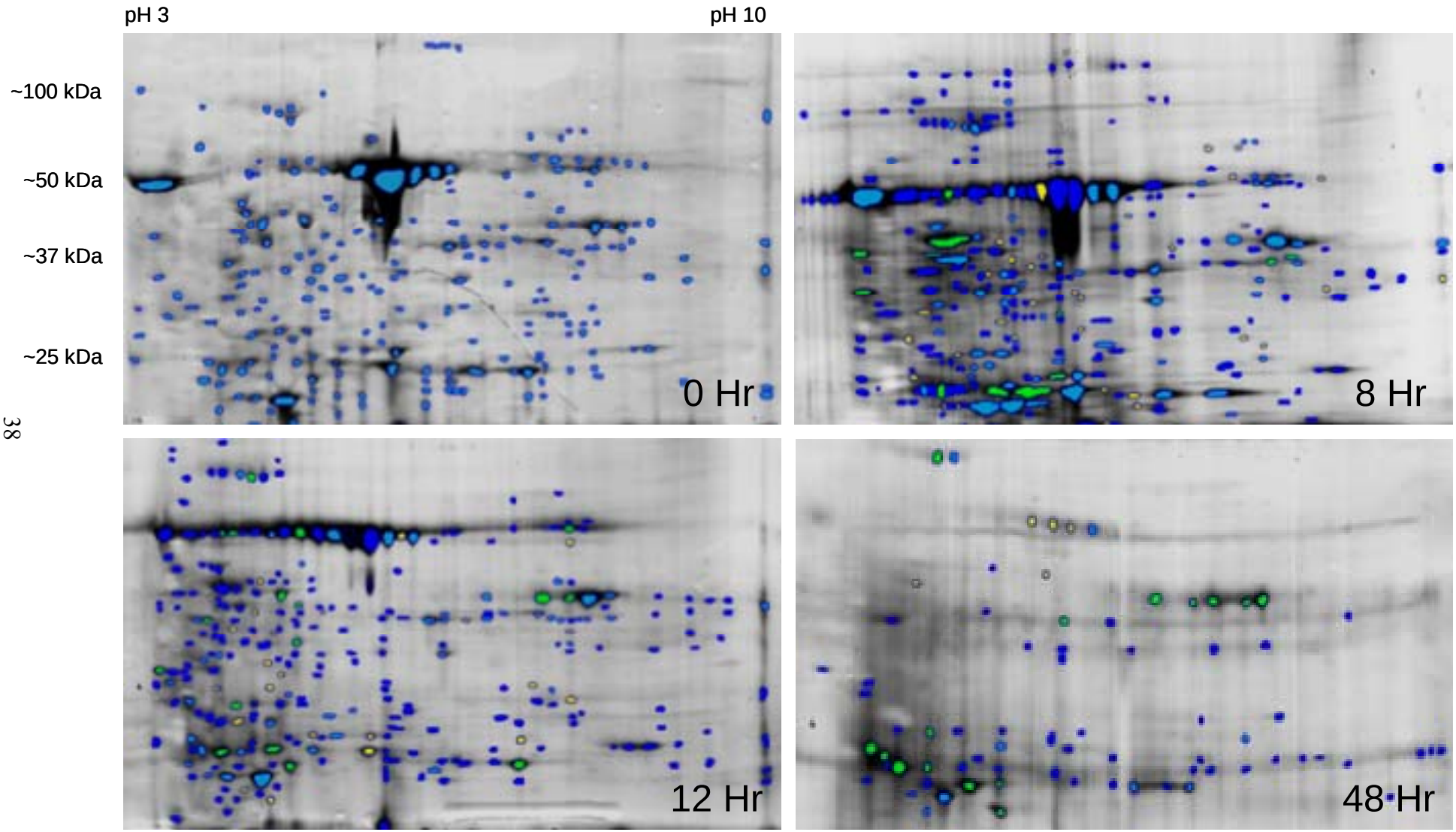

Figure 3.7: Differential expression of resolved HMW proteins from extracts of hydroponically-grown WT *A. thaliana* shoots in response to copper over time. ● – unmatched protein spot; ● - upregulated protein spot; ● - downregulated protein spot; ● - non-selected protein spot.

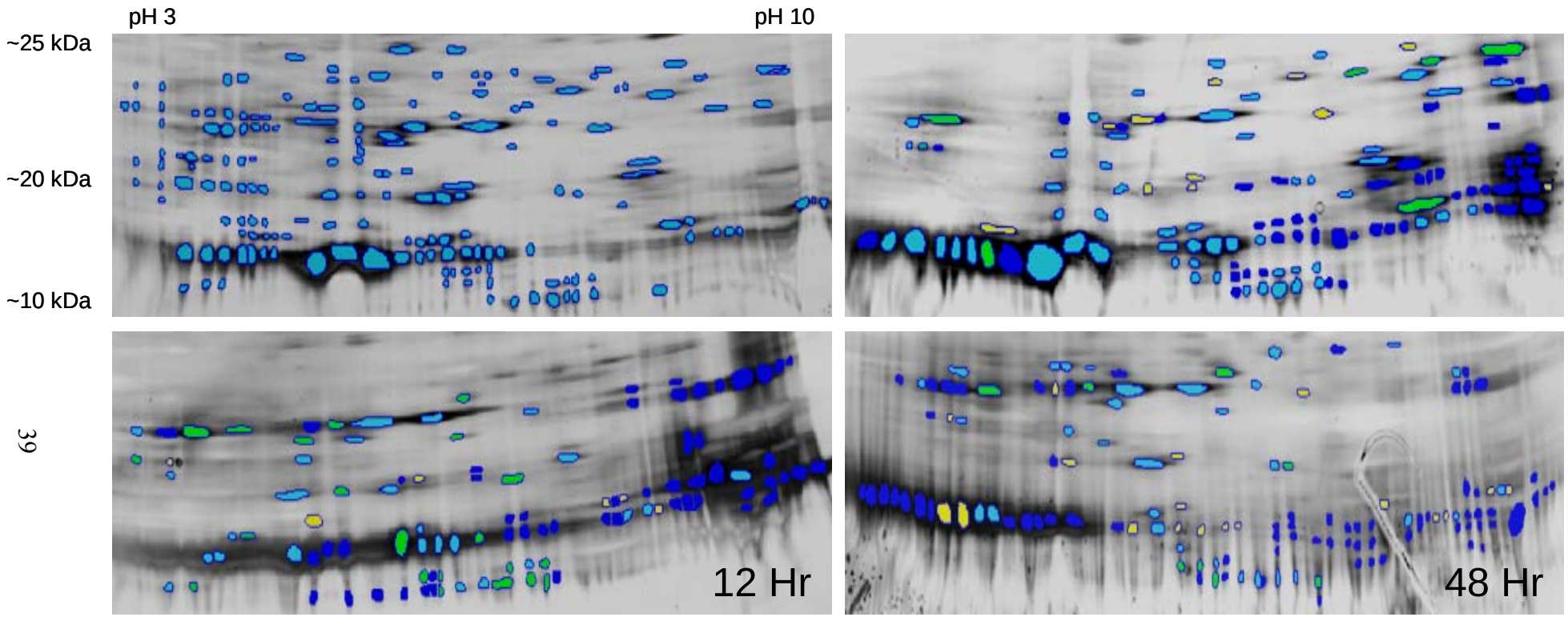

Figure 3.8: Differential expression of resolved LMW proteins from extracts of hydroponically-grown WT *A. thaliana* shoots in response to copper over time. – unmatched protein spot; - upregulated protein spot; - downregulated protein spot; - non-selected protein spot.

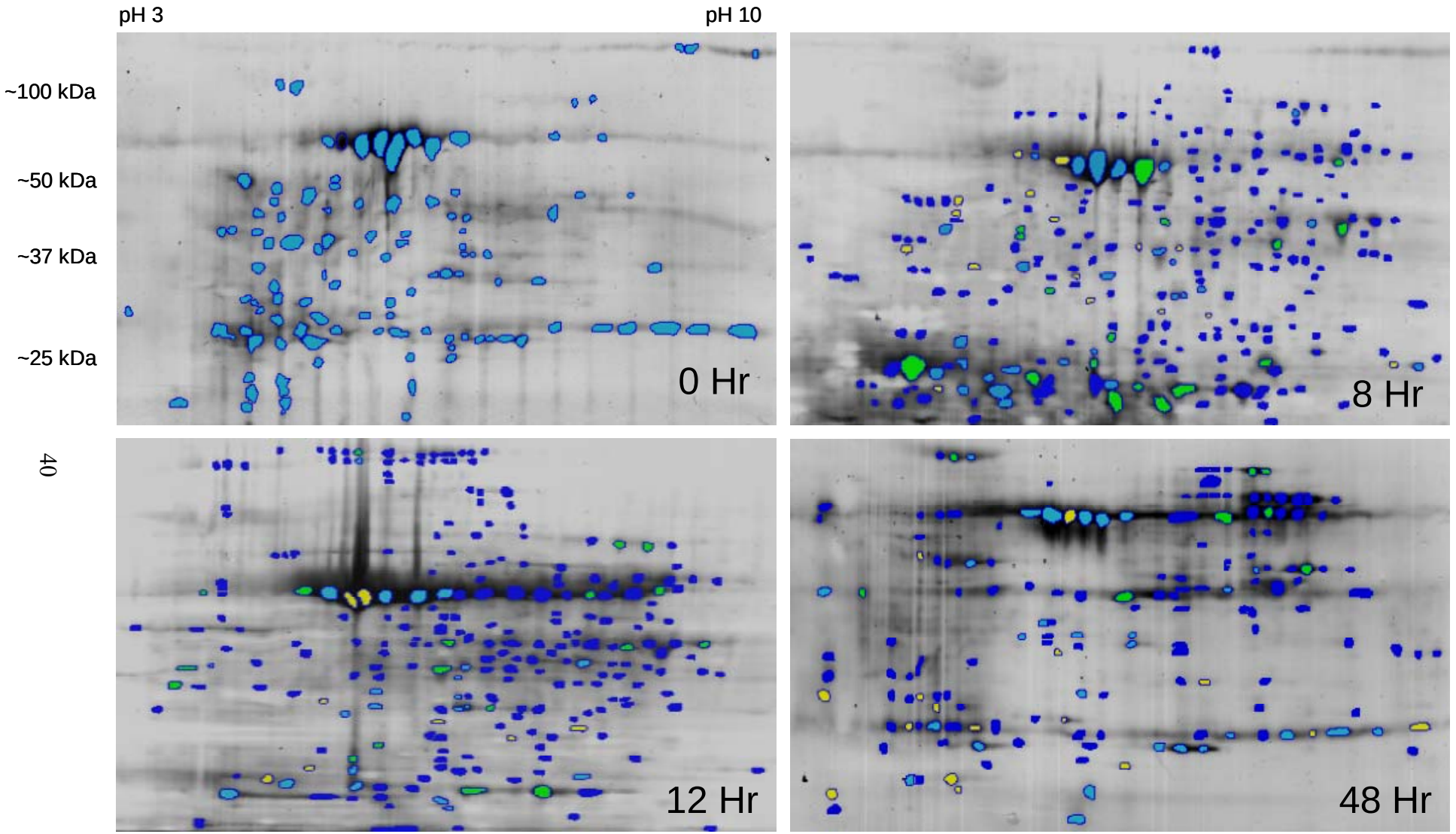

Figure 3.9: Differential expression of resolved HMW proteins from extracts of hydroponically-grown mutant *A. thaliana* shoots in response to copper over time. – unmatched protein spot; - upregulated protein spot; - downregulated protein spot; - non-selected protein spot.

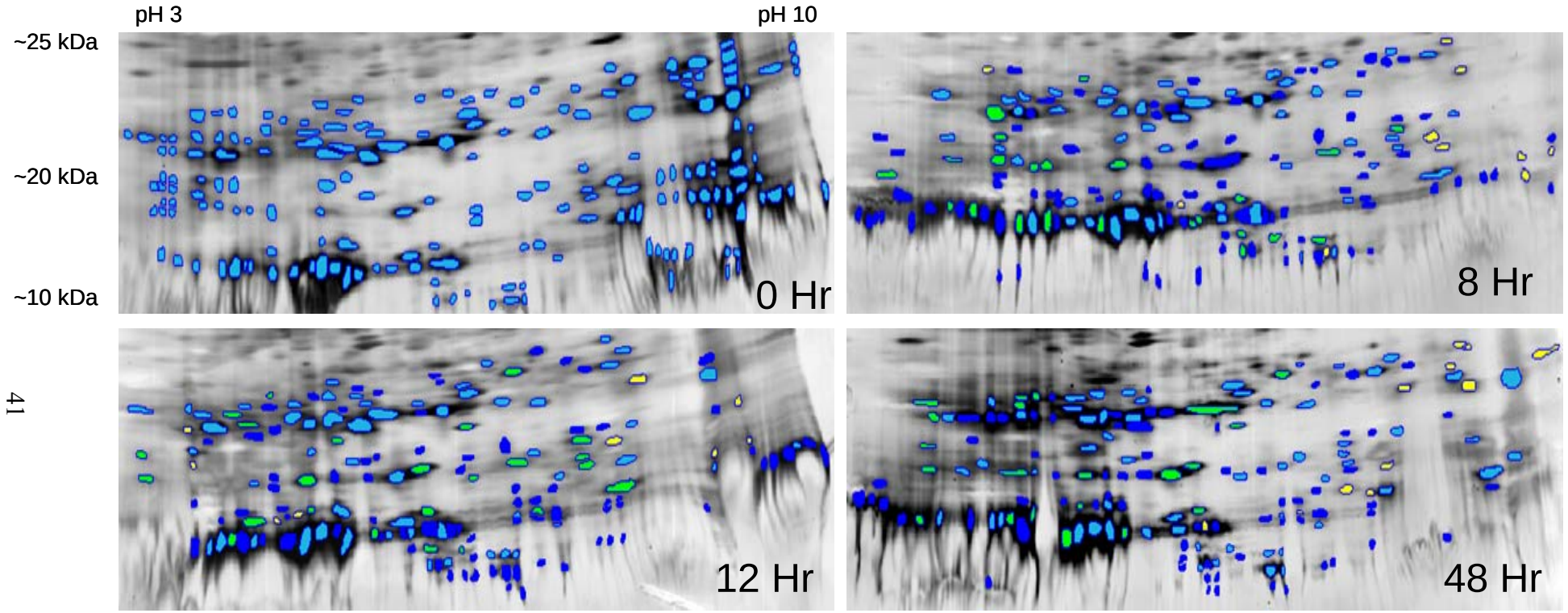

Figure 3.10: Differential expression of resolved LMW proteins from extracts of hydroponically-grown mutant *A. thaliana* shoots in response to copper over time. – unmatched protein spot; - upregulated protein spot; - downregulated protein spot; - non-selected protein spot.

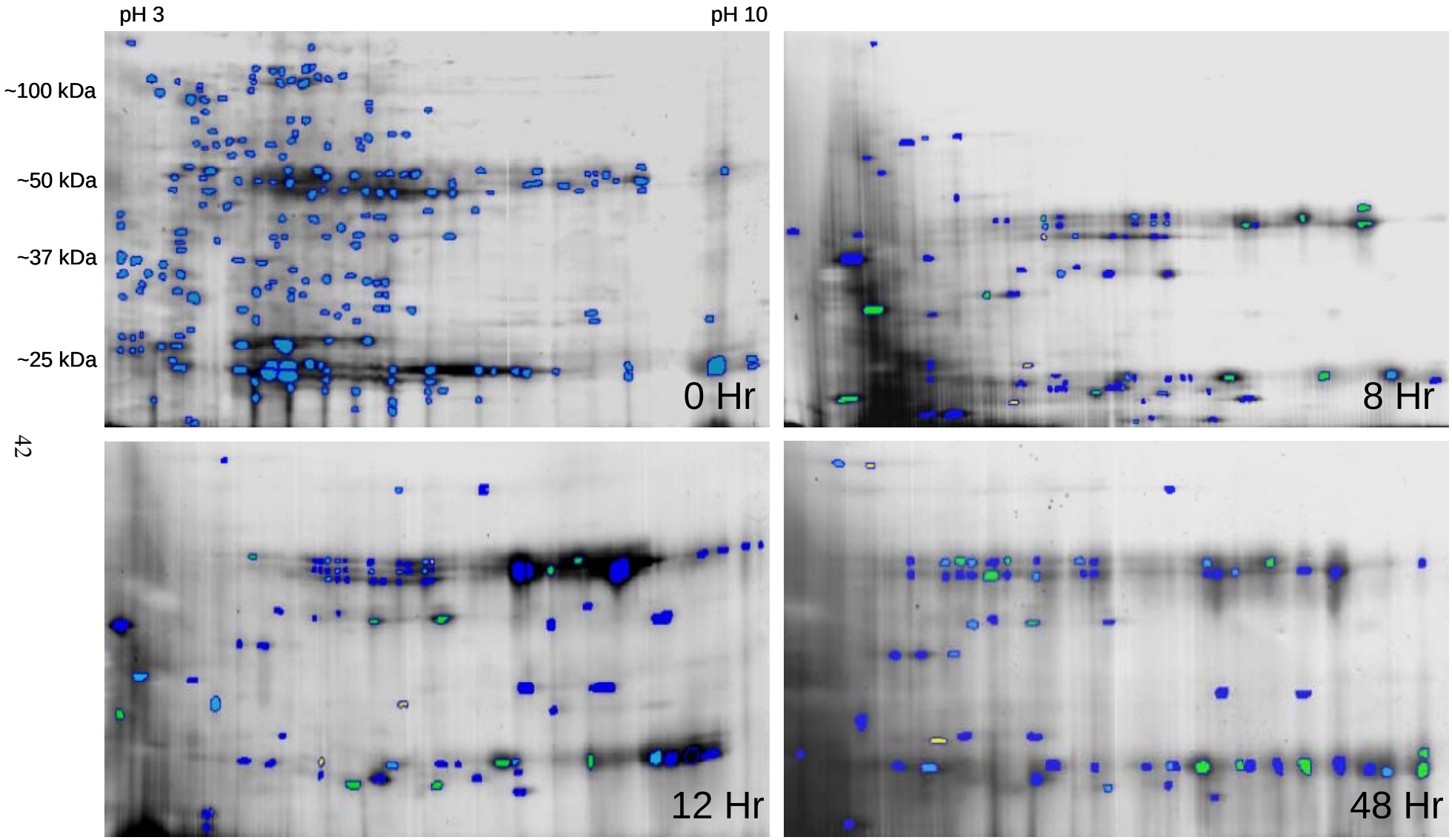

Figure 3.11: Differential expression of resolved HMW proteins from extracts of hydroponically-grown WT *A. thaliana* roots in response to copper over time. – unmatched protein spot; - upregulated protein spot; - downregulated protein spot; - non-selected protein spot.

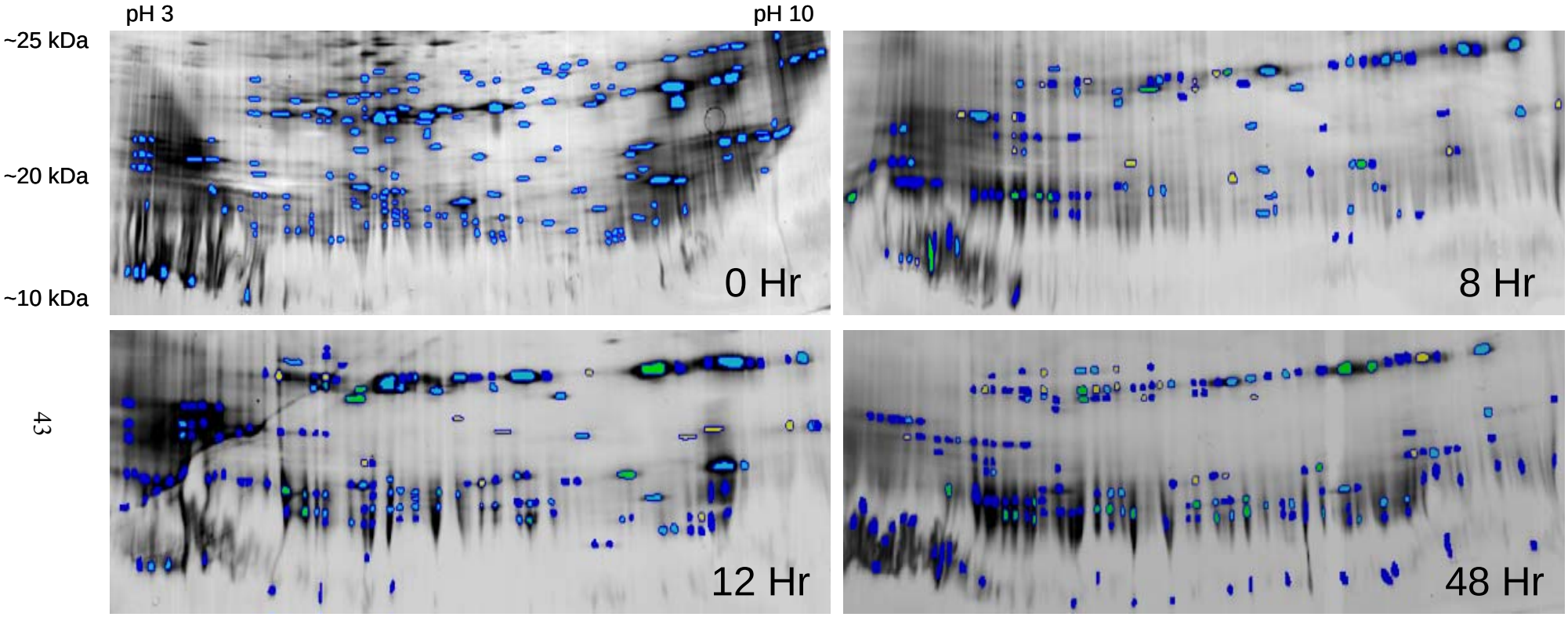

Figure 3.12: Differential expression of resolved LMW proteins from extracts of hydroponically-grown WT *A. thaliana* roots in response to copper over time. ● – unmatched protein spot; ● - upregulated protein spot; ● - downregulated protein spot; ● - non-selected protein spot.

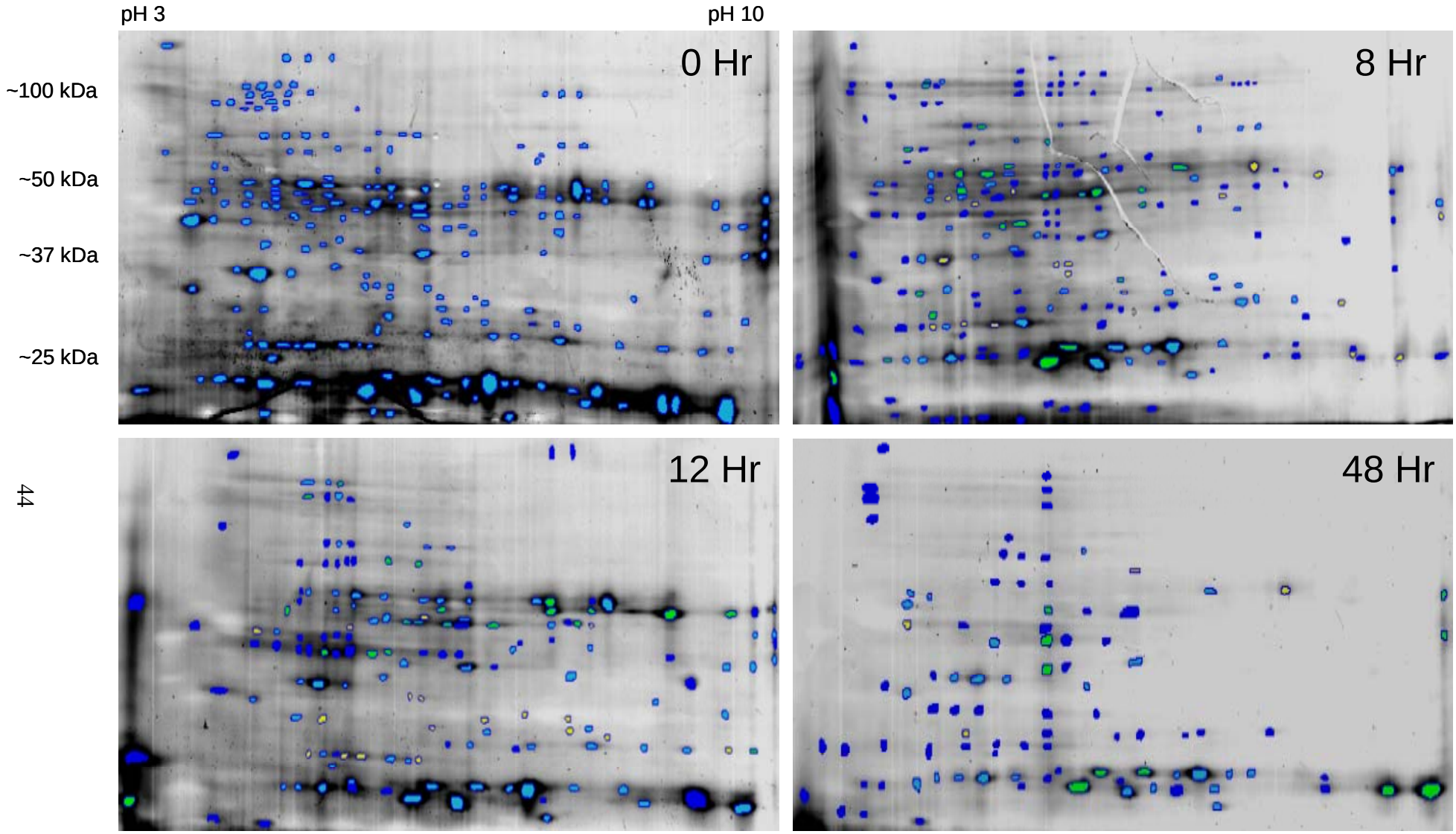

Figure 3.13: Differential expression of resolved HMW proteins from extracts of hydroponically-grown mutant *A. thaliana* roots in response to copper over time. ● – unmatched protein spot; ● - upregulated protein spot; ● - downregulated protein spot; ● - non-selected protein spot.

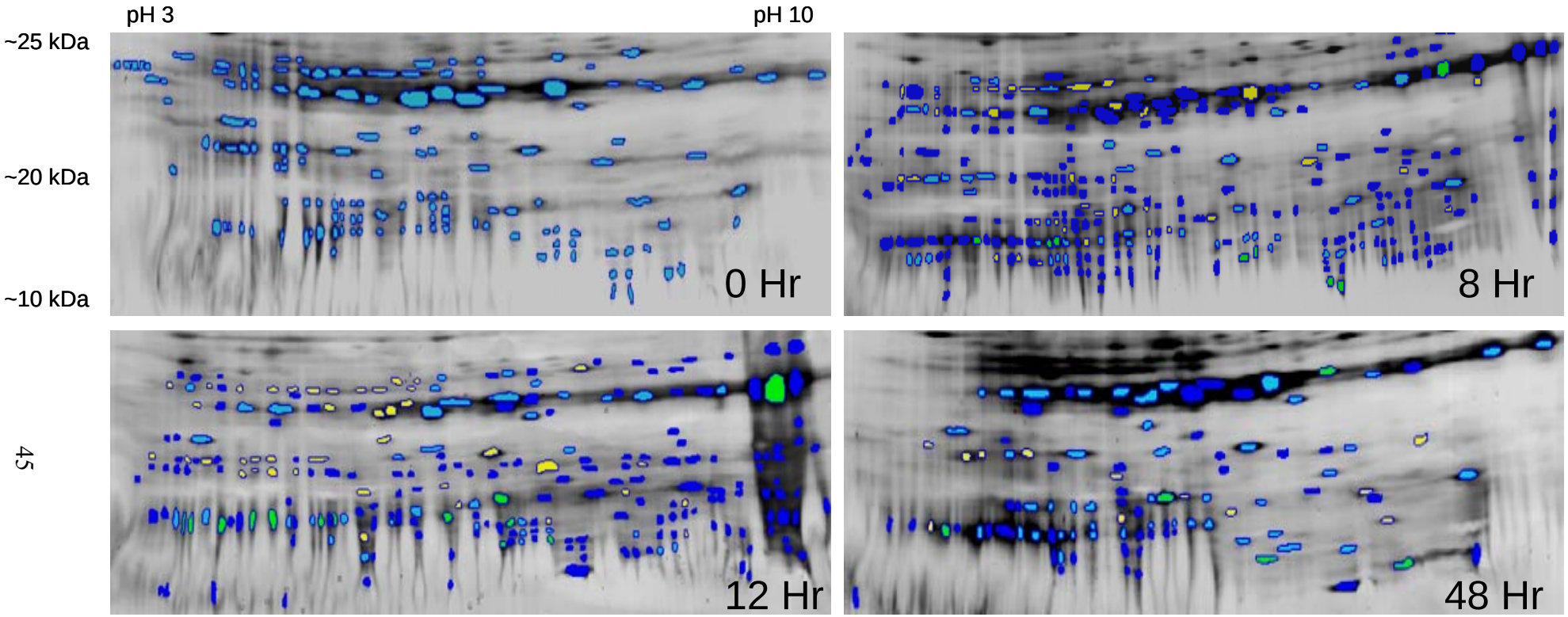

Figure 3.14: Differential expression of resolved LMW proteins from extracts of hydroponically-grown mutant *A. thaliana* roots in response to copper over time. – unmatched protein spot; - upregulated protein spot; - downregulated protein spot; - non-selected protein spot.

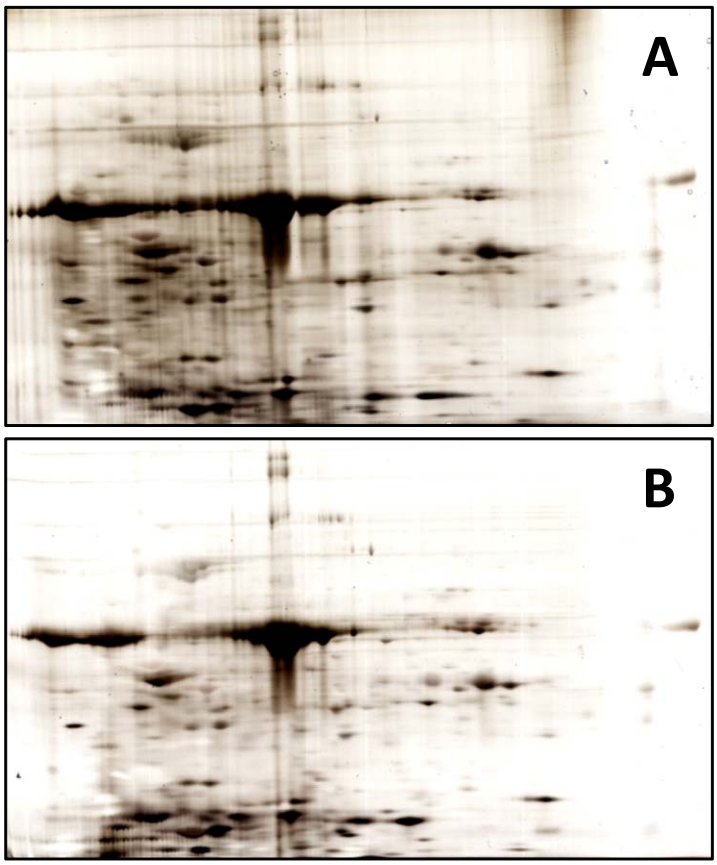

Figure 3.15: Example duplicate 2DE gel images of soluble proteins resolved from independent protein extracts of independent pooled samples of hydroponically-grown WT *A. thaliana* shoots following an 8-hour exposure to Cu.

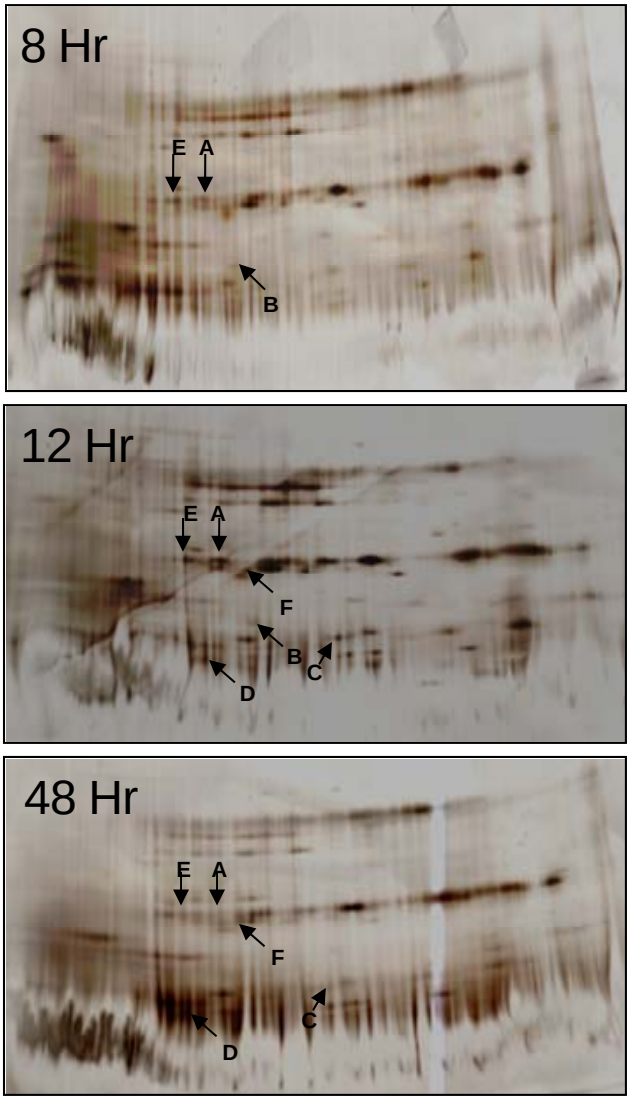

Figure 3.16: 2DE gels of protein extracts from hydroponically-grown WT *A. thaliana* roots exposed to 30 μM Cu over a 48 hour timecourse. Lettered arrows indicate co-localized protein spots through time. Identified proteins are listed in Table 3.3.

Table 3.3: MS data for co-localized protein spots isolated from 2DE gels of soluble protein extracts from hydroponically-grown WT *A. thaliana* roots following exposure to 30 μM Cu over a 48 hour timecourse. Spot ID correlates to lettered arrows indicated on Figure 3.16; MOWSE Threshold = the score over which an identified protein is considered a non-random event; Score = the individual protein score for the protein identified through MASCOT™ Deamon; # Pep. Matched = the number of unique peptides identified in the protein sequence; % Cov. = percentage of the total identified protein sequence represented by the identified peptides.

				8 Hour			12 Hour			48 Hour		
Spot ID	**Accession #**	**Protein ID**	**MOWSE Threshold**	**Score**	**# Pep. Matched**	**% Cov.**	**Score**	**# Pep. Matched**	**% Cov.**	**Score**	**# Pep. Matched**	**% Cov.**
A	gi|15241583	nutrient reservoir	37	42	1	3	55	2	6	68	2	6
B	gi|30693971	universal stress protein	37	66	1	8	206	5	43			
C	gi|322551	nucleoside-diphosphate kinase	36				241	4	36	51	1	1
D	gi|9294558	unnamed protein product	37				76	3	23	176	4	30
E	gi|8778432	glutathione-S-transferase	37	121	4	10	141	3	7	160	4	10
	gi|15241583	nutrient reservoir	37	50	2	6	75	2	6	65	2	6
F	gi|18202452	germin-like protein subfamily T member 1 precursor	37				147	2	10	158	2	10
	gi|4098968	germin-like protein	37				40	1	4	50	1	4

Table 3.4: Total number of protein spots resolved on 2DE gels from total protein extracts of roots and shoots of two hydroponically-grown *A. thaliana* genotypes in response to 30 µM Cu over time.

	0 Hr	8 Hr	12 Hr	48 Hr
WT Shoot	398	428	390	204
WT Root	399	191	250	246
Mutant Shoot	299	440	414	374
Mutant Root	370	507	423	210

concentration (Figure 3.6). A recent study found the global proteomic response of *A. thaliana* suspension cell cultures to 24-hour cadmium exposure to be a pattern of protein induction (Sarry *et al.,* 2006). Likewise, several other studies have also observed an increase in protein abundance in response to heavy metal exposure either over 24 hour or 48 hour periods (Ingle *et al.,* 2005a; Requejo & Tena, 2005; Roth *et al.,* 2006). The general decrease in protein expression at 48 hours can be attributed to the high levels of Cu *in situ* and the resulting inhibitory effects on protein biosynthesis (Dameron & Howe, 1998). The initial drop and subsequent stabilization in the number of proteins expressed in WT roots following addition of Cu are consistent with the susceptibility of the non-accumulating genotype to the toxic effects of Cu at the initial site of exposure. In contrast, the high number of proteins expressed in mutant roots at 8 hours suggest a rapid induction of certain proteins in response to Cu exposure, with WT and mutant shoots showing a similar response in terms of the number of additional protein spots expressed during the first 8 hours of exposure. The number of proteins expressed in mutant shoots remains higher than in WT shoots even as both numbers decrease over time, despite the fact that more proteins were expressed in WT than in mutant shoots prior to Cu exposure. This, together with the relatively slow decline in the number of proteins in mutant roots over time, suggests that the proteome of the frd3-3 mutant is indeed altered from the WT state and suggests a modified response to elevated metal concentrations.

3.3.2.2 Functional Categorization of Identified Proteins

To identify those proteins that are most likely to be involved in the plants' responses to Cu exposure, protein spots in 8, 12 or 48 hour gels that showed differential expression when compared to the corresponding control (0 hour) gel were selected for trypsin digestion and analysis by nanoLC-MS/MS. The proteins in each spot were subsequently identified by database searching. Spots of molecular weight greater than approximately 23kD on the LMW gels were not selected for further analysis as they had already been accounted for in the analysis of the HMW gels. Differentially expressed proteins included spots matched to control gel spots that changed in relative abundance by a factor of ±2. Visible spots on the 8, 12 and 48 hour gels that could not be matched to the corresponding control gel were also included for analysis as

thesewere judged to have been differentially expressed by at least a factor of +2. Identified proteins were then grouped into seven categories (Table 3.5) based on function.

Where possible, proteins were categorized according to GO (gene ontology) classification. In cases where GO annotations were not available, proteins were categorized according to putative functions as listed by the NCBI (National Center for Biotechnology Information), UniProtKB/Swiss-Prot (The Universal Protein Resource) or InterPro protein databases. Proteins with multiple functions were categorized by the function of highest specificity. For example, a protein identified as cobalamin-independent methionine synthase was placed in the *sulfur metabolism* category, although it also has functionality that could place it in the *protein modification* or the *defense* categories.

A protein "master-list" was created for each of the four genotype/tissue combinations. These lists contained all proteins identified in all analyzed gel spots selected from the 8-, 12- and 48-hour gels. As such, the master-lists are representative of all the differentially expressed proteins identified in each genotype/tissue during Cu exposure, categorized according to protein function. These master-lists provide an overview of differential expression within the plant proteome and a framework for the interpretation of the global changes in the functional metabolism of the plant in response to Cu exposure.

A total of 3242 and 2666 proteins were identified in differentially expressed WT and mutant shoot gel spots respectively (Appendix 2 and 3). The majority of these proteins (89% in the WT and 92% in the mutant) were induced. Several proteins were identified in multiple spots, and probably represent multiple isoforms of the same protein. Other proteomic studies have noted multiple spots corresponding to the same protein (Dai *et al*., 2006; Sheoran *et al.,* 2007; Wan *et al.,* 2007). As isoforms can represent different stages of control and post-translational modification of a particular protein, all identified isoforms were included in the master-lists. The use of appropriate protease inhibitors during protein extraction further suggests that the majority of the identified isoforms are true isoforms and not simply the degradation products of larger proteins. The master-lists of the WT and mutant shoots are remarkably similar (Figure 3.17) in terms of functional classification, implying similar responses to Cu exposure that reflect the comparable and relatively low Cu concentrations found in WT and mutant shoot tissues as opposed to roots (Figure 3.6). Approximately half of the proteins identified were involved in

Table 3.5: Description of the seven protein function categories used in this study.

Category Name	Protein Function
Protein Modification	Protein and amino acid synthesis and assembly.
General Metabolism	Proteins involved in photosynthesis and carbohydrate and energy metabolism.
Transport and Storage	Proteins involved in inter- and intra-cellular transport and storage.
Defense	Proteins involved in plant defense, stress response, metal binding and signaling.
Sulfur Metabolism	Proteins involved in the metabolism of sulfur.
Unknown	Proteins with no known function.
Other	Proteins of known function that do not belong to the previous functional categories.

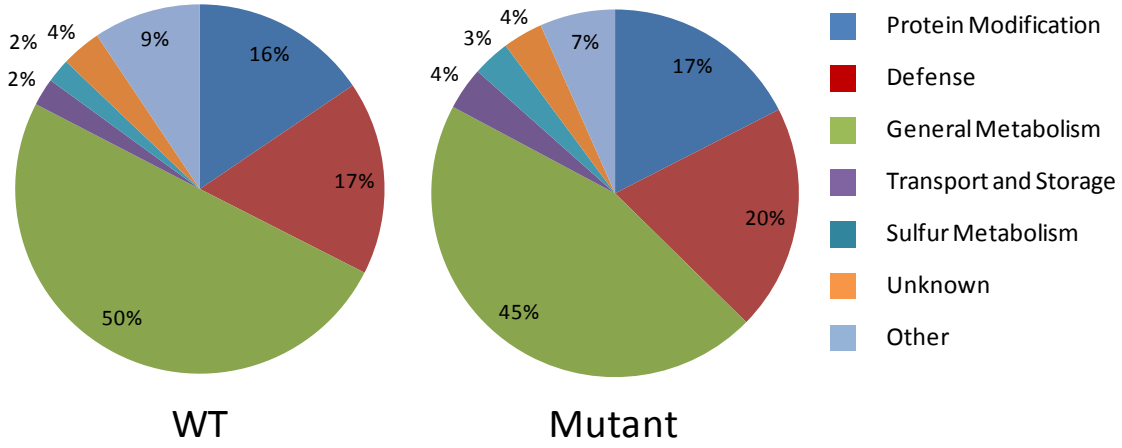

Figure 3.17: Functional categorization of proteins in shoot master-lists for both *A. thaliana* genotypes.

general metabolism, with *defense* and *protein modification* each accounting for approximately 20%. About 10% of proteins in the master-lists were classified as having functions *other* than those specified in the rest of the categories. Proteins with *sulfur metabolism, transport and storage* and *unknown* functions comprised the remainder of the shoot master-lists, being more or less equally divided between these remaining categories.

As with the shoot master-lists, the total number of proteins included in the WT and mutant root master-lists were similar between genotypes: the number of differentially expressed proteins being 651 and 573, respectively (Appendix 4 and 5). Again, most of these (91% in the WT and 88% in the mutant) were induced. Unlike the shoots, however, these root master-lists showed significant differences between the WT and mutant in terms of protein distribution between functional categories (Figure 3.18). This is consistent with the immediate exposure of the roots to Cu in the hydroponic growth medium, as opposed to the delayed exposure of the shoot tissues, and the correspondingly rapid, and contrasting responses from the two different genotypes.

The distributions of proteins belonging to the *protein modification* and *other* categories were roughly similar between WT and mutant roots. The main divergence between genotypes was in the proportions of *defense, general metabolism* and *transport and storage* proteins. In the WT, 25% of the identified differentially expressed proteins were members of the *transport and storage* category whereas these represented only 7% of all root proteins in the mutant. The balance (approximately 20%) appears to be distributed evenly between the *defense* and *general metabolism* categories, with 11% fewer *defense* proteins and 10% fewer proteins attributed to *general metabolism* in the WT. Another notable difference was observed within the *sulfur metabolism* category, with 4% representation in the mutant and only 1% in the WT.

The genetic background of the two genotypes is very similar; differing in only one locus, implying that the mutant phenotype results largely from altered regulation of mechanisms inherent to the WT. The metal accumulation phenotype of the mutant is consistent with the increase in differential expression of defense and sulfur metabolism proteins in roots of the mutant relative to WT, whereas repression of transport proteins in the mutant is consistent with the lower level of metal translocation indicated by total Cu measurements in root and shoot tissues (Figure 3.6; Table 3.2). Since roots are the first point of contact for heavy metal exposure, it appears that the mutant may control the expression of transport and storage proteins

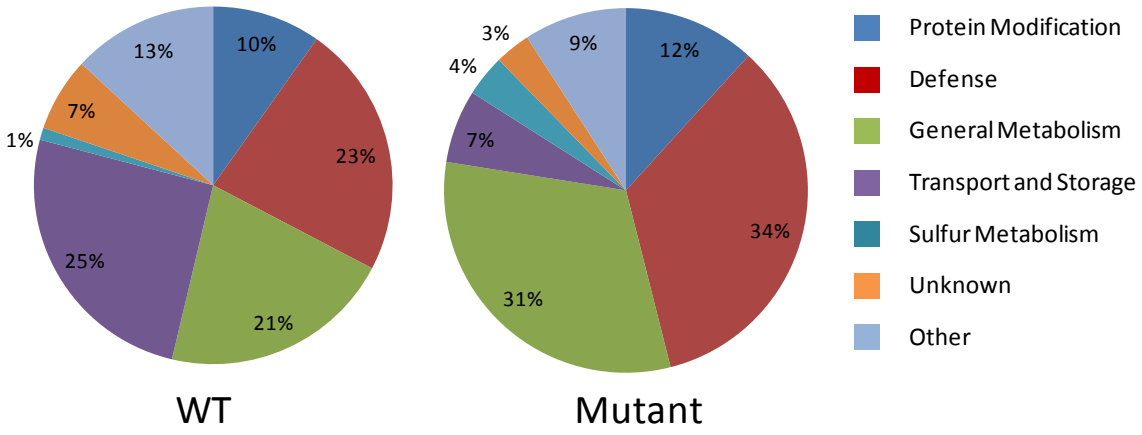

Figure 3.18: Functional categorization of proteins in root master-lists for both *A. thaliana* genotypes.

in the roots to moderate Cu uptake and limit intracellular levels of Cu in the shoots. This in turn would allow the defense mechanisms to counter the elevated metal concentration, thereby avoiding or delaying toxicity. This could also explain why the functional proportions in the shoots between the two genotypes are similar; if the roots of the mutant limit the uptake and more importantly, the delivery of metal ions to the shoots then the WT distribution of metabolically-active proteins between the various functional groups does not need to be modified in the mutant.

When comparing the root master-lists with those of the shoots, the main difference appears to be the number of differentially expressed proteins involved in *general metabolism*. The proportions of these proteins were much larger in shoots than in roots, as expected based on the number of proteins involved in photosynthesis alone. For example, the thylakoid membrane contains more than 100 proteins (van Wijk, 2006) and the chloroplast lumen may contain up to 80 different proteins (Kieselbach & Schroder, 2003). This expected difference demonstrates the validity of this approach to carrying out a global protein survey of these two tissue types. It also imparts validity to the differences observed between genotypes and at different timepoints, as discussed below.

The master-lists provide an integrated overview of the plants' responses to Cu exposure at the proteome level. However, the investigation of those proteins that were induced in root and shoot tissues of each genotype over time, was of particular interest since (a) most of the differentially expressed proteins identified were induced with respect to the control timepoint, and (b) protein induction is a clear indication of plant response, whereas apparent repression could be an artifact of the inhibitory effect of Cu toxicity on overall protein biosynthesis, especially at later timepoints. Again, the identified proteins were grouped into functional categories (Table 3.5), and the number of proteins in each category at each timepoint expressed as a percent of the total number of induced proteins observed in that genotype/tissue during the entire experiment (*i.e.* over all timepoints), effectively normalizing the data and thus allowing for a direct comparison between different timepoints. The *not represented* category represents the percentage of the total number of induced proteins of that genotype/tissue that were not observed in the timepoint under discussion. Because all induced proteins (including all isoforms) were used for data normalization and because it is the proportional changes that are being compared,

any over-representation of specific proteins due to possible inclusion of redundant isoforms is accounted for.

The total number of induced proteins in WT shoots decreased over time (Figure 3.19), as indicated by the increasing size of the *not represented* category (60% at 8 hours to 86% at 48 hours), the most notable change occurring between 12 and 48 hours (Δ 23%). The overall distribution of induced shoot proteins between different functional categories is similar between genotypes, and interestingly, the relative proportions of these categories were remarkably similar in both genotypes at each timepoint. Considering the similarity of the shoot master-lists, it seems that little difference exists between the shoots of the two genotypes either in functional protein representation or in the induction of those proteins.

Unlike the shoots, the induction pattern of root proteins changed considerably over time with respect to total numbers and categorical representation (Figure 3.20). In the mutant, the greatest amount of protein induction occurred at 8 hours (45%), the number of induced proteins subsequently decreasing steadily over time. In the WT, however, the greatest level of protein induction occurred at 12 hours (44%), with slightly fewer proteins showing induction at 8 hours than at 48 hours. This is consistent with the increase (from 191 to 250) in the number of differentially expressed proteins identified in WT roots between 8 and 12 hours, during which period the number of such proteins identified in all other tissues decreased (Table 3.4).

The distributions of proteins by function in mutant and WT roots at each timepoint also differed from one another, with major changes occurring in different functional categories depending on the genotype. In the mutant, for example, the number of induced proteins in the *protein modification* category fell between 12 and 48 hours, whereas induction of such proteins in the WT increased slightly between 8 and 12 hours and then decreased again between 12 and 48 hours, a trend mirrored by all protein categories in the WT. The largest changes occurred in the *protein modification, general metabolism* and *other* functional categories, with moderate changes in the *defense* and *transport and storage* categories, and minor changes in the *unknown* and *sulfur metabolism* categories. In the mutant, large changes in protein numbers were observed between 8 and 12 hours in the *defense, general metabolism*, *transport and storage* and *other* categories.

Perhaps the two most interesting differences between WT and mutant roots are those in the *defense* and *transport and storage* categories. The mutant, at 8 hours, had a much higher

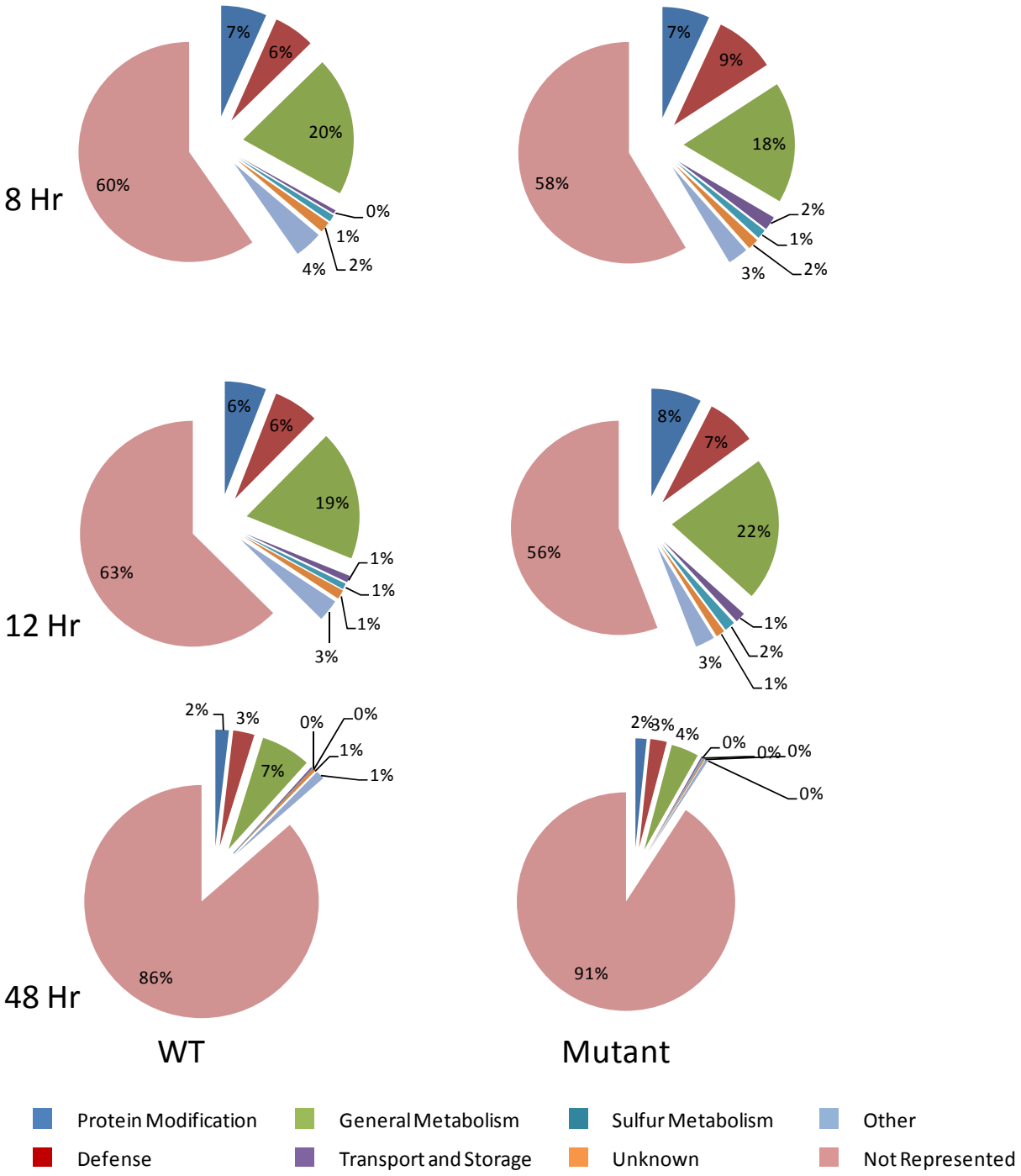

Figure 3.19: Functional categorization of induced shoot proteins in both *A. thaliana* genotypes.

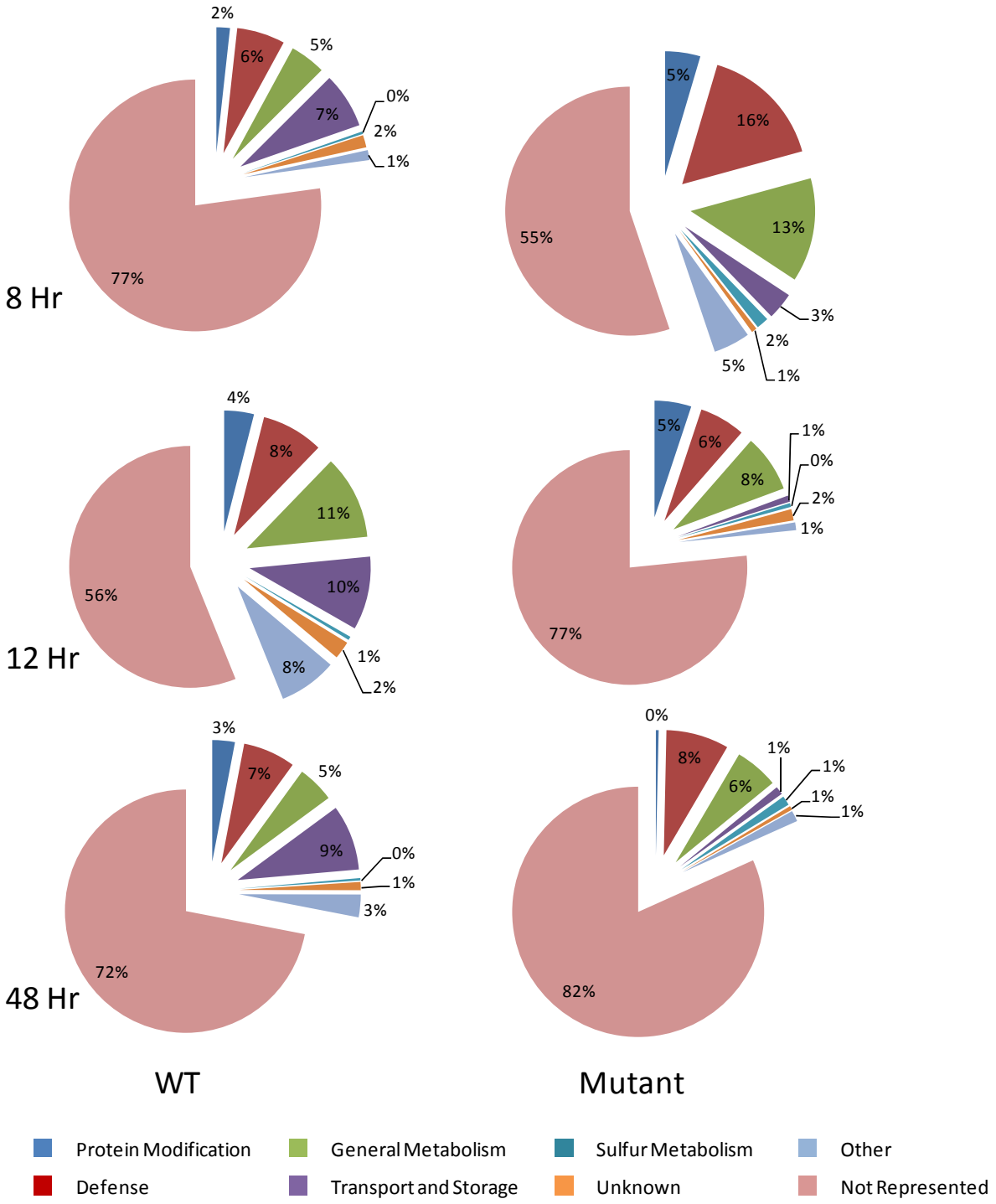

Figure 3.20: Functional categorization of induced root proteins in both *A. thaliana* genotypes.

proportion of induced *defense* proteins than the WT (16% and 6% respectively). Even comparing the WT roots at 12 hours with the mutant roots at 8 hours, when the total number of induced proteins in the two genotypes was similar, the proportion of *defense* related proteins in the WT was lower (8%) compared with that in the mutant at 8 hours (16%). Conversely, the proportion of *transport and storage* proteins in the WT was higher in all timepoints (7%, 10% and 9%) than in the mutant (3%, 1%, and 1%). Because it is the roots that initially encounter heavy metals, it is likely that the management of root proteins would be different between a metal accumulator and a non-accumulating plant. The metal accumulating phenotype could prepare the plant system for elevated metal exposure by having a high initial complement of defense- and stress-related proteins. Although this may seem energetically wasteful, this "state-of-readiness" would afford the plant a quick response, accounting for the apparent lack of adverse effects (*e.g.* the greater biomass of the mutant plants) despite elevated internal concentrations of Cu. Another possible compensatory mechanism of the mutant phenotype is the general repression of *transport and storage* proteins in the root. This would provide a means of regulating any increase in intracellular Cu resulting from exposure, allowing other protein-based defense mechanisms (*e.g.* chelation) to deal with free Cu ions and prevent adverse effects. This could explain why the WT roots and shoots had higher Cu concentrations after 8 and 12 hours exposure than the mutant plant, despite the fact that the mutant plants produced more biomass.

In WT roots, the number of induced proteins increased from 8 to 12 hours, whereas in mutant roots the greatest extent of induction occurred during the first 8 hours. The apparent delayed response in the WT plant makes sense given that the resources required for synthesis of defense, stress and metal binding proteins and/or their precursors in anticipation of metal exposure would be energetically costly, whereas this modification might be expected in the mutant. Upon encountering elevated Cu concentrations, however, an induction of such proteins could be expected in WT roots, the time required for biosynthesis accounting for the apparent lag in response compared with the mutant.

3.3 Conclusion

The comparison of the functionally-characterized global proteomes of two genotypes of *A. thaliana* that differ in their reported accumulation of metals, has allowed for interpretations of

the differential protein responses that could be at least partially responsible for the metal accumulation mechanism in plants. Not unexpectedly, it appears that the proteins involved in defense reactions and transport and storage are particularly involved in the mechanism as a whole. Further investigation into these specific categories of proteins and the control of these proteins may provide further insights into this complex mechanism.

Based on visual observation, Cu toxicity likely began to affect plants of both genotypes between 24 and 48 hours of exposure in this study. It is perhaps due to this that the extent of the reported differences in metal accumulation between these two genotypes was not fully observed. Possibly the reported differences in metal accumulation between WT and mutant are more fully demonstrated when plants are not undergoing extreme exposure. Consequently, future work should include the study of these genotypes subsequent to lower levels of Cu exposure (*e.g.* 1, 2, 5, 10 μM). An investigation of the time involved for various concentrations of Cu to cause toxicity and/or lethality in conjunction with the proteomic response would also provide interesting insights into the metal accumulation phenomenon.

Although small format gels have been used successfully in proteomic profiling and comparative proteomic studies in plants (Watson *et al.,* 2003; Dani *et al.,* 2005; Franklin et al., 2005; Sheoran *et al.,* 2005; Yan *et al.,* 2005, 2006; Hashimoto & Komatsu *,* 2007), the use of larger format gels would likely improve the resolution of more proteins into individual spots. In turn, this could simplify protein identification and interpretation. Alternatively, or in combination with larger format gels, the use of more sophisticated 2DE gel analysis techniques such as DIGE (differential in-gel electrophoresis), that allow for a more simplified and co-ordinated comparison of proteomic responses to various treatments, could prove extremely valuable. Finally, although protein precipitation by acetone is an acceptable and well-used method of washing protein samples (Watson *et al.,* 2003; Jiang *et al.,* 2004; Méchin *et al.,* 2004; Sheoran *et al.,* 2005; Kung *et al.,* 2006), alternative precipitation methods or the use of commercial 2D clean-up kits, exist and have recently become common. The efficacy of desalting methods can be affected by organism, tissue type and tissue age and is largely guided by downstream analyses (Rose *et al*., 2004). In future studies, optimization of desalting/precipitation methods could likely improve or alleviate problems with protein streaking and again facilitate downstream identification efforts.

4.0 ISOLATION AND IDENTIFICATION OF COPPER-BINDING PROTEINS IN COPPER-TREATED *ARABIDOPSIS THALIANA* GENOTYPES

4.1 Introduction

Due to anthropogenic activities such as mining, agriculture and the burning of fossil fuels, heavy metals are accumulating in soils and waters to levels that threaten environment quality and potentially, human health. Phytoremediation, the use of plants for the reclamation of contaminated sites, has been proposed as a mechanism to alleviate contaminated environments. An emerging technology, phytoremediation is a passive, cost-effective method that requires few inputs and enjoys a high level of public approval. Through the use of hyperaccumulators, plants capable of concentrating heavy metals in their tissues, metal contaminated soils and waters can be reclaimed to acceptable levels (Salt *et al.,* 1998; McGrath *et al.,* 2002).

Plants require metals as essential micronutrients (Marschner & Romheld, 1995; Salt *et al.,* 1995) however, metal concentrations in excess of the natural requirements can lead to toxicity and so their homeostatic levels must be tightly controlled (Cunningham *et al.,* 1995; McGrath *et al.,* 1995; Garbisu & Alkorta, 2001). Plants therefore produce a range of proteins and other compounds for the acquisition, translocation, chelation and sequestration of metal ions within the plant (Colangelo & Guerinot, 2006). Recently, a number of specific highly-expressed metal transporters and chelators have been identified in hyperaccumulating plant species (Himelblau *et al.,* 1998; Lasswell *et al.,* 2000; Maser *et al.,* 2001; Baxter *et al.,* 2003; Delhaize *et al.,* 2003; Andrés-Colás *et al.,* 2006). However, while significant progress has been made regarding the identification and physiological characterization of hyperaccumulating species, the molecular and biochemical basis for this phenomenon remains largely unknown (Ingle *et al.,* 2005a). Many hyperaccumulating plants are closely related to non-accumulating species, implying that regulation and differential expression of genes and proteins common to both species are likely responsible for much of the hyperaccumulation effect (Cobbett, 2003). Furthermore, single mutations such as in the *frd3* gene in *Arabidopsis thaliana* is reported to produce a general enhancement in metal accumulation (Delhaize, 1996; Rogers & Guerinot, 2002), suggesting that changes in the expression of several genes and proteins are involved.

Proteomics, which involves the large-scale identification and expression analysis of individual proteins, facilitates the examination of the actively translated part of the genome

(Ingle *et al.,* 2005). The majority of proteomic studies to date have focused on the description of proteomes of organelles, sub-cellular fractions and tissues (Kamo *et al.,* 1995; Santoni *et al.,* 1999; Chivasa *et al.,* 2002; Calikowski *et al.,* 2003; Herranen *et al.,* 2004; Giavalisco *et al.,* 2005). Proteomics however, can also be an effective tool for the comparison of proteomes in response to different environmental stimuli such as metal stress (Leymarie *et al.,* 1996; Ingle *et al.,* 2005a; Roth *et al.,* 2006). By examining the differential protein response of plants to metal stress, insights into the mechanisms of metal homeostasis, transport, and detoxification may be achieved. Additionally, by comparing the proteomic responses of accumulating and closely related non-accumulating plants, the hyperaccumulation response can be described more completely.

Immobilized metal affinity chromatography (IMAC) was originally developed as a tool for the fractionation of biological samples (Porath *et al.,* 1975). It can also be an efficient means of selectively enriching a specific metalloproteome; that is, a sub-proteome consisting of proteins with affinity for a particular metal ion immobilized by chelation to a solid support (Smith *et al.,* 2004; Sun *et al.,* 2005). By exploiting the selectivity afforded by IMAC, the expression of selected metalloproteins in response to different stimuli can be examined. In this study, copper(II)-IMAC was used to examine the differential expression of proteins with an affinity for copper in two *A.thaliana* genotypes exposed to divalent copper (Cu) in the growth medium over a timecourse.

Two genotypes of *A. thaliana* (the metal accumulating frd3-3 mutant, and the closely related non-accumulating wildtype (Columbia)) were grown hydroponically and exposed to copper over a 48 hour period. Cu(II)-IMAC was used to isolate copper-binding proteins from total protein extracts of root and shoot material collected from mutant and wildtype (WT) plants harvested following different periods of copper exposure (0, 8, 12 and 48 hours). Following separation of the IMAC samples by one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (1D SDS-PAGE), the predominant gel bands were excised, treated with trypsin, and the proteins in each trypsin digest identified by nanoLC-MS/MS using the MASCOT™ search engine and the NCBInr protein sequence database.

4.2 Materials and Methods

All reagents, unless otherwise noted were obtained from Sigma (St. Louis, MO, USA), and all solutions prepared using Milli-Q water (Millipore, Billerica, MA, USA).

4.2.1 Plant Material

Hydroponically-grown *Arabidopsis thaliana* (Columbia ecotype and frd3-3 mutant) plants were grown in sequential growth chamber experiments under identical conditions as described previously (*Section 3.2.1 Plant Material and Growth Conditions*). Root and shoot material was collected at pre-determined timepoints (0, 8, 12 and 48 hours) for tissue-specific analysis of Cu-binding proteins. Like-tissues from Growth Experiments #2 and #3 (Figure 4.1) were combined as outlined in *Section 3.2.1 Plant Material and Growth Conditions* and used for protein extractions.

4.2.2 Protein Extraction

Root and shoot tissues were ground in liquid nitrogen with a mortar and pestle. Total protein was extracted according to Murphy *et al.* (1997) with modifications. Approximately 5-6 g of tissue was combined with 0.25g polyvinylpolypyrrolodone (PVPP) and 18 mL of ice-cold homogenization buffer (0.25 M sucrose, 20 mM Tris (pH 8.2), 5 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 µg/mL leupeptin). Samples were homogenized 3 times for 2 minute intervals and 5 minute incubations on ice using a PRO200 homogenizer (Pro Scientific, Oxford, CT, USA). The homogenate was strained through 4 layers of cheesecloth to remove cellular debris and centrifuged (16,000 x *g*, 15 minutes, 4^{o}C). Supernatant was volume adjusted to 20 mL with homogenization buffer. Ice-cold ethanol (24 mL) and chloroform (2 mL) were added to the supernatant and gently mixed prior to centrifuging (500 x *g*, 30 minutes, 4^{o}C). The supernatant was again decanted and 2 volumes of ice-cold ethanol were added. The sample was incubated at -20^{o}C for 20 minutes and centrifuged (6,000 x *g*, 20 minutes, 4^{o}C). The supernatant was removed and the remaining pellet was air-dried to remove residual ethanol. The

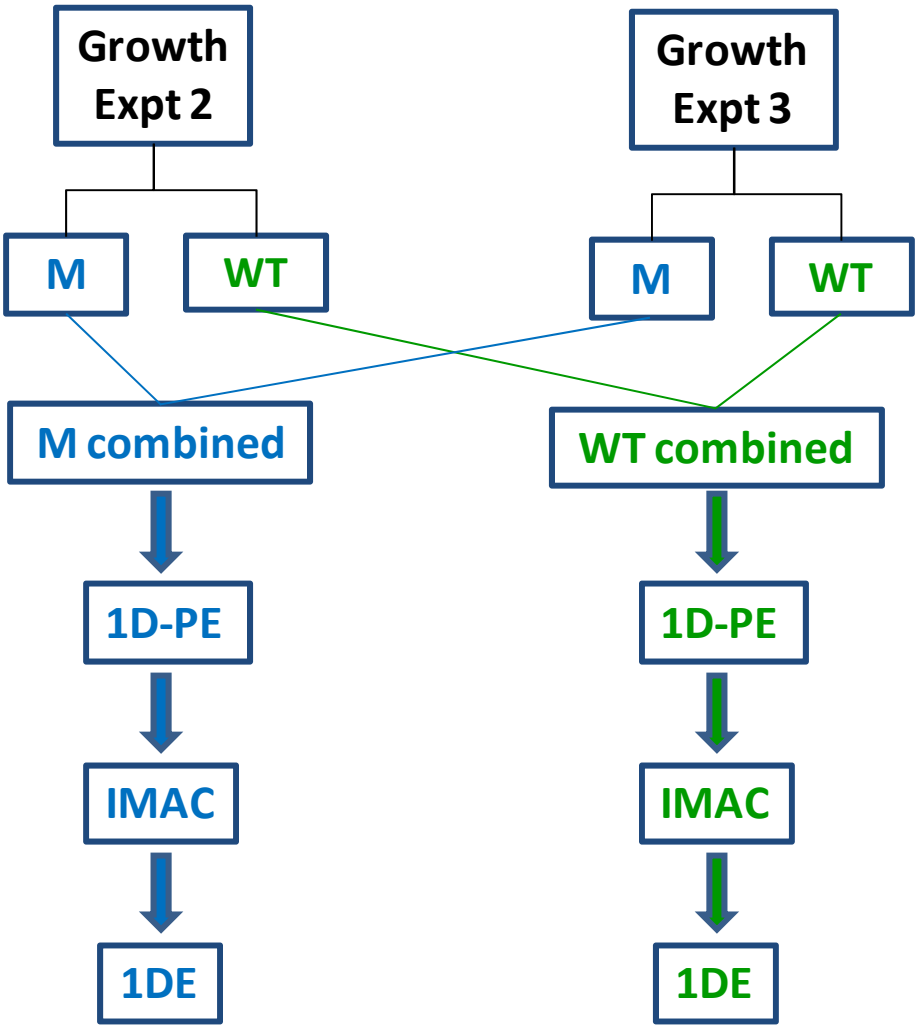

Figure 4.1: Schematic representation of sequential hydroponic growth experiments and the subsequent experimental procedures. M = mutant; WT = wildtype; 1D-PE = protein extraction for 1DE gels. Mutant tissues and wildtype tissues produced in Growth Experiment #2 were combined with like-tissues produced in Growth Experiment #3.

pellets were re-suspended in 2 mL of 100 mM DTT, 0.2% CHAPS, and 50 μM ethylenediaminetetraacetic acid (EDTA), centrifuged (100,000 x *g*, 15 minutes, 4°C), the supernatants decanted and the amount of recovered protein determined using the Bradford Assay (Bradford, 1976).

4.2.3 Enrichment of Copper-Binding Proteins

Individual protein samples, each representing a specific genotype, tissue and timepoint combination were desalted using PD-10 Desalting Columns (GE Healthcare) and IMAC column buffer (10 mM HEPES (pH 7.8), 0.5 M $(NH_4)_2SO_4$). Cu(II)-IMAC (Porath *et al.,* 1975) was performed using 5 mL of Chelating Sepharose™ Fast Flow (GE Healthcare) loaded into 10-mL gravity-fed Poly-Prep Chromatography Columns (Bio-Rad) capped with frits (Figure 4.2). The packing was rinsed with 6 column volumes (cv's) of Milli-Q water and charged with 8 mL of 10mM $CuSO_4$. The column was then washed with 2 cv's of water to remove any unbound Cu and equilibrated with 4 cv's of IMAC column buffer. The desalted protein extract was then loaded onto the column and washed with 4 cv's of column buffer to remove any non-specifically bound proteins. Cu-bound proteins were eluted by the addition of 5.5 mL of elution buffer (10 mM HEPES (pH 7.8), 0.5 M $(NH_4)_2SO_4$, 50 mM EDTA (pH 7.8)). Samples were desalted, concentrated and free Cu was removed using YM-3 Centricon Tubes (Millipore) according to manufacturer's instructions. The amount of recovered protein was determined using the Bradford Assay (Bradford, 1976).

4.2.4 Analysis and Identification of Copper-Binding Proteins

Normalized amounts of Cu(II)-IMAC-enriched protein extracts were separated by 1D SDS-PAGE using a Criterion PreCast Gel System (Bio-Rad) and 10-20% Tris-Cl ReadyCast Gradient Gels (Bio-Rad). Running conditions were 200V for 55 minutes with a starting current range of 90-120 mA/gel and a current of 35-55 mA/gel at completion. Gels were fixed overnight at 4°C in 50% aqueous methanol containing 12% acetic acid and 0.05% formalin. Subsequently gels were silver stained with a MS-compatible silver staining protocol (Shevchenko *et al.,*

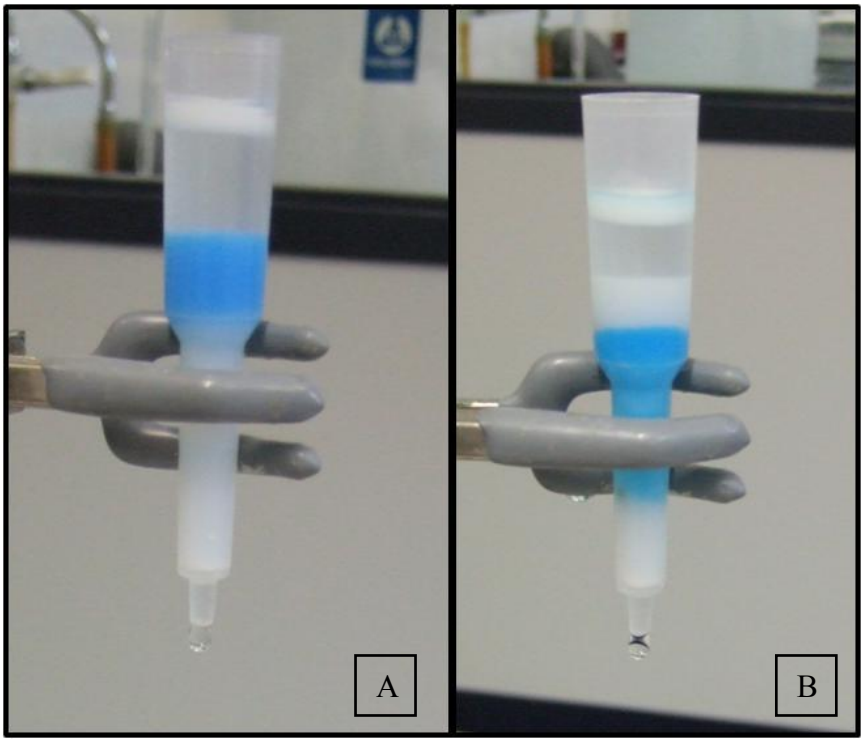

Figure 4.2: Cu(II)-IMAC column demonstrating (**A**) the charged pre-sample application stage and (**B**) a partially-eluted stage.

1996) using a Hoefer Processor Plus™ (GE Healthcare) as detailed previously (*Section 3.2.5 - Visualization*). Visible bands were excised by hand and subjected to an in-gel tryptic digestion protocol using a MassPrep system (Waters) as detailed in *Section 3.2.7- Proteomic Analysis)*. Peptide digests were dried in a DNA 120 Speed Vac vacuum centrifuge (Thermo Scientific) and reconstituted in 6 µL of 1% triflouroacetic acid (TFA).

The peptide digests were analyzed by reversed-phase nanoLC-MS/MS using a Q-Tof™ Ultima™ Global™ quadrupole-time of flight (Q-TOF) instrument and nano-ACQUITY™ ultra-performance liquid chromatography (UPLC) system (Waters). Peptide samples (6 µL) were separated using a flowrate of 400 nL/min and a 1 hour solvent gradient (10-45% acetonitrile and 0.1% formic acid). Peptides were desalted using a Waters Symmetry™ C_{18} trapping column (180 µm x 20 mm, 5 µm particle size) and separated on a Waters BEH130 C_{18} analytical column (100 µm x 100 mm, 1.7 µm).

Data-dependent acquisition using a *m/z* range of 400-1900 for MS and 50-1990 for MS/MS scans was employed. Data were processed using MASCOT™ Distiller (Matrix Sciences) and searched against *Arabidopsis* protein sequences within the NCBInr database using MASCOT™ Daemon (Matrix Sciences). Searches were performed with MS and MS/MS tolerances of 0.8 and 0.6 Da, respectively; fixed and variable modifications of carbamidomethylation of cysteines and oxidation of methionines respectively; and peptide charge states of +2 and +3.

4.3 Results and Discussion

4.3.1 Detection of Differentially Expressed Copper-Binding Proteins

Total protein extracts obtained from root or shoot tissue at each timepoint were selectively enriched for Cu-binding proteins using Cu(II)-IMAC. IMAC is an effective method of enriching protein samples for protein species with specific metal affinities, in this instance, Cu. It should be noted that Cu(II)-IMAC can also enrich protein samples for those proteins with a general ability to bind Cu. For example, proteins that specifically bind Cu either as a co-factor (eg. Cu/Zn-superoxide dismutase) or as a substrate (eg. Uclacyanin) were isolated via Cu(II)-IMAC. Additionally, proteins that do not have a specificity for Cu per se, but do have an ability to bind

it (eg. Glutathione-*S*-transferase) were likewise isolated. However all proteins identified from the Cu(II)-IMAC enriched samples may accurately be referred to as Cu-binding. Figure 4.3 clearly depicts the enrichment for the Cu-binding proteome when using Cu(II)-IMAC. The IMAC-enriched samples were normalized for total protein concentration and separated using 1D SDS-PAGE. Several distinct bands were visualized, as shown in Figure 4.4 and were excised and an in-gel digestion performed using trypsin. The resulting peptides were analyzed by nanoLC-MS/MS and the corresponding proteins identified by database searching. The following discussion applies to identified proteins that showed differential expression over time, as determined by comparing the confidence with which the same protein (or a functional homolog matched by common tryptic peptides) was identified at different timepoints, an established method of determining relative protein abundance (see below). There was an observed protein drift such that not all like-proteins migrated to the same position either within or between gels. This is likely due to pH differences and/or the dynamic range within individual protein extractions. As such, no efforts have been or should be made to interpret the banding patterns either through time within a genotype/tissue combination or between genotypes at similar times and in similar tissues.

4.3.1.1 Shoot Proteins

Of the IMAC-enriched proteins identified in shoot tissue extracts at different timepoints, six were found in both WT and mutant shoots; a putative glutaredoxin (GRX)/arsenate reductase, thioredoxin (TRX3), pathogenesis-related protein (PR), methionine sulfate reductase (MSR), cyclophilin and Cu/Zn superoxide dismutase (SOD) (Figure 4.5; Table 4.1).

The expression profiles of these and other proteins discussed below, are represented by comparative histograms of the respective protein scores for each protein/timepoint/genotype combination. MASCOT™ employs a probability-based implementation of the MOWSE algorithm (Pappin *et al.,* 1993) where protein scores based on peptide matches above the significance threshold are considered non-random events (Matrix Science, 2007). Protein scores are an established measure of relative abundance with an adequately high correlation factor (r=.72) (Ishihama *et al.,* 2005). To confirm this, replicate 1D SDS-PAGE and nanoLC-MS/MS analyses were performed using different concentrations of a bovine serum albumin (BSA)

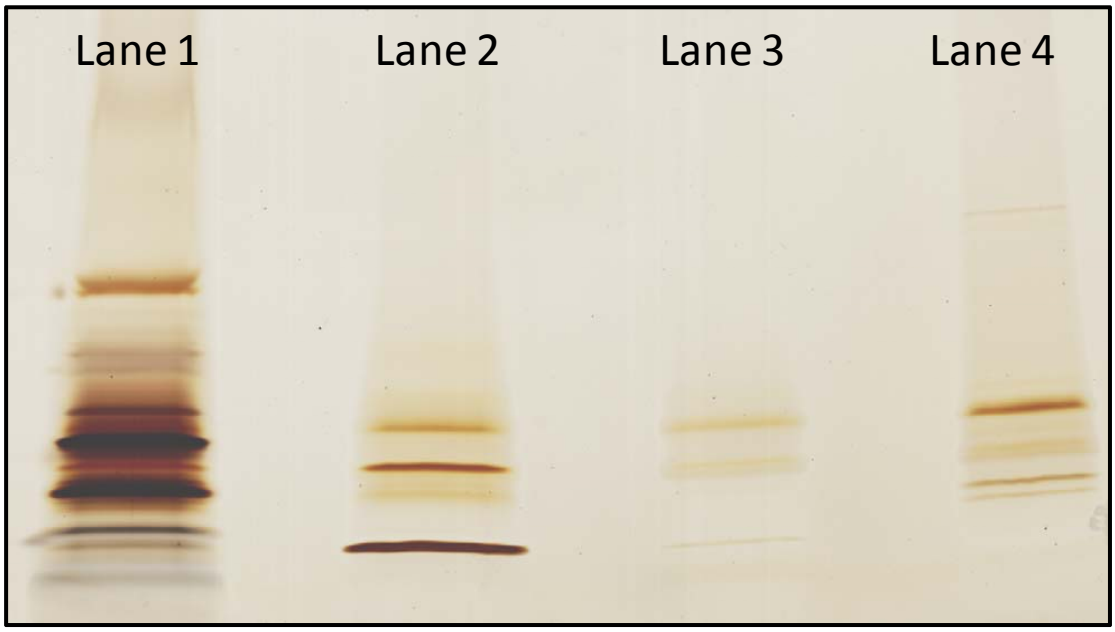

Figure 4.3: Enrichment for Cu-binding protein species using Cu(II)-IMAC. Lane 1 = desalted total soluble protein extract prior to IMAC; Lane 2 = 1st column volume wash following application of protein extract to IMAC column; Lane 3 = 3rd column volume wash following application of protein extract to IMAC column; Lane 4 = Cu-binding proteins eluted from IMAC column.

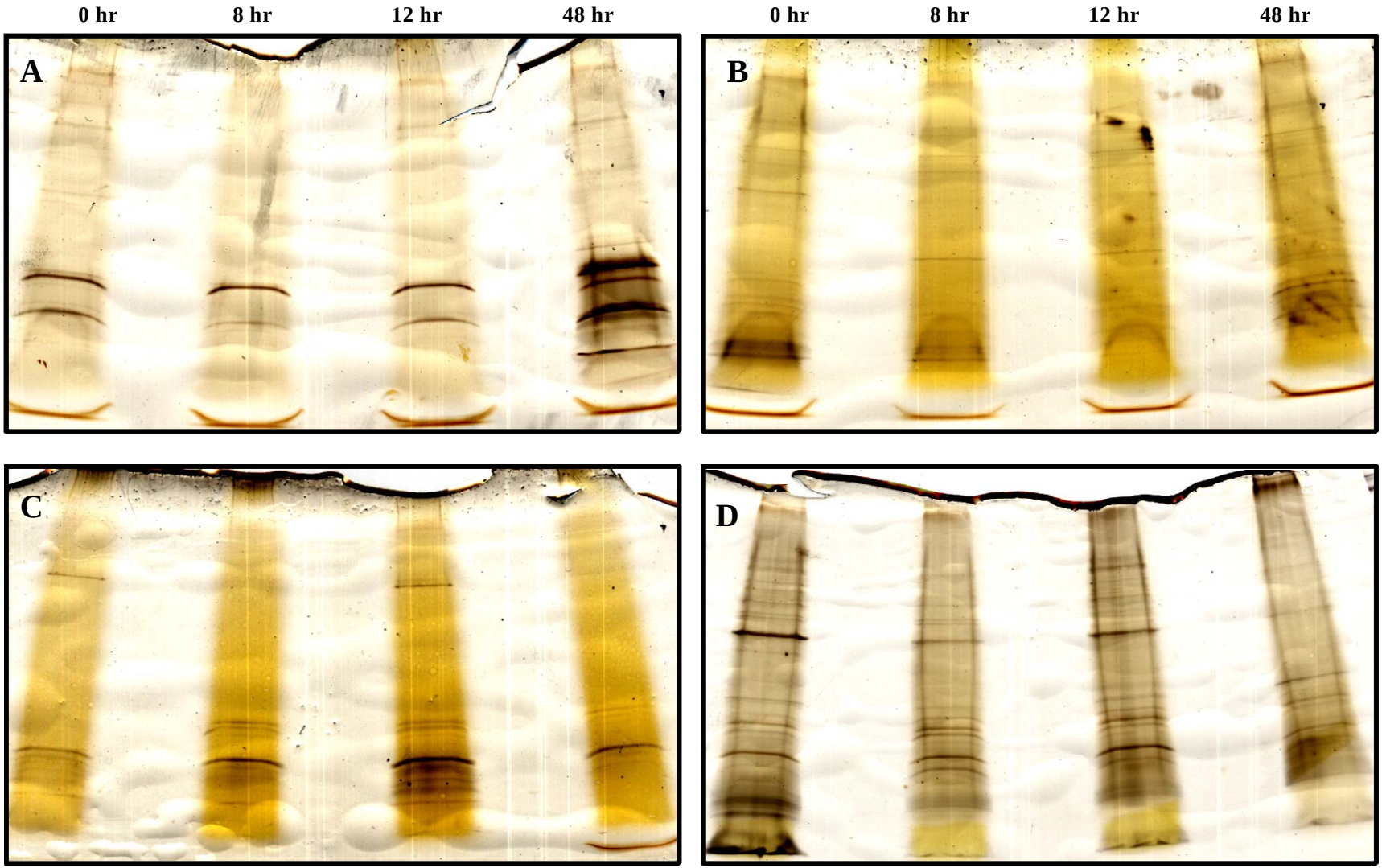

Figure 4.4: 1D SDS-PAGE separation of Cu(II)-IMAC enriched protein extracts from roots and shoots of two hydroponically-grown *A. thaliana* genotypes exposed to 30 µM Cu over 48 hours. A = wildtype shoot; B = mutant shoot; C = wildtype root; D = mutant root.

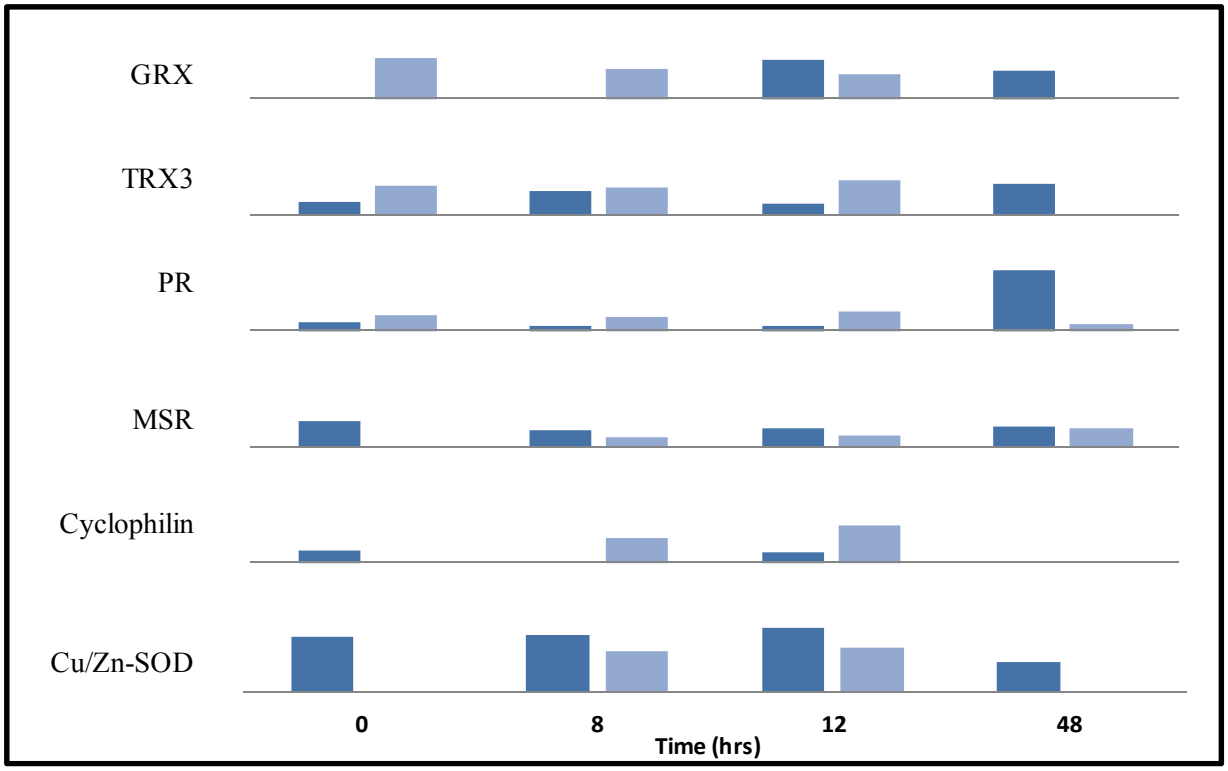

Figure 4.5: Expression trends of *A. thaliana* proteins identified from Cu(II)-IMAC enriched shoot protein extracts prepared from hydroponically-grown plants exposed to 30 µM Cu over a 48 hour timecourse.
(■ = WT; ■ = mutant)

Table 4.1: MS data for proteins identified in Figure 4.5. MW = molecular weight; pI = isoelectric point; Threshold = the score over which an identified protein is considered a non-random event; # Peptides = the number of unique peptides identified in the protein sequence; % Cov. = percentage of the total identified protein sequence represented by the identified peptides.

Protein ID	Genotype	Timepoint	Accession #	MW	pI	Threshold	# Peptides	% Cov.
GRX	**WT**	**12 Hour**					4	39
		48 Hour					2	20
	Mutant	**0 Hour**	gi|15242674	11920	6.71	37	3	27
		8 Hour					2	20
		12 Hour					2	20
TRX3	**WT**	**0 Hour**					2	16
		8 Hour					5	38
		12 Hour					3	23
		48 Hour	gi|15239136	13272	5.06	37	4	35
	Mutant	**0 Hour**					5	44
		8 Hour					4	38
		12 Hour					6	44
PR	**WT**	**0 Hour**	gi|15222089	26148	4.75		1	5
		8 Hour	gi|15225974	18008	9.08		2	9
		12 Hour					2	9
		48 Hour	gi|15222089	26148	4.75	37	9	42
	Mutant	**0 Hour**					7	27
		8 Hour	gi|15225974	18008	9.08		4	19
		12 Hour					6	19
		48 Hour					2	9
MSR	**WT**	**0 Hour**					4	16
		8 Hour					2	8
		12 Hour					2	7
		48 Hour	gi|1279212	28892	8.97	37	4	16
	Mutant	**8 Hour**					2	7
		12 Hour					2	7
		48 Hour					3	12
Cyclophilin	**WT**	**0 Hour**	gi|2443757	18666	8.33		2	9
		12 Hour					2	9
	Mutant	**8 Hour**	gi|15234781	18589	7.68	37	6	25
		12 Hour					7	45
Cu-ZnSOD	**WT**	**0 Hour**					6	15
		8 Hour					5	15
		12 Hour	gi|3273753	22332	6.28	37	5	15
		48 Hour					2	12
	Mutant	**8 Hour**					3	12
		12 Hour					3	18

standard under identical conditions to those used in this study (Figure 4.6), with excellent results (Table 4.2; Appendix 6). The amount of protein in a nanoLC-MS/MS sample can be directly correlated with protein sequence coverage (Winkler *et al.,* 2007) which, along with individual peptide scores, contributes to the overall protein score, confirming the validity of the latter as a measure of relative protein abundance. It should be noted, however, that such comparisons are applicable only to the expression of a given protein over time, and not to comparisons between different proteins. Consequently, no y-axis was used in the comparative histograms.

It is also important to note that the absence of a bar in a comparative histogram does not necessarily indicate that the protein was not expressed, only that the protein was not detectable under experimental conditions. In this study, only visible bands could be submitted for analysis. Those proteins identified during this study had to be of sufficient abundance to form distinct bands in order to be selected for analysis, however, that does not preclude the possibility of the expression of a protein, merely that it was not abundant enough for visualization. Furthermore, an apparent drift in the migration of certain proteins on the 1D SDS-PAGE gels was observed, and so again the certainty that a particular protein was not expressed at a given timepoint cannot be assured. It is also important to note that all plants, both mutant and WT, were grown simultaneously in the same growth chamber with similar results, and that the preparation and analysis of samples from a particular timecourse experiment were performed simultaneously, minimizing the impact of experimental variability on apparent differences in protein expression.

Glutaredoxin was initially expressed in shoots of the mutant at relatively high levels. As Cu exposure persisted, and Cu concentration likely approached toxic levels however, the expression of the protein decreased. In contrast, GRX expression in the WT shoots appeared to be induced following Cu exposure. GRX/arsenate reductase catalyses the reduction of arsenate to produce arsenite and oxidized glutaredoxin (Duan *et al.,* 2005). Putative evidence locates some GRX isoforms in the plastid or as part of the secretory pathway (Rouhier *et al*., 2004). GRX also functions as an intermediate in thiol-disulfide exchange reactions and, as such is involved in many cellular functions including DNA synthesis, signal transduction and defense against oxidative stress (The Arabidopsis Information Resource (TAIR): gi|15242674). Thioredoxin 3 expression was evident initially in both genotypes although at a higher level in the mutant than in the WT. In the mutant, TRX3 levels remained similar through time until 48 hours when the protein was no longer detectable. In the WT, TRX3 expression

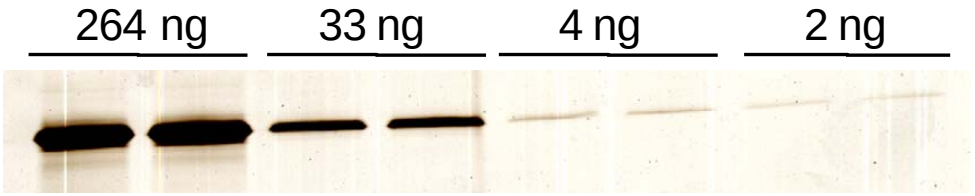

Figure 4.6: Silver-stained 1D SDS-PAGE gel of four concentrations of BSA in duplicate.

Table 4.2: MOWSE protein scores of four concentrations of BSA in duplicate.

BSA (ng)	Replicate	MOWSE Score	Averaged Score
264	A	2789	**2709**
	B	2629	
33	A	2153	**2028**
	B	1903	
4	A	1004	**939**
	B	873	
2	A	655	**608**
	B	560	

fluctuated but the protein was detected at all timepoints. Like GRX, TRX3 functions as an intermediate with oxidoreductase activity in thiol-disulfide exchange reactions but, unlike GRX is localized in the cytosol (Schürmann & Jacquot, 2000; Gelhaye *et al.,* 2005). It is thought that the cytosolic thioredoxins are important regulators of membrane-bound, receptor-like kinases (Bower *et al.,* 1996). Evidence has shown that cytosolic TRXs may be mobile elements and as such could function in regulating membrane receptors (Schürmann & Jacquot, 2000). The cytosolic TRXs may also be involved in sulfate assimilation and in conferring resistance to hydrogen peroxide (H_2O_2) (Mouaheb *et al.,* 1998).

As exchange intermediates in the thiol-disulfide exchange reactions, both TRX3 and GRX participate in the reduction of disulfide bridges in proteins and other small molecules to reduced thiols. This reduction of protein thiols could allow for the subsequent chelation of metal ions. Since the frd3-3 mutant is known to exhibit enhanced metal accumulation, it is logical that this mutant would have altered mechanisms for dealing with the excess metals. Both GRX and TRX3 expression were evident in the mutant prior to Cu inoculation which could be a modification of the plant due to the mutation. Interestingly, while GRX expression in the mutant appears to decrease over time, the expression of TRX3 increases slightly. Between 12 and 48 hours of exposure, Cu uptake began to overload the plant system and likely became toxic (*Section 3.3.1 – Copper Uptake and Plant Performance*). During this time, it is possible that excess Cu from the overloaded organelles started to accumulate in the cytoplasm which could in turn cause an induction of the TRX3 protein. In the WT, GRX expression seemed to be induced. This could be a result of the time lag involved in Cu delivery to the organelles whereas TRX3 expression was evident at the initial sampling and continued through time as Cu was transported through the cytoplasm to the organelles. As in the mutant, TRX3 expression increased slightly as GRX expression decreased in WT perhaps as a result of the overloading of organelles with Cu ions.

The expression of PR (pathogenesis-related) proteins in mutant shoots was consistent over time, decreasing slightly at 48 hours. In WT shoots, PR protein expression was slightly lower than in the mutant until 48 hours, at which time expression increased sharply. PR proteins are localized to the endomembrane system and may also be secreted (Uknes *et al.,* 1993). They function in the defense response of plants and are involved in systemic acquired resistance (SAR) although their exact molecular function is still unknown. PR proteins are defined as being

induced by various types of pathogens (viruses, bacteria, fungi) as well as chemicals that mimic infection or induce similar stresses (Bol *et al.,* 1990) while PR genes have been found to be both constitutively expressed and inducible (Agrios, 1997). The production of reactive oxygen species (ROS) such as H_2O_2, superoxide (O_2^-), and hydroxyl (OH^-), is concomitant with pathogen attack (Auh & Murphy, 1995; Desikan *et al.,* 1996; Keller *et al.,* 1998). Due to this, several roles have been suggested for PRs, including activation of defense genes and antimicrobial agents, and induction of the hypersensitive response (HR), cell death, salicylic acid, and SAR (Doke *et al.,* 1996; Low & Merida, 1996; Lamb & Dixon, 1997). Excess intracellular Cu can also lead to the formation of ROS (Schiavon *et al.,* 2007). It is therefore conceivable that the inducible response of PR proteins is due to the formation of intracellular ROS due to Cu exposure. The enhanced internal metal concentration that is consistent with the mutant phenotype could act as a deterrent to pathogen attack and herbivory. It is possible therefore that the mutant plant does not have the same requirement for an oxidative burst that usually occurs following physical attack. Consequently, the increase of proteins normally induced in response to oxidative burst would not occur in the mutant. In contrast, WT plants are not modified for the abnormally high *in situ* amount of metals, and so this elevated metal content could cause ROS production and hence, induction of defense proteins such as the PRs in the WT.

MSR expression in the WT remained fairly consistent over time, but showed a gradual increase in the mutant from its initial detection at 8 hours. Peptide-bound methionine is readily oxidized to methionine sulfoxide (MetSO) by ROS, which can inactivate an entire protein (Dann & Pell, 1989; Vogt, 1995). Metals can also cause the oxidation of methionine (Gao *et al.,* 1998). This facile oxidation however, is also readily reversible by MSR. Several isoforms of MSR exist and are localized to the chloroplast, the secretory pathway and the cytosol (Sadanandom *et al.,* 2000; Romero *et al.,* 2004). It was previously determined that expression of some MSR isoforms did not change upon exposure to heat, cold, or wounding by pathogen attack (Sadanandom *et al.,* 2000) indicating a constitutive level of expression. The results here indicate a similar result in that the expression of MSR did not change notably in the WT in response to Cu exposure. This could indicate that MSR provides a "housekeeping" function and therefore steady state levels are maintained. However, the initial expression of MSR in the mutant is again consistent with metal exposure, and a concomitant delay in the onset of Cu-induced oxidative damage requiring repair/reversal by MSR.

Cyclophilin expression was evident in WT at 0 hours and detected at similar levels at 12 hours. It was not, however, detected at 8 or 48 hours. In the mutant, cyclophilin was not initially detectable. Expression increased slightly from 8 to 12 hours but again, the protein it was not detectable at 48 hours. Cyclophilins are ubiquitous proteins localized to all subcellular compartments (Romano *et al.,* 2004) and are involved in catalyzing a rate-limiting step in protein folding (Brandts *et al.,* 1975). As such, they are believed to be involved in potential stress response related functions such as receptor complex stabilization (Leverson & Ness, 1998), receptor signaling (Yurchenko *et al.,* 2002), and apoptosis (Lin & Lechleiter, 2002). Expression of cyclophilins has been shown to be inducible by a variety of biotic and abiotic stresses (Marivet *et al.,* 1992, 1994, 1995; Chou & Gasser, 1997; Kong *et al.,* 2001; Sharma & Singh, 2003). While the expression trend of the mutant may have demonstrated induction, this was not apparent in the WT and the low levels of the protein overall did not indicate involvement in the stress response even though the plants by 48 hours were clearly experiencing stress conditions.

SOD, another protein expressed in response to oxidative damage, was also expressed at all timepoints in the WT, but decreased between 12 and 48 hours. SOD was not initially detectable at 0 hours in the mutant and, while detected at 8 and 12 hours, the expression was lower than in the WT. At 48 hours SOD expression was again not detectable. SODs are integral enzymes involved in the oxidative stress response of plants (Kliebenstein *et al.,* 1998). Together with catalase, SOD is responsible for the detoxification of a majority of the ROS (Beyer & Fridovich, 1987) that occur either as a consequence of metabolism or as a result of biotic and abiotic stressors (Van Breusegem *et al.,* 1999; Alscher *et al.,* 2002). SODs catalyze the conversion of superoxide radicals to molecular oxygen (O_2) or H_2O_2 and are differentiated from one another by their metal co-factors (Fe, Mn, and Cu-Zn) (Kliebenstein *et al.,* 1998). Cu-Zn SODs can be localized in the chloroplast, the cytosol or in extracellular spaces (Alscher *et al.,* 2002). A hydroponic-growth chamber environment could cause low-level stress to plants that otherwise appear visually healthy. This might explain the initial and persistent high level of SOD expression in the WT. As a consequence of the mutant phenotype, frd3-3 plants may be constitutively prepared for elevated metal concentrations whereas the WT plants would not be. It follows, therefore, that SOD induction in the mutant would require higher levels of ROS then are necessary in the WT. Perhaps, the additional stress of the growth conditions was enough to induce SOD production in the mutant. The decrease in SOD expression at the 48 hour timepoint

in the WT and the lack of detection altogether in the mutant could be an indication of overwhelming Cu concentrations and the shutdown of cellular machinery and protein inactivation, resulting in the necrosis observed by 72 hours.

Glutathione transferase (GST) was identified in WT shoots but not in the mutant (Figure 4.7; Table 4.3). Conversely, three proteins of interest were identified in the shoots of the mutant only; cystatin-1 (CYS1), non-specific lipid transfer protein 1 (LP1), and plant defensin 1.3 (PDF1.3) (Figure 4.8; Table 4.4).

GST expression in the WT was initially evident, although at a low level. The protein was not detectable through the middle timepoints; however, at 48 hours GST expression levels increased greatly from that evident at 0 hours. GSTs are involved in cellular detoxification via interaction of glutathione with environmental toxins, ROS and alkylating agents (Hayes & McLellan, 1999) and are generally believed to be cytosolic (Wagner *et al.,* 2002). Several isoforms of GST exist and each may be differentially expressed in response to varying stresses. Induction has been demonstrated in response to infection, ROS, phytohormones, heat, drought and exposure to heavy metals (Marrs, 1996). As with PR proteins, if the oxidative burst in mutant plants is effectively suppressed as a result of their anticipated elevated *in situ* metal concentrations*,* it is perhaps not surprising that GSTs apparently are not expressed at high levels in mutant shoots, as the need for detoxification is much reduced. In the WT, however, the observed expression pattern is consistent with induction and accumulation of GST to high levels by 48 hours.

CYS1 expression in the mutant shoots was similar at 0 and 12 hours, but was not detectable at 8 or 48 hours. CYS1 functions as a cysteine protease inhibitor. Differential expression of cystatins has been shown in response to developmental stage as well as to biotic and abiotic stress (Felton & Korth, 2000). Expressed constitutively in roots and siliques, induction of cystatins by wounding also occurs in shoots. In cell suspension cultures of *A. thaliana*, CYS1 has been shown to inhibit cell death caused by oxidative stress (Belenghi *et al.,* 2003). The expression pattern observed in this study mirrors to some extent the pattern of metal uptake in both mutant and WT shoots (*Section 3.3.1 – Copper Uptake and Plant Performance*), in that periods showing the greatest increase in Cu uptake (between 0 and 8 hours, and between 12 and 48 hours) correspond to an apparent reduction in CYS1 expression. This suggests that, despite its role in stress responses, synthesis of CYS1 is particularly susceptible to Cu inhibition,

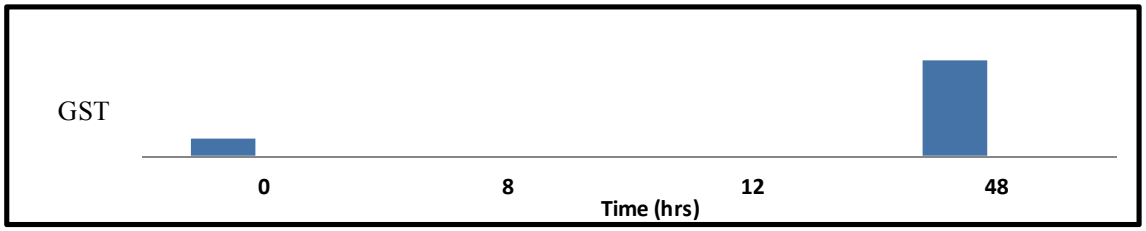

Figure 4.7: Expression trend of GST from Cu(II)-IMAC enriched shoot protein extracts prepared from hydroponically-grown plants exposed to 30 µM Cu over a 48 hour timecourse. (■ = WT).

Table 4.3: MS data for proteins identified in Figure 4.7. MW = molecular weight; pI = isoelectric point; Threshold = the score over which an identified protein is considered a non-random event; # Peptides = the number of unique peptides identified in the protein sequence; % Cov. = percentage of the total identified protein sequence represented by the identified peptides.

Protein ID	Genotype	Timepoint	Accession #	MW	pI	Threshold	# Peptides	% Cov.
GST	WT	0 Hour	gi|8778432	50585	6.82	37	1	4
		48 Hour					4	9

.

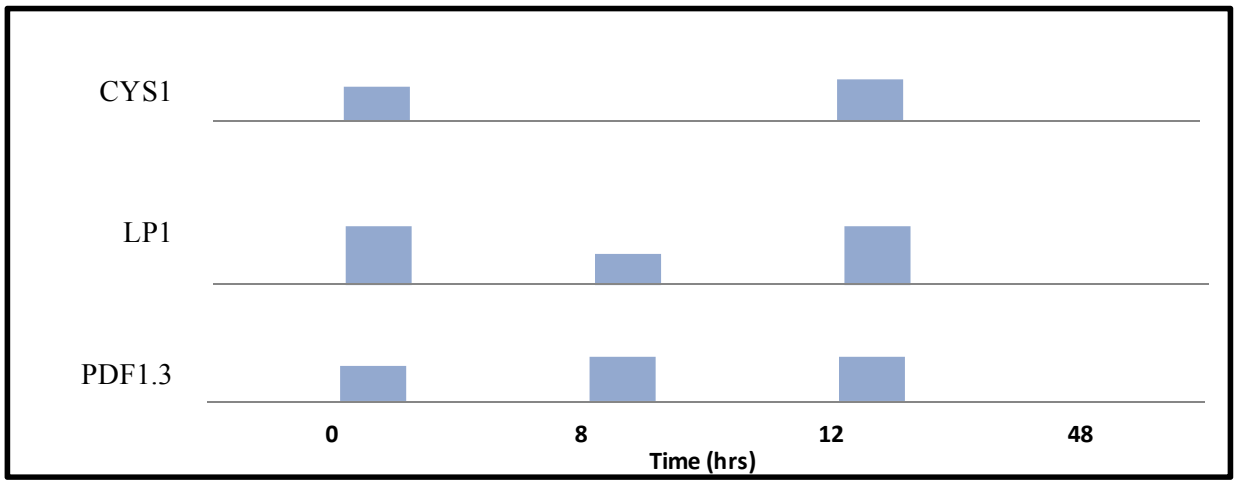

Figure 4.8: Expression trends of proteins from Cu(II)-IMAC enriched shoot protein extracts prepared from hydroponically-grown plants exposed to 30 µM Cu over a 48 hour timecourse. (= mutant).

Table 4.4: MS data for proteins identified in Figure 4.8. MW = molecular weight; pI = isoelectric point; Threshold = the score over which an identified protein is considered a non-random event; # Peptides = the number of unique peptides identified in the protein sequence; % Cov. = percentage of the total identified protein sequence represented by the identified peptides.

Protein ID	Genotype	Timepoint	Accession #	MW	pI	Threshold	# Peptides	% Cov.
CYS1	Mutant	0 Hour	gi|15239883	11249	5.07	37	1	15
		12 Hour					1	15
LP1	Mutant	0 Hour					2	18
		8 Hour	gi|15224899	12317	9.30	37	1	8
		12 Hour					2	18
PDF1.3	Mutant	0 Hour					1	12
		8 Hour	gi|15225238	9030	8.14	37	1	12
		12 Hour					1	12

the slightly higher levels of Cu in WT shoots being sufficient to reduce expression to below detectable levels.

The expression levels of LP1 and PDF1.3 in the mutant were similar in that expression levels were relatively consistent over time until 48 hours, at which point both proteins ceased to be detectable. LP1 is localized in the cell wall and is involved in lipid transport and calmodulin binding. LPs are ubiquitous proteins capable of transporting lipids between membranes (Thoma *et al.,* 1994) and as such, are believed to be involved in myriad biological activities such as embryogenesis, plant adaptation to environmental conditions, cutin formation and defense reactions (Kader, 1996). Calcium (Ca^{+2}), an important secondary messenger, is often bound by calmodulin. The ability of LPs to also bind calmodulin suggest a contributive role in the Ca^{+2} signaling cascade that controls a variety of physiological processes (Zielinski, 1998) Multiple isoforms of LPs exist that are differentially controlled and expressed although expression is rare in roots and occurs mainly in the epidermis of leaves and flowers (Arondel *et al.,* 2000).

PDF1.3 and other plant defensins are important proteins in the innate immune response of plants (Thomma *et al.,* 2002). Originally PDFs were believed to have only antifungal activity but they have also been found to inhibit α-amylase and protein synthesis (Colilla *et al.,* 1990; Osborn *et al.,* 1995). Plant defensins are localized mainly in the peripheral cell layers of vegetative tissue (Thomma & Broekaert, 1998; Thomma *et al.,* 2002). While plant pathogen attack induces plant defensins, it is also thought that induction may occur in response to biotic and abiotic stress although the potential of their role in stress tolerance is unknown (Mirouze *et al.,* 2006). As PDFs are part of the innate immune response, relatively high steady-state levels may be maintained for rapid deployment, if required. The similar expression patterns observed for LP1 and PDF1.3 in mutant shoots suggest relatively high background levels lacking in the WT that are consistent with modification of the mutant to metal exposure, while lack of expressed protein at 48 hours may be due to the toxic effects of prolonged Cu exposure.

4.3.1.2 Root Proteins

Seven proteins of interest were identified in the roots (Figure 4.9; Table 4.5) of both the mutant and the WT; lipid-associated family proteins, MD-2- related lipid recognition domain-

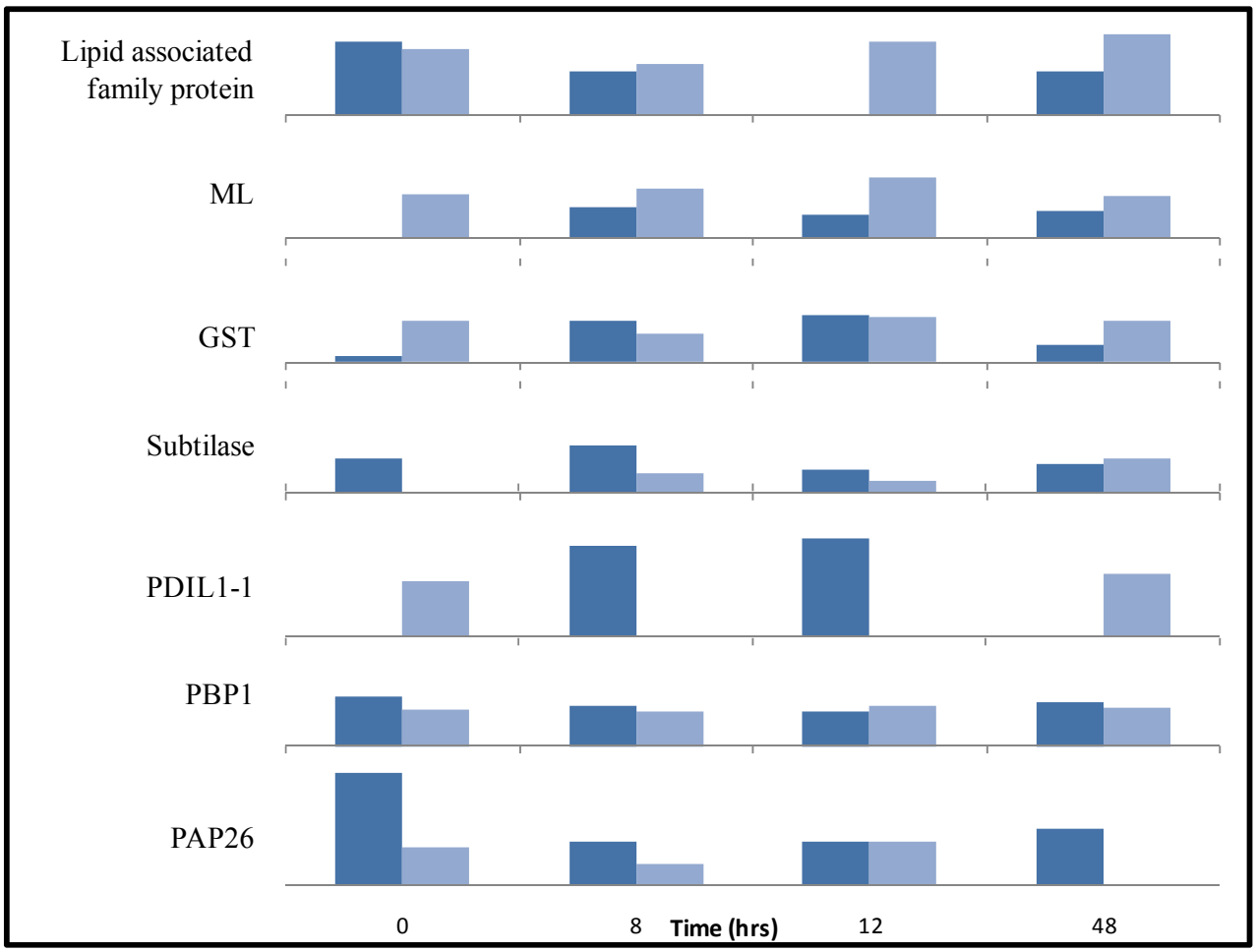

Figure 4.9: Expression trends of *A. thaliana* proteins identified from Cu(II)-IMAC enriched root protein extracts prepared from hydroponically-grown plants exposed to 30 µM Cu over a 48 hour timecourse. (■ = WT; ■ = mutant).

Table 4.5: MS data for proteins identified in Figure 4.9. MW = molecular weight; pI = isoelectric point; Threshold = the score over which an identified protein is considered a non-random event; # Peptides = the number of unique peptides identified in the protein sequence; % Cov. = percentage of the total identified protein sequence represented by the identified peptides.

Protein ID	Genotype	Timepoint	Accession #	MW	pI	Threshold	# Peptides	% Cov.
Lipid	WT	0 Hour					3	14
Associated		8 Hour					2	10
family		48 Hour					2	9
protein	Mutant	0 Hour	gi|18399899	20402	5.12	37	3	14
		8 Hour					3	14
		12 Hour					3	14
		48 Hour					3	14
ML	WT	8 Hour	gi|15237840	18739	4.55		2	8
		12 Hour	gi|15237838	18362	4.41		1	5
		48 Hour	gi|15237840	19739	4.55		2	8
	Mutant	0 Hour	gi|15237838	18362	4.41	37	2	11
		8 Hour					2	11
		12 Hour	gi|15237836	18124	4.57		4	23
		48 Hour	gi|15237838	18362	4.41		2	11
GST	WT	0 Hour					1	1
		8 Hour					5	10
		12 Hour					5	10
		48 Hour	gi|8778432	50585	6.82	37	2	4
	Mutant	0 Hour					4	8
		8 Hour					3	7
		12 Hour					4	11
		48 Hour	gi|2554769	23983	5.93		5	26
Subtilase	WT	0 Hour					2	3
		8 Hour					4	6
		12 Hour	gi|22331076	82450	6.29		3	4
		48 Hour				37	3	5
	Mutant	8 Hour					3	5
		12 Hour	gi|4115919	74667	6.53		2	3
		48 Hour	gi|22331076	82450	6.29		3	5
PDIL1-1	WT	8 Hour					3	5
		12 Hour	gi|15219086	55852	4.81	37	2	3
	Mutant	0 Hour					2	3
		48 Hour					2	3
PBP1	WT	0 Hour					1	3
		8 Hour					1	3
		12 Hour					1	3
		48 Hour	gi|15228198	32138	5.46	37	1	3
	Mutant	0 Hour					1	3
		8 Hour					1	3
		12 Hour					1	3
		48 Hour					1	3
PAP26	WT	0 Hour					6	14
		8 Hour	gi|15242870	55032	6.80		2	3
		12 Hour					3	6
			gi|15222978	74012	5.78	37	2	3
		48 Hour					3	6
	Mutant	0 Hour	gi|15242870	55032	6.80		2	5
		8 Hour					1	1
		12 Hour	gi|15222978	74012	5.78		3	4

containing protein (ML), GST, subtilase family protein, protein disulfide isomerase-like protein 1-1 (PDIL1-1), PYK-10 binding protein 1 (PBP1), and purple acid phosphatase 26 (PAP26).

The expression of the lipid associated family protein was initially high in the WT and decreased at 8 and 48 hours. It was not detected at 12 hours. In the mutant, expression was initially high and remained so over the sampling period. The lipid associated family protein is localized to the endomembrane system (TAIR; At2g22170) and may be further localized to the thylakoid membrane (TAIR; At4g39730). Its molecular function is presently unknown although it does contain the PLAT (polycystin-1/lipoxygenase and alpha toxin)/LH2 (lipoxygenase homology) domain, the proposed function of which is to mediate the interaction of membrane-bound proteins with lipids. Many of the proteins containing PLAT/LH2 domains are stress induced (TAIR; At2g22170). The protein sequence contains a predicted transmembrane domain (DAS Transmembrane Prediction Server (Cserzo *et al.,* 2002)) as well as two predicted disulfide bridges (DIpro 2.0 (Baldi *et al.,* 2005; Cheng *et al.,* 2006)). The lipid association in conjunction with the presence of a transmembrane domain suggest that this protein may be a previously undocumented transporter or receptor.

Expression of the ML-protein was not initially detectable in WT but was evident at 8 hours and remained at a similar level through to 48 hours. Its expression was, however, initially evident in the mutant and while it was expressed at a similar level it also remained higher comparatively to the WT throughout the timecourse. The function of ML-proteins remains unknown although they have been implicated in lipid recognition or metabolism as well as in host response to pathogen attack (Inohara & Nuñez, 2002). Containing a putative lipid binding cavity, it has been suggested that ML functions by shielding the hydrophobic portion of a lipid ligand thereby facilitating its solubility and recognition by enzyme receptor membrane sites (Sandhoff & Kolter, 1996; Record *et al.,* 1999). ML-proteins contain a transmembrane domain (DAS Transmembrane Prediction Server) and are thought to be localized to the endomembrane system (TAIR: At5g23840). They also however, contain an N-terminal signal peptide indicating that they are secretory or luminal proteins (Inohara & Nuñez, 2002). ML may act as a transporter on the inside of the ER to facilitate lipid entry to, or exit from, the ER. Steady-state levels of the protein appear to be maintained even during severe Cu toxicity. Interpretations of the expression trends of the lipid associated family protein and ML are difficult without further characterization of protein function. However, the observed expression patterns are consistent

with induction in the WT and relatively high background levels in the mutant, suggesting a role in response to metal stress.

GST expression in WT roots was very low initially, increased through the 8 and 12 hour timepoints and decreased again at 48 hours. Conversely, GST expression in the mutant was high initially and remained at similar levels throughout the timecourse. For the discussion of GST localization and function see previous section (*Section 4.3.1.1 - Shoot Proteins*). For many external stimuli, roots are the first point of contact for a plant. Consequently, it is not surprising that GST expression was high in the roots despite no evidence for its expression in shoots of the mutant. The mutant plants may tightly regulate the transport of metals ions from roots to shoots so as not to overwhelm the sequestration mechanisms in the shoots. This could prevent or retard the formation of ROS in the shoots, which could in turn lead to little or no induction of GST in shoots. Roots, on the other hand, may be required to deal with elevated metal-ion levels that in turn cause ROS formation. A high steady state level of GST expression when compared to shoots could be one strategy employed. In the WT, GST expression appears to be induced in the roots, as was the case with shoots. The apparent decrease in expression level at 48 hours could again be indicative of the deleterious effect that Cu toxicity has on plant protein biosynthesis.

WT subtilase expression varied through time with what appeared to be a slight induction from 0 to 8 hours following inoculation with Cu and subsequently decreased in expression. In the mutant, expression was not initially apparent and once observed remained low with an apparent slight induction at 48 hours. Belonging to the S8 serine protease family, subtilases are believed to be involved in general protein turnover as well as highly specific regulation of plant development or response to environmental stimuli (Berger & Altmann, 2000). Subtilases are generally regarded as secretory pathway proteins and are localized to the extracellular spaces, although some are predicted to occur in chloroplasts and mitochondria (Tripathi & Sowdhamini, 2006). S8 proteases possess a PA (protease-associated) domain, the predicted function of which is to promote substrate specificity and/or protein-protein interaction (Mahon & Bateman, 2000; Luo & Hoffmann, 2001). The PA domain is also found in two classes of transmembrane proteins thought to function as lytic or storage vacuolar receptors (Luo & Hoffmann, 2001). Perhaps subtilases, in response to an increased *in situ* concentration of metals, act as a form of chaperone by which metal-chelating proteins are transported and deposited in vacuoles. A cyclic pattern, whereby a period of induction would be followed by a decrease in protein abundance as

reserves are used up, might be expected in this scenario. Since mutant plants are likely modified for higher tissue concentrations of metals, the induction threshold in such plants could be set higher, which might explain the later occurrence of the cyclic pattern in the mutant.

The expression of PDIL1-1 was evident in the mutant at 0 and 48 hours. It was not detectable at the two intermediate timepoints. Conversely, in the WT, while not detected at either the 0 or the 48 hour timepoints, PDIL1-1 expression was evident at similar levels at both 8 and 12 hours. PDIL1-1 is a member of the thioredoxin superfamily and is localized to the endoplasmic reticulum (ER) (Houston *et al.,* 2005), the entry point to the secretory pathway. These proteins function generally in cellular redox homeostasis as they catalyze the formation, isomerization and reduction/oxidation of disulfide bonds (Wilkinson & Gilbert, 2004) and thereby assist protein folding. As such they are especially important in protein secretion and storage (Houston *et al.,* 2005). The pattern of PDIL1-1 expression in WT roots suggests induction in response to the elevated levels of Cu, the drop in expression at 48 hours resulting from Cu toxicity. In contrast, the relative abundance of this protein in mutant roots at 0 and 48 hours is characteristic of a more constitutive expression, possibly involving chelation of excess Cu by thiol groups, although the reason for the apparent drop in abundance at intermediate timepoints is not readily apparent unless metal-ion complexes of PDIL1-1 are translocated and/or degraded soon after formation.

PBP1 expression was similar at all timepoints in both the mutant and the WT. Cytosolic PBP1 binds PYK-10, a β-glucosidase localized to ER-bodies thought to be involved in defense systems (Hayashi *et al.,* 2001; Nitz *et al.,* 2001). PBP1 may also act as a molecular chaperone for the correct polymerization of PYK-10 (Nagano *et al.,* 2005). It has been speculated that the PBP1/PYK-10 complex may participate in the resistance of plants to the counter-adaptations of herbivores and pathogens (Nagano *et al.,* 2005). The steady-state levels observed in the roots of both genotypes suggests that, at least in this case, metal accumulation does not stimulate a response similar to pathogen/herbivore attack, as there was no apparent induction of PBP1. PYK10 accumulates mainly in roots and so it has also been speculated that the PBP1/PYK10 complex may exist to prevent the diffusion of PYK10 into the soil water (Nagano *et al.,* 2005). As these plants were grown hydroponically, the relatively high steady-state levels observed could therefore be a result of the growth environment.

PAP26 expression was high initially in the WT and decreased at all subsequent timepoints. In the mutant, PAP26 expression was comparatively low throughout until 48 hours at which time it was no longer detectable. PAP26 is a vacuolar acid phosphatase that catalyzes the hydrolysis of inorganic phosphate (Pi) (Veljanovski *et al.,* 2006) the expression of which appears to be induced in response to Pi starvation. It is also speculated that PAP26 may be involved in the metabolism of ROS following the loss of vacuolar integrity caused by programmed cell death and may also be involved in the mobilization of intracellular Pi in senescing tissues (Veljanovski *et al.,* 2006). Based on the above speculations, an induction of PAP26 might be expected; however, this was not observed in the data here. Metal ions have been shown to inhibit acid phosphatases (Tabaldi *et al.,* 2007) and so the low level of expression in the mutant and the suspected repression of PAP26 in the WT may be a consequence of metal uptake. As speculated above, and in keeping with the higher metal loads associated with the mutant phenotype, the production of expected ROS may not occur as rapidly or to the same extent as in the WT under the same metal challenge. Consequently, the mutant may have a lower endogenous expression of PAP26 than the WT.

One protein of interest was identified in the roots of WT plants that was not also identified in the mutant (Figure 4.10; Table 4.6); glutathione reductase (GR). The expression of GR was initially evident at a high level but was not detectable again until 48 hours at which time the expression level had dropped in comparison to the 0 hour timepoint. Different isoforms of GR are localized in the chloroplast, the mitochondria and the cytosol (Rouhier *et al.,* 2006) and catalyze the conversion of glutathione disulfide (GSSG) to glutathione (GSH) (Kubo *et al.,* 1993), an important scavenger of H_2O_2 and oxidized ascorbate (Xiang *et al.,* 2001). GSH is the major source of non-protein thiols in plant cells (Bergmann & Rennenberg, 1993) and in addition to its important function protecting plants against oxidative stress, its thiol group is also capable of forming disulfide bonds with metal ions (Xiang *et al.,* 2001). GR activity has been shown to increase in response to a variety of stresses; ROS, low temperature, drought and xenobiotic chemicals (Xiang & Oliver, 1989). When the GR expression pattern is considered along with that of GST in WT an interesting pattern emerges. At 0 hours there was a high level of GR expression and a low level of GST expression. This situation should result in a large amount of reduced GSH as there would be adequate GR to reduce GSSG to GSH and low levels of GST would chelate xenobiotics with the available GSH at a high rate. At 8 and 12 hours however,

Figure 4.10: Expression trend of GR proteins from Cu(II)-IMAC enriched root protein extracts prepared from hydroponically-grown plants exposed to 30 µM Cu over a 48 hour timecourse. (■ = WT).

Table 4.6: MS data for proteins identified in Figure 4.10. MW = molecular weight; pI = isoelectric point; Threshold = the score over which an identified protein is considered a non-random event; # Peptides = the number of unique peptides identified in the protein sequence; % Cov. = percentage of the total identified protein sequence represented by the identified peptides.

Protein ID	Genotype	Timepoint	Accession #	MW	pI	Threshold	# Peptides	% Cov.
GR	WT	0 Hour	gi|15232559	61327	7.97	37	7	15
		48 Hour					1	1

there was no detectable GR expression and high GST expression. This should result in the opposite situation in which there would be low levels of free GSH. Low GR levels would not be able to reduce large amounts of GSSG and high levels of GST might be required for the chelation of the prevalent Cu ions. At 48 hours the low levels of both GR and GST could be attributed to the perturbation of protein synthesis and protein inactivation by excess Cu ions.

A total of 17 proteins of interest were identified in mutant roots but not identified in the roots of the WT (Figure 4.11A; Table 4.7A and Figure 4.11B; Table 4.7B); germin-like protein and (GLP), vegetative storage protein (VSP) , uclacyanin 2 (UCC2), calreticulin 1 (CRT1), cystatin family protein, cobalamin-independent methionine synthase (CIMS), *S*-methionine adenosyltransferase (MAT3), peroxidase (POX), Cu/Zn-SOD, L-galactose-1-phosphate phosphatase (VTC4), thioredoxin 3 (TRX3), PR proteins, glyceraldehyde-3-phophate dehydrogenase C (GAPC), osmotin and three unknown proteins referred to in text as Unknown 1, Unknown 2 and Unknown 3.

The expression of the GLP was high initially and decreased following Cu exposure. Expression levels increased again at the subsequent timepoints. Germin-like proteins function as nutrient reservoirs and are part of the endomembrane system although they may also be located to the apoplast (Carter *et al.,* 1998). They possess oxalate oxidase activity and catalyze the oxidative breakdown of oxalate which produces H_2O_2. Because of the production of H_2O_2, GLPs are thought to be involved in plant defense (Lane, 1994). H_2O_2 however is also required for the catalysis of cell wall component crosslinking reactions (Varner & Lin, 1989) and may also function as a secondary messenger that induces genes involved in plant protection or defense (Levine *et al.,* 1994; Chen *et al.,* 1995). Due to the expected high internal metal concentration of the mutant phenotype GLPs may be induced early in development either to act as metal scavengers or to compensate for expected ROS species. The decrease in protein at 8 hours could be a result of the depletion of the GLP pool in response to the increasing Cu content. At the later timepoints, GLP synthesis could have recovered from the initial depletion at 8 hours explaining the increase of protein at those timepoints.

The VSP expression was initially high but decreased at the 8 hour timepoint and was not subsequently detected. VSPs store carbon and nitrogen inside the vacuoles of plants in the vegetative tissues for use in the growth and development of plants (Utsugi *et al,.* 1998). Some VSPs have been shown to have acid phosphatase activity (Rapp *et al.,* 1990; DeWald *et al.,*

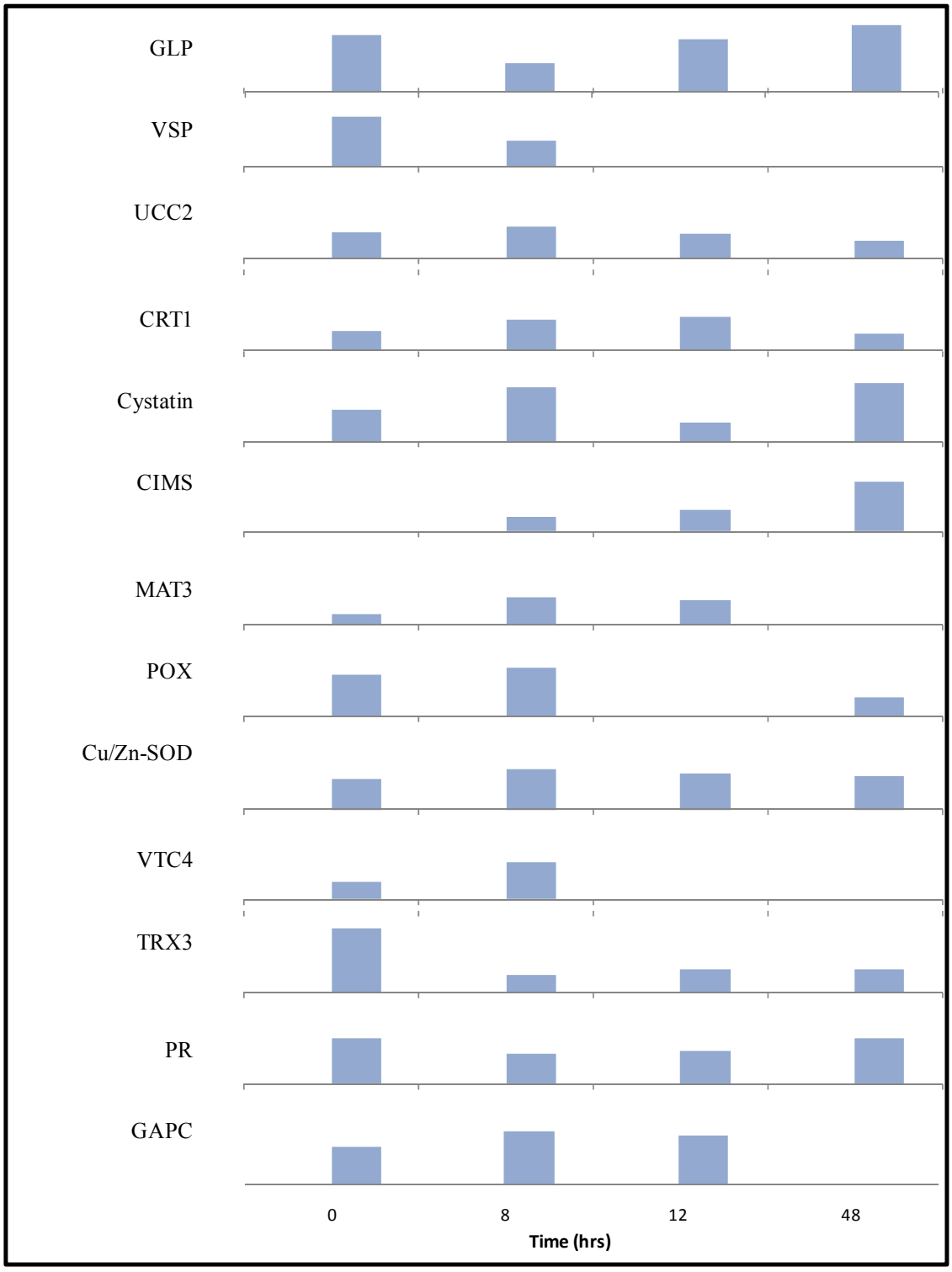

Figure 4.11A: Expression trends of proteins from Cu(II)-IMAC enriched root protein extracts prepared from hydroponically-grown plants exposed to 30 µM Cu over a 48 hour timecourse. (= mutant).

Table 4.7A: MS data for proteins identified in Figure 4.11A. MW = molecular weight; pI = isoelectric point; Threshold = the score over which an identified protein is considered a non-random event; # Peptides = the number of unique peptides identified in the protein sequence; % Cov. = percentage of the total identified protein sequence represented by the identified peptides.

Protein ID	Genotype	Timepoint	Accession #	MW	pI	Threshold	# Peptides	% Cov.
GLP	**Mutant**	**0 Hour**	gi|15241638	23965	6.27	38	4	19
		8 Hour	gi|18202452	23370	6.81		1	5
		12 Hour	gi|15241638	23965	6.27		3	11
		48 Hour	gi|1755160	21950	5.81		4	18
VSP	**Mutant**	**0 Hour**	gi|2373399	29977	6.17	38	4	15
		8 Hour					2	6
UCC2	**Mutant**	**0 Hour**	gi|3399769	19822	7.18	38	4	22
		8 Hour					4	22
		12 Hour					4	22
		48 Hour					3	11
CRT1	**Mutant**	**0 Hour**	gi|15223517	48668	4.46	37	5	8
		8 Hour					5	10
		12 Hour					7	13
		48 Hour					4	8
Cystatin	**Mutant**	**0 Hour**	gi|15235771	12605	9.16	38	2	18
		8 Hour					3	29
		12 Hour					3	24
		48 Hour					3	29
CIMS	**Mutant**	**8 Hour**	gi|15238686	84646	6.09	37	3	4
		12 Hour					3	3
		48 Hour					8	10
MAT3	**Mutant**	**0 Hour**	gi|15228048	42927	5.76	38	2	6
		8 Hour					5	13
		12 Hour					5	14
POX	**Mutant**	**0 Hour**	gi|166807	38718	5.95	37	3	8
		8 Hour					3	11
		48 Hour	gi|21592888	39438	6.21		1	2
SOD	**Mutant**	**0 Hour**	gi|3273753	22332	6.28	37	2	12
		8 Hour					2	12
		12 Hour					2	12
		48 Hour					2	12
VTC4	**Mutant**	**0 Hour**	gi|15232993	29388	5.21	38	2	6
		8 Hour					3	10
TRX3	**Mutant**	**0 Hour**	gi|15239136	13272	5.06	38	4	35
		8 Hour					1	9
		12 Hour					1	9
		48 Hour					1	9
PR	**Mutant**	**0 Hour**	gi|1228950	19659	7.62	37	1	5
		8 Hour					1	5
		12 Hour					1	5
		48 Hour					1	5
GAPC	**Mutant**	**0 Hour**	gi|15229231	37005	6.62	37	6	21
		8 Hour					6	18
		12 Hour					7	16

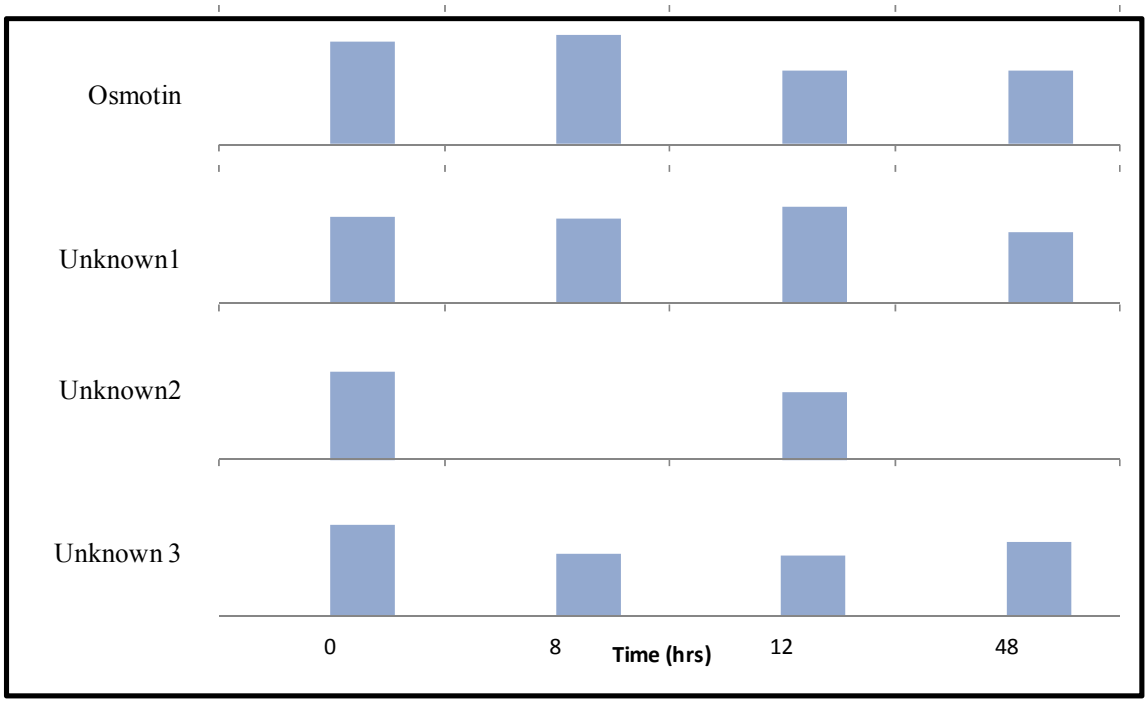

Figure 4.11B: Expression trends of proteins from Cu(II)-IMAC enriched root protein extracts prepared from hydroponically-grown plants exposed to 30 µM Cu over a 48 hour timecourse. (= mutant).

Table 4.7B: MS data for proteins identified in Figure 4.11B. MW = molecular weight; pI = isoelectric point; Threshold = the score over which an identified protein is considered a non-random event; # Peptides = the number of unique peptides identified in the protein sequence; % Cov. = percentage of the total identified protein sequence represented by the identified peptides.

Protein ID	Genotype	Timepoint	Accession #	MW	pI	Threshold	# Peptides	% Cov.
OSM	**Mutant**	**0 Hour**	gi|887390	27498	6.67	38	1	4
		8 Hour					1	4
		12 Hour					1	4
		48 Hour					1	4
UNK 1	**Mutant**	**0 Hour**	gi|18420052	20601	4.62	38	1	5
		8 Hour					1	5
		12 Hour					1	5
		48 Hour					1	5
UNK 2	**Mutant**	**0 Hour**	gi|15236948	20298	5.01	37	2	12
		12 Hour					2	12
UNK 3	**Mutant**	**0 Hour**	gi|9958068	19896	5.62	38	5	28
		8 Hour					4	20
		12 Hour					4	20
		48 Hour					4	20

1992). Genetic induction occurs in response to environmental stress (Staswick, 1990, 1994). It has been suggested that VSPs are required as a nitrogen source for the synthesis of defense products and/or the repair of damaged tissue (Utsugi *et al*., 1998). High initial expression of VSPs in the mutant, not observed in the WT, is consistent with the production or "stockpiling" of defense products and/or their components for rapid deployment/assembly in response to metal-induced oxidative damage. As VSPs are thought to have acid phosphatase activity, the apparent decrease in expression could be attributed to the increased Cu concentration, as metals have been shown to inhibit acid phosphatase activity (Tabaldi *et al.,* 2007) as was discussed previously in relation to PAP26.

UCC2 expression in mutant root tissues was consistent throughout the timecourse. UCC2 is a poorly characterized member of the blue Cu protein family known as phytocyanins (Nersissian *et al.,* 1998). Uclacyanins possess a domain that resembles a cell wall structural protein leading to speculation that they are themselves cell wall proteins. Additionally, they contain secretory signal peptides but lack an ER retention signal (Pelham, 1990; Napier *et al.,* 1992) suggesting that they are transported via the golgi apparatus. Evidence suggests that uclacyanins may be involved in the stress response of plants (Chang *et al.,* 1996) and other members of the phytocyanin family have been shown to be induced by oxidative stress (Richards *et al.,* 1998). The observed steady state levels of UCC2 are suggestive of constitutive expression. The putative localization to the cell wall as well as Cu-binding properties, leads to speculation that UCC2 could function in a mechanism for sequestration of metal ions via storage in the cell wall, particularly as this protein was not detected in WT roots.

Expression levels of CRT1 were lower at the 0 and 48 hour timepoints than at the two intermediate timepoints. CRT1 is localized to the ER and functions in calcium signaling and as a chaperone (Nakamura *et al.,* 2001). CRTs are inducible by stress (Denecke *et al.,* 1995; Jaubert *et al.,* 2002) and, as Ca^{+2} is important in signal transduction and stress responses, it is not surprising that its molecular chaperone would have a constitutively high expression pattern. This pattern was also exhibited by LP1, which has calmodulin binding activity and was discussed previously. The fact that Ca^{+2} and Cu^{+2} are both divalent cations leads to speculation that, if exposed to Cu concentrations significantly in excess of calcium levels, CRT1 might also bind, transport and/or mediate sequestration of Cu ions. Based on both the Pauling and Goldschmidt determinations of cationic radii, which are, respectively, 0.96 Å and 0.94 Å for Cu^{+2} and 0.99 Å

and 1.06 Å for Ca^{+2} (Cotton & Wilkinson, 1980), it is feasible that Cu^{+2} could occupy a Ca^{+2} binding site, as the two cations are of similar size. This hypothesis also explains the constant expression levels of CRT1 in mutant roots, as the internal Cu concentrations were very high in these tissues.

The expression of cystatin fluctuated throughout the experiment, rising between 0 and 8 hours, decreasing sharply at the 12 hour timepoint, and increasing sharply again at 48 hours. Cystatins are cysteine protease inhibitors that are localized to the endomembrane system (TAIR; At4g16500). They are involved in regulating apoptotic cysteine proteases that have been induced by pathogen attack or during oxidative stress (Solomon *et al.,* 1999) and so function to prevent unwanted cell death. The observed pattern of the cystatin protein is consistent with induction of the protein in response to the significant increase in root Cu concentration between 0 and 8 hours, and between 12 and 48 hours, during which times the corresponding rapid increase in oxidative stress might be expected to trigger such a response, which is apparently absent or much reduced in WT roots.

While not initially detected at 0 hours, expression levels of CIMS increased through the remaining timepoints. CIMS is an integral member of the methionine synthesis cycle and catalyses conversion of homocysteine to methionine with the transfer of the terminal methyl group (Eichel *et al.,* 1995). CIMS is localized to the cytosol (Ravanel *et al.,* 1998). Methionine and cysteine are the only two amino acids that contain sulfur and so both have critical roles in general metabolism and protein synthesis. As the plant is challenged with increasing levels of Cu, the synthesis of compensatory proteins, many of which are sulfur-containing, are expected to increase. It follows then that those proteins responsible for the synthesis of requisite building blocks would also increase, and that higher overall expression might be expected in the mutant than in the WT if such proteins contribute to enhanced metal accumulation.

MAT3 levels in mutant root tissues were initially low, increasing at the 8 and 12 hours timepoints but becoming undetectable at 48 hours. MAT3 catalyses the production of *S*-adenosylmethionine (AdoMet) from methionine and so participates in methionine cycling. AdoMet is the primary methyl donor for transmethylation reactions and is also the substrate for the biosynthesis of ethylene, an important phytohormone (Lindermayr *et al.,* 2006) which itself is a regulator of several plant defense genes (Ecker & Davis, 1987). As has already been discussed, methionine plays a pivotal role in protein synthesis, sulfur assimilation and cellular

metabolism. Therefore the proteins involved in methionine cycling likely follow an induction pattern as discussed earlier in relation to CIMS.

Peroxidase expression was initially high, rising slightly at the 8 hour timepoint. Expression levels dropped sharply at the 48 hour timepoint and the protein was not detected at 12 hours. POX is a secretory protein that acts as an H_2O_2 scavenger and therefore participates in the detoxification of ROS (Kvaratskhelia *et al.,* 1999). POXs are localized in the vacuoles or extracellular spaces and are induced in response to stress (National Center for Biotechnology Information (NCBI); CDD: 29388.). The high levels of POX in the mutant could be again a proteomic modification due to the phenotype in which high metal concentrations are expected and consequently, a high level of ROS could also be expected. By having a constitutively high level of ROS scavengers *in situ*, the deleterious effects of ROS production would be minimized. The decrease in protein at the later timepoints can again be attributed to the interference of regular metabolism and cell death caused by Cu toxicity.

Cu/ZnSOD expression in mutant plant roots was essentially constant over time. For the description of SOD function and localization refer to the previous section (*Section 4.3.1.1 - Shoot Proteins*). A similar pattern of expression was observed in WT shoots, whereas SOD expression in mutant shoots was apparently induced following exposure to Cu. SOD is the major scavenger of O_2^- radicals, and high Cu concentrations in root tissues might be expected to result in the production of ROS such as these. The relatively high background levels of SOD, maintained throughout Cu exposure (despite the apparent suppression of many other proteins by 48 hours), therefore suggest that this family of proteins plays a critical role in the metal accumulation mechanisms possibly by conferring tolerance of ROS to the plant. The apparent lack of SOD expression in WT roots, on the other hand, suggests that the metabolic machinery required for the induction and synthesis of SOD may have been overwhelmed by such high levels of Cu, whereas the lower concentrations of Cu in shoot tissues had much less affect on expression of SOD, for which background levels appear to be much higher than in the roots of WT plants.

VTC4 expression levels were low initially and increased slightly at 8 hours. The protein was not detected at the subsequent timepoints. VTC4 is involved in L-ascorbic acid biosynthesis (Conklin *et al.,* 2006). Ascorbic acid, while a co-factor in many enzymes (de Tullio, 2004), is also well known as an antioxidant able to detoxify ROS and so functions in the maintenance of

homeostatic ROS levels (Conklin *et al.,* 2006). As a consequence, it is also likely to have a role in ROS-signaling cascades (Conklin *et al.,* 1996; Conklin & Barth, 2004). An induction of the ascorbic acid biosynthesis proteins could be expected in response to increasing metal concentration, as increasing amounts of ROS could also be produced or expected. The lack of detectable amounts of protein at 12 and 48 hours could suggest that VTC4 is especially sensitive to metal concentrations and that the elevated levels have surpassed an upper integrity threshold for VTC4. This would also explain why this protein was not detected in roots of the WT plant, as it is not tolerant of elevated metal concentrations and so is presumably less able to prevent damage of such proteins by Cu.

Unlike the expression in mutant shoots, TRX3 expression levels began high and decreased sharply at the 8 hour timepoint. Expression levels at the subsequent timepoints were similar to that at 8 hours. For the description of TRX3 refer to the previous section (*Section 4.3.1.1 - Shoot Proteins*). The difference in localized expression between root and shoot could be due to the fact that roots represent the initial point of contact and the expected high concentrations of metals *in situ*. Although the primary function of TRX3 is not one of metal binding it is involved in thiol reduction, the products of which can participate in metal binding reactions. A high level of TRX3 expression could therefore be expected at early timepoints whereby the protein could be reducing available thiols for chelation of the high metal content expected. The decrease in TRX3 levels could be caused by a lag in the re-synthesis/re-generation of the proteins. Alternatively, it could be another Cu-sensitive protein that shows an early susceptibility to elevated presence of Cu ions.

As in mutant shoots, the expression levels of the PR proteins in mutant roots were consistent through time. For a detailed description of PRs, refer to the previous section (*Section 4.3.1.1 - Shoot Proteins*). The relatively constant levels of PRs imply a non-induced expression pattern. This is consistent with the expression of PR in the shoots and, as in shoots, could result from the lack of oxidative burst in response to an elevated metal concentration. That PR was undetected at 0, 8 and 12 hours in WT roots is consistent with the lower levels of PR expression observed in WT relative to mutant shoots, while the much higher Cu concentrations observed in root tissues were apparently sufficient to suppress PR synthesis at 48 hours and hence, any induction similar to that observed in WT shoots.

GAPC expression was initially high at 0 and 8 hours, but decreased at 12 hours and was no longer detected at 48 hours. GAPC is a cytosolic member of the glycolytic pathway that catalyzes the reduction of 1,3-diphosphoglycerate to glyceraldehyde-3-phosphate (Petersen *et al.,* 2003). Environmental stresses such as heat shock, salt stress and anaerobiosis have been shown to stimulate the expression of the *GAPC* gene (Russell & Sachs, 1989; Yang *et al.,* 1993). Protein expression is localized to the roots (Petersen *et al.,* 2003) and, more specifically, to the cytosol and mitochondria (Sweetlove *et al.,* 2002). As GAPC interacts with H_2O_2 it may also participate in the response to ROS (Hancock *et al.,* 2005). The high initial expression levels of GAPC in mutant roots could be part of a modification to high levels of metals and, consequently, ROS (specifically H_2O_2), particularly since the protein was not detected in WT roots, while the subsequent decrease in protein levels at later timepoints could be due to the impairment of protein biosynthesis due to high Cu levels.

Osmotin showed high levels of expression in mutant plant roots, dropping from a maximum at 0 hours to slightly lower levels at 8, 12 and 48 hours. Osmotin is a member of the thaumatin-like family of proteins, which also contains the PR-5 class of pathogenesis-related proteins (Capelli *et al.,* 1997). Osmotin is a vacuolar protein (Melchers *et al.,* 1993) and, although the mode of action is unknown, is thought to be involved in SAR and the stress response of plants (Hu & Reddy, 1997). According to information provided by the DAS Prediction and DIpro Servers, osmotin contains a transmembrane domain and seven putative disulfide bonds. Perhaps osmotin functions in the delivery of stress compounds for vacuolar sequestration/destruction, in which case the high initial levels of osmotin may be part of the proteomic modifications of the mutant to elevated metal ion and/or ROS concentrations. Alternatively, the relatively high number of thiol groups that could potentially be generated upon reduction of osmotin disulfide bonds suggests a direct role for this protein in chelation, transport and/or vacuolar sequestration of metals.

Expression levels of Unknown 1, the first of three unknown proteins identified as being present in mutant roots, were high and constant throughout the timecourse experiment. According to The Arabidopsis Information Resource, Unknown 1 is an endomembrane protein with a conserved DUF538 domain whose function is also unknown (TAIR; At5g19860). Peptide sequence analysis shows that this protein contains a transmembrane domain (DAS Prediction Server) and a signal peptide sequence indicating a secretory protein (p=0.976) (Bendtsen, 2004).

It also possesses one potential disulfide bond and contains seven possible phosphorylation sites (Bendtsen, 2004). This protein may, therefore, function as a receptor or signaling protein, as the phosphorylation sites suggest that there are both active and inactive forms of the protein. Localization to the secretory pathway, along with the presence of a transmembrane domain, further suggest that this is a membrane-bound protein and/or that it may interact with such proteins, while its expression in mutant but not WT roots implies a role in metal accumulation. Further study of this protein may help to establish whether or not it is involved in stress responses.

The level of expression of the Unknown 2 protein in mutant plant roots was similar at 0 and 12 hours; however, the protein was not detected at 8 or 48 hours. Although the molecular function of the protein is not known, Unknown 2 contains the ubiquinone-binding protein domain QP-C of the cytochrome bc1 complex (TAIR; At4g10860). QP-C functions in the Q-cycle by reducing cytochrome c with the consequent oxidation of ubiquinone (Bechmann *et al.,* 1992). The QP-C domain and the presence of three strong phosphorylation sites (Bendtsen, 2004) suggests a function in signal transduction; however, peptide sequence analysis shows no transmembrane domains (DAS Prediction Server) nor signal peptides that would be required to localize the protein to the chloroplast, mitochondrion or the secretory pathway (Bendtsen, 2004). Ubiquinone is also known to have antioxidant activity (Bentinger *et al.,* 2007). These qualities lead to the speculation that perhaps Unknown 2 functions as a molecular chaperone in transporting cytosolic ROS to membrane bound ubiquinone for detoxification. Whatever its function, the notable amount of this protein present at 0 hours in mutant but not WT roots suggest that it may contribute to the metal accumulation phenotype in the mutant, while its apparent suppression at timepoints corresponding to rapid increases in root Cu concentration suggest that, like CYS1 expressed only in mutants shoots, synthesis of this protein may be particularly susceptible to Cu toxicity.

Unknown 3 expression levels were relatively constant throughout the timecourse. According to TAIR (gi|9958068), the protein sequence corresponds to allene oxide cyclase (AOC). These proteins are involved in the biosynthesis of jasmonates, which are important signaling molecules and have been shown to be involved in the plant stress response (Wasternack & Hause, 2002). Significant background levels of AOC might, therefore, be expected in the mutant either as a modification resulting from the mutation or as a result of the

hydroponic growth environment, which may impart a certain amount of stress to plants accustomed to growing in soil.

4.4 Conclusion

There are several mechanisms by which a plant may respond to potentially toxic levels of Cu, including regulation of Cu uptake, chelation, efflux, sequestration/compartmentalization, and detoxification of ROS (Wintz & Vulpe, 2002). The mutant, frd3-3, has constitutive ferric chelate reductase activity and accumulates a variety of metals (Delhaize 1996). Any of the *frd* alleles studied to date have constitutively expressed strategy I responses to iron deficiency, where proton release is accompanied by ferric chelate reductase activity and ferrous transport even under non-limiting conditions. FRD, shown to be induced in the roots, is a member of the MATE (multi-drug and toxin efflux) family of transmembrane efflux proteins (Rogers & Guerinot, 2002). FRD may not transport iron itself but rather may function as a transporter for a metal chelator such as nicotianamine (NA) (Rogers & Guerinot, 2002) and thereby participate in the whole-plant localization of iron (Pittman, 2005). Ferric chelate reductase activity may be attributable to IRT1 (iron-regulated transporter1) (Eide *et al.,* 1996, Vert *et al.,* 2002) or FRO2 (ferric reductase oxidase2) (Robinson *et al.,* 1999), which interestingly also exhibits Cu(II) chelate reductase activity (Rogers & Guerinot, 2002). Rogers & Guerinot (2002) have speculated that the frd3-3 phenotype is a result of impaired iron sensing or incorrect localization of iron which could in turn lead to erroneous signaling from shoot to root.

The frd3-3 mutation specifically affects only one gene in the *A. thaliana* genome. However, compensatory mechanisms in the transcriptome and/or proteome may occur as a result. With constitutive ferric chelate reductase activity, metals in excess of requirements are transported into roots and translocated to shoots in the mutant (Delhaize, 1996). This is likely to cause changes in the regulation and expression of transportation, chelation, sequestration and signaling, a hypothesis strongly supported by the results of this study, in which many proteins involved in such processes were found to show high background expression and/or rapid induction in, respectively, mutant and WT tissues. Furthermore, only one of the proteins found in shoot extracts (glutathione transferase) and one of those found in root extracts (glutathione reductase) were detected only in the WT, whereas numerous Cu-binding root and shoot proteins

were found in the mutant tissue but not in the WT, suggesting that such proteins are involved in metal accumulation.

Various oxygen radicals are produced as a consequence of aerobic metabolism and plants have a variety of mechanisms to detoxify those radicals not required as metabolites. The abundance of intracellular ROS increases upon Cu stress (Drążkiewicz *et al.,* 2004). To deal with this, the mutant could have altered the regulation and expression of its suite of antioxidants to have constitutive expression. A corollary to this is the possibility that, due to the reported propensity of the mutant for metal accumulation (Delhaize, 1996), it should likewise have an altered internal amount of ROS. This could result in the re-setting of the thresholds for induction of antioxidant synthesis or activation.

Although it was hoped to isolate and characterize known, thiol-containing Cu transporters and chelators expressed in response to Cu exposure, these compounds were largely unable to be identified. One reason for this is that the techniques required for (metallo)proteome analysis are not optimal for isolation of compounds such as metallothioneins and phytochelatins, which are of relatively low molecular weight and readily oxidized. Specifically, the methodologies used here do not utilize non-oxidizing conditions or make use of thiol affinity technology (Murphy *et al.*, 1997), and are unlikely to isolate proteins below 10 kDa. In future it would be interesting to see the variation in the protein species isolated if these alterations were made. However, a significant number of Cu-binding proteins were isolated and identified using Cu(II)-IMAC, 1D SDS-PAGE and UPLC-MS/MS, most of which are apparently involved in various ways with plant defense responses to oxidative stress.

This general overview of the Cu proteomic response of plants is to the best of the author's knowledge a first. It has provided insights into the defense response and oxidative stress response of plants and moreover has pointed the way to many interesting and key questions for future investigation.

5.0 CHANGES IN THE GLOBAL AND COPPER PROTEOMES OF TWO *ARABIDOPSIS THALIANA* GENOTYPES IN RESPONSE TO COPPER EXPOSURE

5.1 Introduction

Proteomic analysis allows for the examination of changes in protein complement associated with different species, developmental stages, tissue locations, or responses to specific stimuli. Global proteomic surveys have been conducted for particular tissues (Kamo *et al,* 1995; Giavalisco *et al.,* 2005) and organelles (Kruft *et al.,* 2001; Friso *et al.,* 2004). Comparative global surveys have also been performed to examine the protein response to stimuli such as cold (Goulas *et al.,* 2006; Yan *et al.,* 2006), heat (Ferreira *et al.,* 2006; Majoul *et al.,* 2004; Sule *et al.,* 2004), salt stress (Abbasi & Komatsu, 2004) and heavy metals (Ingle *et al.,* 2005a; Requejo & Tena, 2005; Roth *et al.,* 2006; Sarry *et al.,* 2006). These global surveys examine the whole proteome. By comparing surveys from different times or in response to different stimuli, the differential control of the proteome can also be studied. By using targeted approaches such as affinity chromatography it is possible to further specify the sub-proteome under investigation based on its reactivity with a particular substrate, antibody or metal ion (Sun *et al.,* 2005; Smith *et al.,* 2004). Using immobilized metal-ion affinity chromatography (IMAC), for example, a specific metallo-proteome can be examined in particular parts of a plant, over time, and/or in response to an external stimulus such as metal exposure.

In previous studies (*Chapters 3 and 4*), hydroponically-grown *Arabidopsis thaliana* plants representing two genotypes (one metal-accumulating and one non-accumulating) were exposed to copper (Cu) over a 48-hour period. The two genotypes examined were (1) the metal accumulating frd3-3 mutant, and (2) the non-accumulating wildtype Columbia ecotype. A global proteomic survey was performed on roots and shoots of both genotypes over time and in response to Cu so as to examine the differential response of each proteome. The Cu-binding (metallo)proteomes of roots and shoots from the two genotypes over time were also isolated using Cu(II)-IMAC. Proteins from both the global and Cu-binding proteomes were analyzed by nanoLC-MS/MS and identified by database searching. By examining both the differential responses of the global proteome as well as the Cu proteome, insights into the mechanism of

metal accumulation will be possible. This study represents the first attempt at a coordinated examination of both the global and Cu proteomes in *Arabidopsis*.

The timed proteomic response of the two genotypes was examined in two ways. Firstly, proteins isolated from each genotype/tissue/timepoint combination were separated by 2D SDS-PAGE so as to examine the effect of Cu uptake over time on the entire plant proteome. Using this approach, it was also possible to examine the differential responses of individual proteins over time. Secondly, protein extracts were enriched for Cu-binding proteins using Cu(II)-IMAC. The resulting Cu-affinity enriched samples were separated by 1D SDS-PAGE. This method allowed for the specific investigation of the response of the Cu proteome in both genotypes over time.

While Cu-binding proteins are of obvious interest when studying the whole-plant proteomic response to Cu exposure, many proteins that are not metal-binding (or, more specifically, Cu-binding) also play pivotal roles in metal accumulation. For example, heavy metal accumulation can be deleterious to the structure and function of membranes, proteins and other biomolecules, either directly or indirectly (Dameron & Howe, 1998). It is also well known that *in situ* Cu accumulation causes the formation of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide (O_2^-), and hydroxyl (OH^-) (Drążkiewicz *et al.,* 2004). These oxygen radicals can likewise have deleterious effects on plant metabolism. Consequently, in addition to mechanisms for tolerance, uptake, translocation and sequestration of metal ions, the metal accumulation response of a plant must also involve tolerance and compensatory mechanisms for stress compounds such as ROS. Because the proteins involved in these mechanisms may or may not be metal-binding, it is important to examine the proteomes in these two ways (*i.e.* global and metal-targeted) in order to gain insights regarding the entire metal accumulation mechanism.

5.2 Results and Discussion

5.2.1 The Dual Approach: Isoforms of Superoxide Dismutase

The importance of using a dual approach to proteome analysis is exemplified by the identification of two very different isoforms of superoxide dismutase (SOD) in *Arabidopsis* plant

tissues. SODs are proteins known to be involved in the oxidative stress response of plants (Kliebenstein *et al.,* 1998). Different isoforms of SOD are characterized by the metal co-factors they contain. Using both the global and targeted methodologies, two SOD isoforms containing Cu and zinc (Cu/ZnSOD: gi|3273753) and manganese (MnSOD: At3g10920; gi|3273751) as co-factors were identified. While both isoforms were identified using the global proteome analysis procedures, only Cu/ZnSOD was identified using the Cu proteome procedure despite the fact that MnSOD was the more abundant isoform, based on global proteome analysis. This result demonstrates both the validity of the Cu(II)-IMAC procedure as well as the importance of both global and targeted methodologies in studying plant proteomic responses to metal exposure.

While neither isoform of SOD is likely to be involved directly in metal translocation, chelation or sequestration mechanisms, both isoforms may participate indirectly in metal accumulation by virtue of their roles as ROS scavengers. On closer inspection it can be seen that the primary sequences (Figure 5.1) of the two SOD isoforms share only 13% identity (Appendix 7) and that consequently, only one tryptic peptide mass is common to both isoforms (Figure 5.2). The fact that the database search results identified Cu/ZnSOD and MnSOD unambiguously, through the matching of several unique tryptic peptides, therefore confirms the presence of each isoform and hence, the specificity of the metal-targeted Cu(II)-IMAC method for Cu-binding proteins.

5.2.2 Hyperaccumulation Arises from the Differential Control of Existing Mechanisms

Cobbett (2003) has suggested that the basic machinery of hyperaccumulators and non-accumulators are practically identical and that it is differential gene regulation and protein network activities that account for the physiological differences. The results discussed previously in this thesis support this hypothesis. From the global, 2DE gel-based proteome analysis results summarized in Chapter 3, it was demonstrated that the distributions between functional categories of differentially expressed proteins in mutant and WT shoots were essentially the same, whereas the distributions in mutant and WT roots were markedly different. Mostly, the same proteins were identified in both mutant and WT plants; but it was the proportional representation of these proteins that differed. In roots, it was found that the mutant

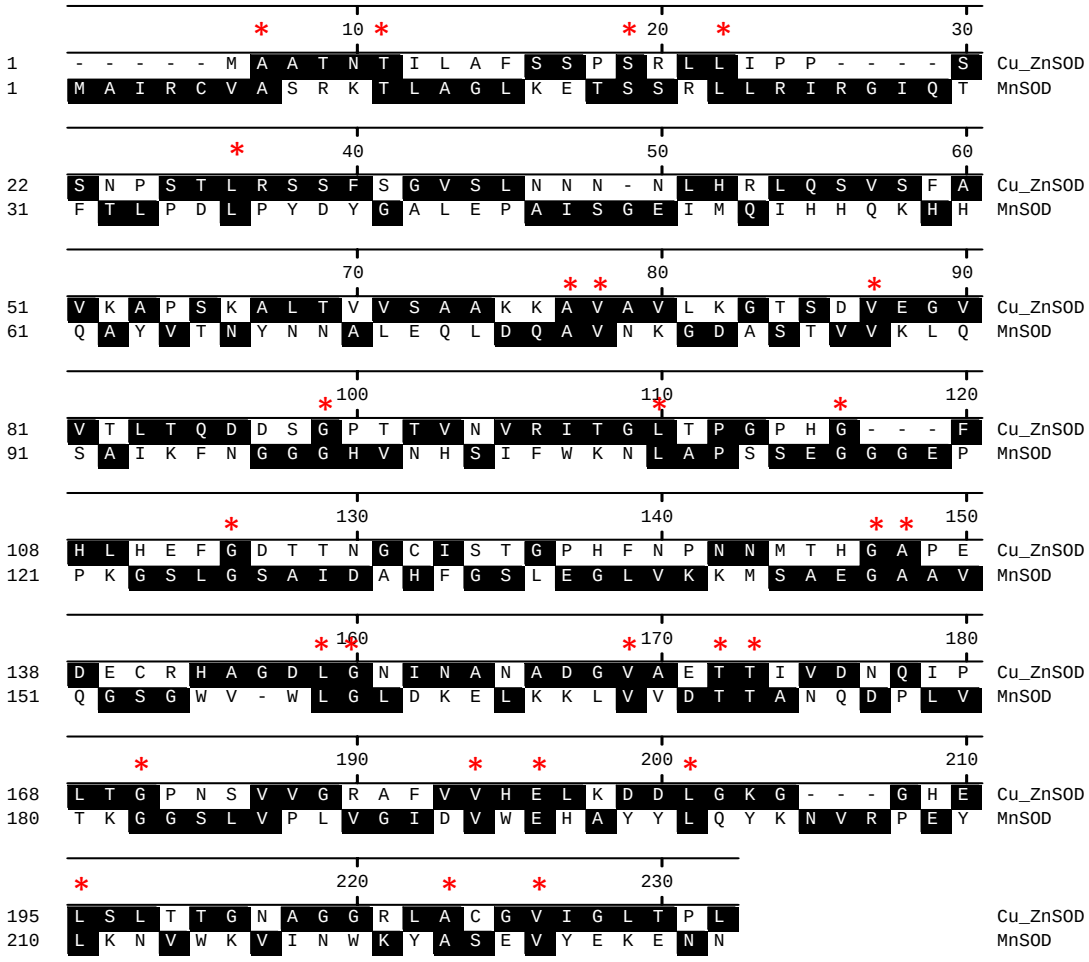

Figure 5.1: Clustal W protein alignment of Cu/ZnSOD and MnSOD. Black boxes indicate amino acids common to the consensus sequence. Red asterisks indicate amino acids common to Cu/ZnSOD and MnSOD.

Cu/ZnSOD

Tryptic Peptide Number	Peptide Sequence	Theoretical Mass (Da)
T7	(K)K(A)	146.11
T5	(K)APSK(A)	401.23
T13	(K)DDLGK(G)	546.26
T8	(K)AVAVLK(G)	599.4
T6	(K)ALTVVSAAK(K)	858.52
T12	(R)AFVVHELK(D)	941.53
T4	(R)LQSVSFAVK(A)	977.55
T15	(R)LACGVIGLYPL(-)	1055.6
T2	(R)LLIPPSSNPSTLR(S)	1393.79
T14	(K)GGHELSLTTGNAGGR(L)	1425.7
T1	(-)MAATNTILAFSSPSR(L)	1565.79
T3	(R)SSFSGVSLNNNNLHR(L)	1644.8
T9	(K)GTSDVEGVVTLTQDDSGPTTVNVR(I)	2446.18
T11	(R)HAGDLGNINANADGVAETTIVDNQIPLTGPNSVVGR(A)	3600.91
T10	(R)ITGLTPGPHGFHLHEFGDTTNGCISTGPHFNPNNMTHGAPEDECR(H)	4815.24

MnSOD

Tryptic Peptide Number	Peptide Sequence	Theoretical Mass (Da)
T18	(K)K(L)	146.11
T3	(R)K(T)	146.11
T15	(K)K(M)	146.11
T7	(R)IR(G)	287.2
T25	(K)ENN(-)	375.14
T17	(K)ELK(K)	388.23
T6	(R)LLR(I)	400.28
T1	(-)MAIR(C)	489.27
T2	(R)CVASR(K)	534.26
T22	(K)NVWK(V)	545.3
T5	(K)ETSSR(L)	578.27
T4	(K)TLAGLK(E)	601.38
T11	(K)LQSAIK(F)	658.4
T23	(K)VINWK(Y)	658.38
T10	(K)GDASTVVK(L)	775.41
T24	(K)YASEVYEK(E)	987.45
T21	(K)NVRPEYLK(N)	1017.56
T13	(K)NLAPSSEGGGEPPK(G)	1338.64
T12	(K)FNGGGHVNHSIFWK(N)	1598.77
T19	(K)LVVDTTANQDPLVTK(G)	1612.87
T14	(K)GSLGSAIDAHFGSLEGLVK(K)	1856.96
T16	(K)MSAEGAAVQGSGWVWLGLDK(E)	2061
T20	(K)GGSLVPLVGIDVWEHAYYLQYK(N)	2506.29
T9	(K)HHQAYVTNYNNALEQLDQAVNK(G)	2569.23
T8	(R)GIQTFTLPDLPYDYGAL	3584.07

Figure 5.2: Tryptic peptides of Cu/ZnSOD and MnSOD. Blue highlight indicates the only common tryptic peptide mass.

plants had a higher proportion of differentially expressed defence/stress response and metal-binding proteins than the WT, whereas the roots of WT plants had a higher proportional representation of proteins involved in transport and storage. Additionally, the mutant plants also showed a larger proportion of sulfur metabolism related proteins in the roots than did the WT plants.

Using the targeted, Cu(II)-IMAC and 1DE gel-based approach described in Chapter 4, a number of Cu-binding proteins were identified. Their expression in response to Cu exposure over time was examined, and their potential roles in either metal accumulation or stress response were discussed. The results from these two studies have directed the focus of this study to particular groupings of proteins; namely those involved in transport and storage, sulfur metabolism, metal binding, and the defence/stress response. Within these four functional categories, 31 proteins identified using both the global and metal-targeted proteomics approaches had identical database accession numbers (Table 5.1). In some cases, the same protein was identified as having several accession numbers. These were treated as different isoforms of the same protein if (1) such proteins were identified in different 2DE gel spots, or (2) if the isoform contained unique peptides that were not also matched to other isoforms in the same band/spot.

5.2.3 Expression Analysis of Individual Proteins Isoforms

During the course of the global proteomic survey (*Chapter 3*), each protein extract was separated twice using 2DE gel compositions chosen to optimize the resolution of high- and low-molecular weight proteins. Protein spots in the treatment (*i.e.* 8, 12 and 48 hour) gels that were found to be differentially expressed by a factor of at least ±2 when compared to the 0 hour control gel were selected for LC-MS/MS analysis and protein identification. Several protein spots in the treatment gels could not be matched with spots in the control gel, despite the successful matching of other spots common to control and treatment gels. Because such spots may well correspond to proteins that are induced by Cu exposure, and in the author's opinion, are therefore induced by a factor greater than +2, they too were selected for analysis and identification by LC-MS/MS.

Table 5.1: Proteins identified in both the global proteome survey and the targeted Cu proteome survey.

Defence/Stress
gi|15219086 ATPDIL1-1; protein disulfide isomerase
gi|15222089 PR5 (PATHOGENESIS-RELATED GENE 5)
gi|15222848 GAPC-2; glyceraldehyde-3-phosphate dehydrogenase
gi|15229231 GAPC (GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE C SUBUNIT)
gi|15229342 PR4 (PATHOGENESIS-RELATED 4)
gi|15232559 GR (GLUTATHIONE REDUCTASE)
gi|166807 peroxidase
gi|21592888 peroxidase
gi|2554769 Chain A, Structure Of Glutathione S-Transferase
gi|3273753 copper/zinc superoxide dismutase
gi|3399769 uclacyanin II
gi|8778432 Glutathione S-transferase
gi|15223517 CRT1 (CALRETICULIN 1)
gi|15231805 carboxylic ester hydrolase
gi|887390 osmotin
gi|15239883 ATCYS1 (A. THALIANA CYSTATIN-1); cysteine protease inhibitor
gi|9958068 Allene oxide cyclase
Sulphur Metabolism
gi|1279212 methionine sulfoxide reductase
gi|15238686 ATCIMS (COBALAMIN-INDEPENDENT METHIONINE SYNTHASE)
Protein and Amino Acid Synthesis
gi|15234781 ROC1; peptidyl-prolyl cis-trans isomerase
gi|22331076 peptidase/ subtilase
gi|2443757 cyclophilin
gi|14423478 glutamate dehydrogenase 2
gi|14517542 Peptidase_C1A
gi|15234360 GLB1; enzyme regulator
gi|18404441 glutamate N-acetyltransferase
Transport and Storage
gi|15241583 nutrient reservoir
gi|1755160 germin-like protein
gi|18202452 Germin-like protein subfamily T member 1 precursor
gi|2373399 vegetative storage protein
gi|512404 vegetative storage product

In many cases, several proteins and/or isoforms were identified in a single resolved gel spot (Table 5.2). In this context, the term *isoform* refers either to like proteins with different accession numbers, or to proteins identified in different gel spots and having the same accession number. Accordingly, different isoforms of a protein may represent different states of post-transcriptional or post-translational modification, either of which can affect the molecular weight and/or pI of a protein. For these reasons all unique isoforms were considered as individual proteins.

Although different gel compositions were used to maximize the resolution of individual proteins in the high and low molecular weight ranges, all gels were necessarily small format, given the total number of experiments involved. Consequently, multiple proteins were often identified in the same resolved spot, making it difficult to examine quantitatively the expression of individual proteins. Nevertheless, a small number of proteins that migrated to the same spot on at least two of the treatment gels were identifiable. Furthermore, it was possible to make inferences regarding the expression profiles using normalized spot volumes as determined by the Phoretix 2D™ software for those co-migrating proteins that met the following criteria:

(1) The protein was the only protein identified in the spot.

(2) The protein represented the top-ranked hit in a list of multiple proteins identified in the same spot. As has been shown previously (*Chapter 4 – Section 4.3.1.1- Shoot Proteins*; Ishihama *et al.,* 2005), the MASCOT™ probability based MOWSE score can be used as an indication of relative protein abundance. Therefore, in an ordered list of multiple protein hits, it can be assumed that the top-ranked hit represents the protein of greatest abundance.

(3) The protein was neither the only nor top-ranked hit in the spot, but the expression level of the entire spot was lower than for corresponding spots meeting criterion (1) or (2) in other gels. In such cases, the expression level of the protein of interest can be regarded as lower than in other gels, although the relative expression level of that individual protein cannot be determined.

Table 5.2: Numbers of protein spots and identified proteins for roots and shoots of two hydroponically-grown *A. thaliana* genotypes exposed to Cu over time.

Genotype/Tissue/Timepoint			Total Number of Resolved Spots	Total Number of Proteins Identified	Total Number of Upregulated Proteins
WT	Shoot	0 Hr	397	---	---
		8 Hr	428	1499	1299
		12 Hr	390	1233	1113
		48 Hr	204	459	411
	Root	0 Hr	399	---	---
		8 Hr	191	156	142
		12 Hr	250	289	264
		48 Hr	246	197	175
Mutant	Shoot	0 Hr	249	---	---
		8 Hr	440	1215	1118
		12 Hr	414	1156	1077
		48 Hr	374	271	236
	Root	0 Hr	370	---	---
		8 Hr	507	286	256
		12 Hr	423	162	136
		48 Hr	210	128	111

5.2.3.1 Root Proteins

Applying the aforementioned criteria, semi-quantitative expression profiling of root proteins was possible for one protein, glutaredoxin (GRX), in both mutant and WT (Figure 5.3; Table 5.3); two proteins, cystatin (CYS) and universal stress protein (USP) in WT only (Figure 5.4; Table 5.4); and a developmentally regulated plasma membrane polypeptides (DREPP) family protein in the mutant (Figure 5.5; Table 5.5).

In the roots of both the WT and the mutant, GRX expression did not become evident until 12 hours, and thus appeared to be induced in response to Cu. In the mutant, GRX expression did not appear to change markedly between 12 and 48 hours. In the WT, however, GRX expression at 12 hours was approximately half that in the mutant, increasing to similar levels at 48 hours. GRX participates in thiol-disulfide exchange reactions and is consequently involved in defense against oxidative stress (TAIR: gi|15242674). GRX activity causes the reduction of protein disulfide bridges (TAIR: gi|15242674), which, for certain proteins, could lead to enhanced metal chelation via reduced thiol groups. The observed increase in root Cu concentration from 0 to 48 hours (*Section 3.3.1 – Copper Uptake and Plant Performance*), and the resulting induction of proteins involved in Cu transport and/or chelation, is consistent with an increase in GRX expression to facilitate reduction and activation of thiol-containing transporters/chelators.

Expression of CYS in the WT roots at 8 hours was high relative to the control, but decreased over time to almost 50% of this maximum at 48 hours. CYS, a cysteine protease inhibitor, is known to be constitutively expressed in roots (Belenghi *et al.,* 2003), and so the elevated levels observed between 8 and 48 hours following exposure suggests induction by Cu. As CYS has been shown to block cell death caused by oxidative stress (Belenghi *et al.,* 2003), a consistently high expression upon continued exposure to Cu might be expected. The apparent decrease in CYS expression after 8 hours could be due to the toxic effects of Cu uptake on protein biosynthesis in general.

Like GRX, expression of USP in WT *A. thaliana* roots increased over time. This localized isoform of USP contains a domain that is similar to a small cytoplasmic bacterial protein (Kerk *et al.,* 2003). While no direct function or expression information exists for USPs in plants, its expression in bacteria has been shown to assist cell survival under conditions of stress such as osmotic shock, nutrient starvation, exposure to UV light and chemical toxicity (Nystrom

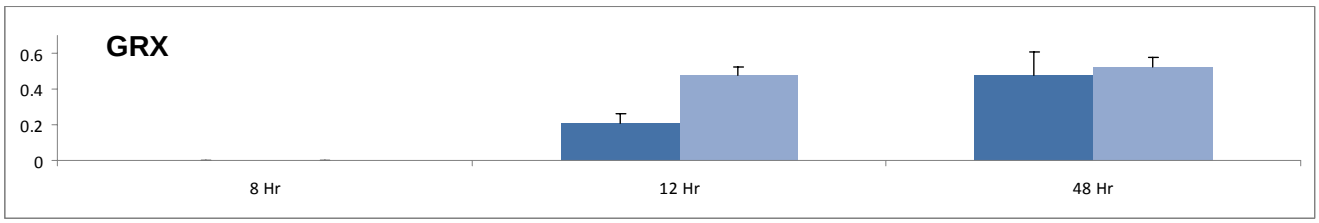

Figure 5.3: Expression profile of GRX in WT and mutant *A. thaliana* roots during Cu exposure. (- WT; - mutant). Data presented are the means of duplicate gels. Error bars represent + standard deviation.

Table 5.3: MS data for proteins identified in Figure 5.3. Threshold = the score over which an identified protein is considered a non-random event; MW = molecular weight; pI = isoelectric point; Score = individual score for the protein identified; # peptides = the number of unique peptides identified in the protein sequence; % Cov. = percentage of the total identified protein sequence represented by the identified peptides.

Protein Name	Genotype	Timepoint	Accession #	Threshold	MW	pI	Score	# peptides	% Cov.
GRX	WT	12 hour	gi|15242674	36	11920	6.71	90	2	20
		48 hour		37			67	1	12
	Mutant	12 hour		37			177	3	32
		48 hour		39			59	2	20

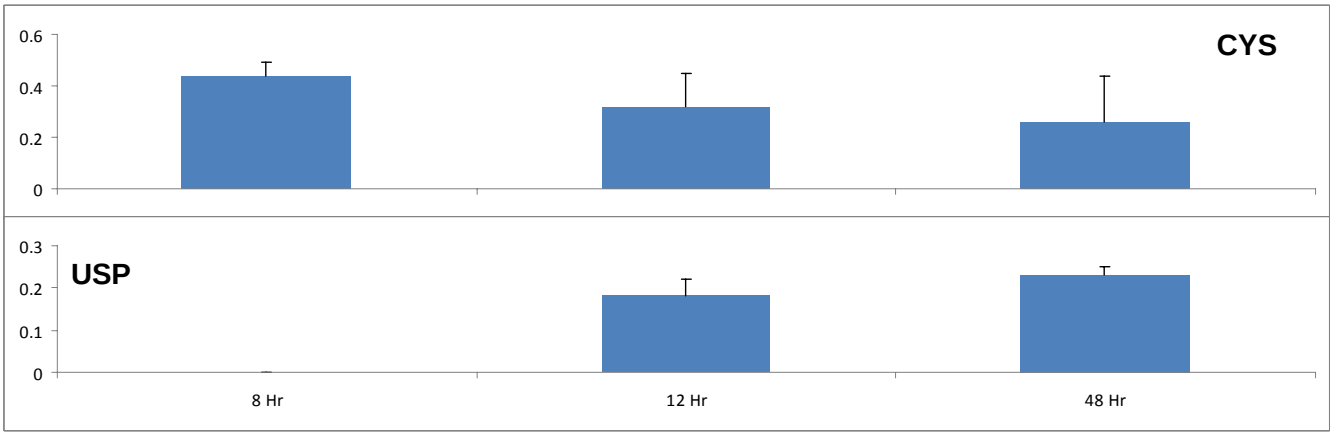

Figure 5.4: Expression profiles of CYS and USP in WT *A. thaliana* roots during Cu exposure. (- WT) Data presented are the means of duplicate gels. Error bars represent + standard deviation.

Table 5.4: MS data for proteins identified in Figure 5.4. Threshold = the score over which an identified protein is considered a non-random event; MW = molecular weight; pI = isoelectric point; Score = individual score for the protein identified; # peptides = the number of unique peptides identified in the protein sequence; % Cov. = percentage of the total identified protein sequence represented by the identified peptides.

Protein Name	Genotype	Timepoint	Accession #	Threshold	MW	pI	Score	# peptides	% Cov.
CYS	**WT**	**8 hour**		36			109	1	15
		12 hour	gi|15239883	37	11249	5.07	100	1	15
		48 hour		37			78	1	15
USP	**WT**	**12 hour**	gi|30693971	37	17896	5.66	166	6	26
		48 hour		37			90	2	13

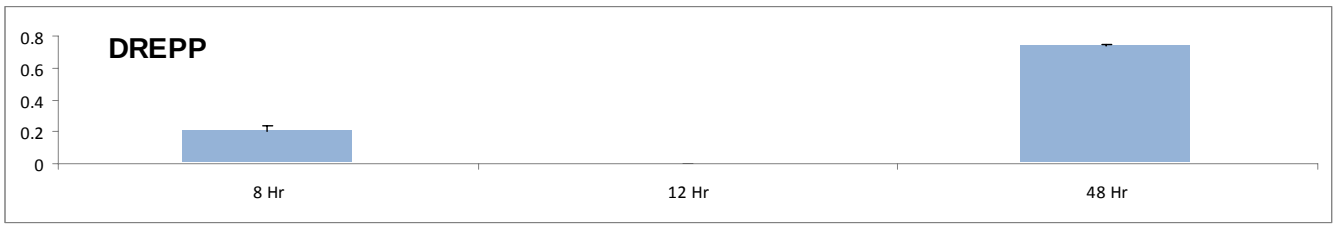

Figure 5.5: Expression profile of DREPP in mutant *A. thaliana* roots during Cu exposure. (- mutant) Data presented are the means of duplicate gels. Error bars represent + standard deviation.

Table 5.5: MS data for proteins identified in Figure 5.5. Threshold = the score over which an identified protein is considered a non-random event; MW = molecular weight; pI = isoelectric point; Score = individual score for the protein identified; # peptides = the number of unique peptides identified in the protein sequence; % Cov. = percentage of the total identified protein sequence represented by the identified peptides.

Protein Name	Genotype	Timepoint	Accession #	Threshold	MW	pI	Score	# peptides	% Cov.
DREPP	**Mutant**	**8 hour**	gi|15235363	38	24569	4.99	47	1	5
		48 hour		37			148	4	16

& Neidhardt, 1992, 1993, 1994). It is possible, therefore, that the apparent induction of USP in WT plants is either a direct response to chemical (*i.e.* Cu ion) toxicity or an indirect response to ROS generated by Cu.

The expression of DREPP was examined in mutant root tissues. Although not evident at 12 hours, expression increased over time between the 8 and 48 hour timepoints. Because it contains a C-terminal Ca^{+2}-binding domain, it has been suggested that DREPP might play a role in Ca^{+2}-signaling (Yuasa & Maeshima, 2000). There is also evidence to suggest that DREPP proteins are involved in cold acclimation (Kawamura & Uemura, 2003). Although DREPP proteins have not been studied extensively, it is possible that induction of DREPP may not be limited to just low temperature stress. Furthermore, its putative role in signal transduction suggests that DREPP might be involved in mediating expression of other defense or stress related proteins. As previously discussed with reference to CRT1 (*Section 4.3.2.2 – Root Proteins*), it is also physically possible for a Cu^{+2} ion to occupy a Ca^{+2} binding site based on ionic radii. In an environment of excess free Cu it is, therefore, conceivable that DREPP might function as a Cu chelator, although if this were the case its isolation and identification using the targeted Cu- proteome methodology (see *Section 5.3.1. – The Dual Approach: Isoforms of SOD*) would have been expected.

5.2.3.2 Shoot Proteins

No co-migrating shoot proteins were found to meet the selection criteria for expression analysis in both WT and mutant gels. Semi-quantitative expression was however, possible for certain proteins identified in WT shoots; namely, one isoform each of DREPP and heat-shock protein (HSP) and two isoforms of pathogenesis-related (PR) protein (Figure 5.6; Table 5.6).

Expression of DREPP in WT shoots increased between 8 and 12 hours, but expression was not evident at 48 hours. The function and possible roles for DREPP have been discussed previously in relation to the mutant root protein expression profiles (*Section 5.3.3.1Root Proteins*). Here it appears that, as in the roots, DREPP is induced in response to Cu exposure.

The expression profiles for the HSP isoform in WT shoots was similar to that for DREPP, increasing from 8 to 12 hours but diminishing to levels at or below the control at 48 hours. HSPs

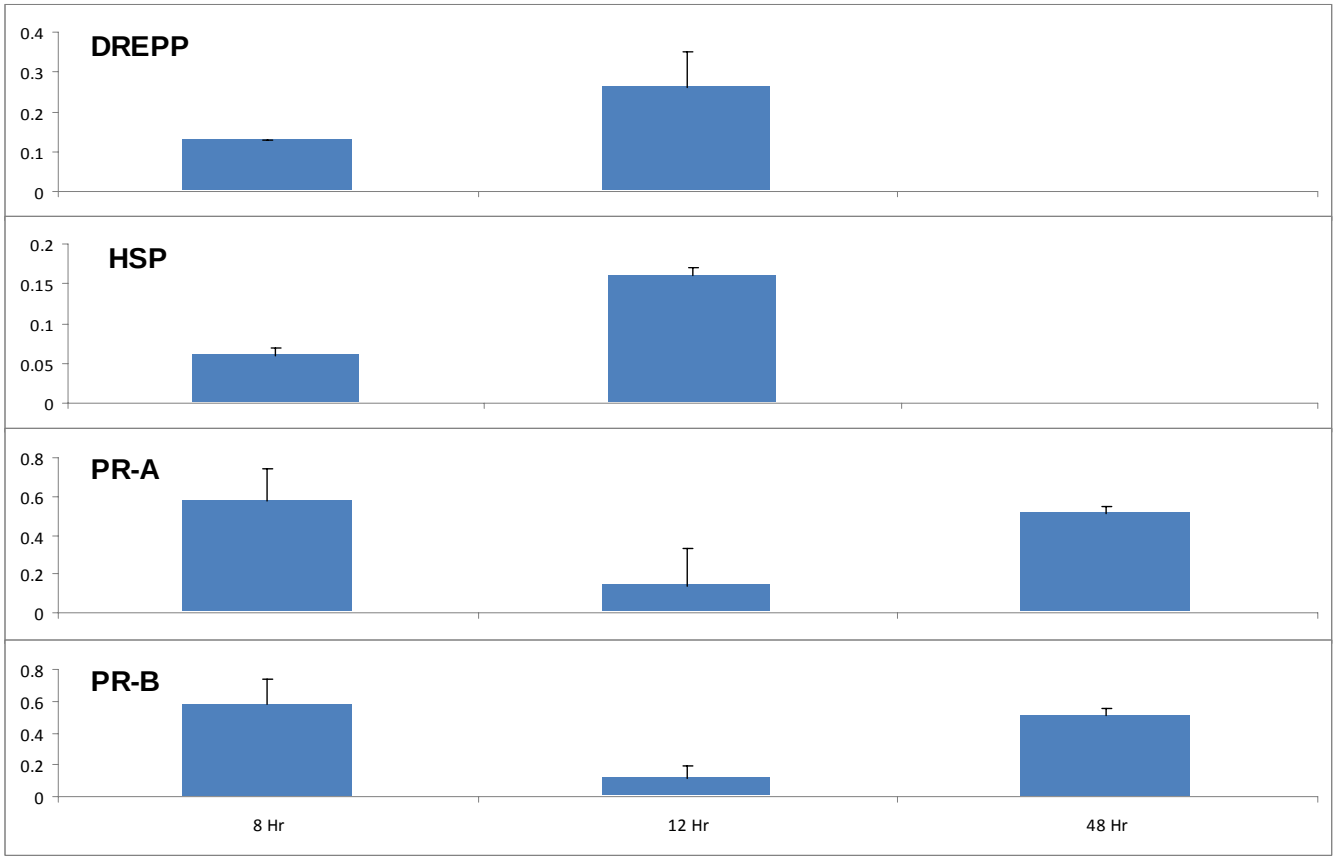

Figure 5.6: Expression profiles of DREPP, HSP and two PR isoforms in WT *A. thaliana* shoots during Cu exposure. (- WT) Data presented are the means of duplicate gels. Error bars represent + standard deviation.

Table 5.6: MS data for proteins identified in Figure 5.6. Threshold = the score over which an identified protein is considered a non-random event; MW = molecular weight; pI = isoelectric point; Score = individual score for the protein identified; # peptides = the number of unique peptides identified in the protein sequence; % Cov. = percentage of the total identified protein sequence represented by the identified peptides.

Protein Name	Genotype	Timepoint	Accession #	Threshold	MW	pI	Score	# peptides	% Cov.
DREPP	**WT**	**8 hour**	gi|15235363	39	24569	4.99	152	4	20
		12 hour	gi|30685040	40			750	21	60
HSP	**WT**	**8 hour**	gi|6746592	40	77230	5.13	488	9	13
		12 hour	gi|397482	39	71726	5.03	568	11	19
PR - A	**WT**	**8 hour**					63	2	7
		12 hour	gi|15230262	40	37373	4.85	296	5	16
		48 hour					211	3	9
PR - B	**WT**	**8 hour**					63	2	7
		12 hour	gi|15230262	40	37373	4.85	517	10	31
		48 hour					211	3	9

function as molecular chaperones and thus participate in protein folding, degradation, localization and accumulation (Feder & Hofmann, 1999). HSPs can be induced by low temperatures (Sabehat *et al.,* 1998), osmotic stress (Sun *et al.,* 2001), salt (Liu *et al.,* 2006), oxidative stress (Banzet *et al.,* 1998; Lee *et al.,* 2000; Desikan *et al.,* 2001; Volkov *et al.,* 2006), desiccation (Liu *et al.,* 2006), exposure to intense light (Hihara *et al.,* 2001; Rossel *et al.,* 2002), wounding (Cheong *et al.,* 2002), and heavy metal exposure (Györgyey *et al.,* 1991). HSPs are thought to protect plants from ROS damage by preventing deleterious protein conformations and/or aggregations, thereby increasing tolerance to environmental stress (Swindell *et al.,* 2007). The observed expression profile is consistent with heavy metal exposure and/or increased levels of ROS, and a general decrease in protein expression after 48 hours of exposure to Cu.

Expression profiles for the two PR isoforms were similar to one another but quite different from those of DREPP and HSP. PR expression appeared to be cyclic, in that induction peaked at 8 and 48 hours of exposure but dropped at 12 hours. This global, isoform-specific analysis contrasts with the pattern of Cu-binding PR expression described in Chapter 4, which appeared to remain low initially but to increase sharply in WT shoots after 48 hours of exposure. Although their specific function remains unknown, PR proteins are involved in the defense responses of plants and are known to participate in systemic acquired resistance (SAR) (Bol *et al.,* 1990). The cyclic expression profiles observed here suggest that PR proteins are induced in response to increasing levels of Cu or oxygen radicals but that, following initial induction, the pool of newly synthesized proteins becomes depleted before more protein can be synthesized.

Eight proteins met the selection criteria for expression profiling in mutant shoots. These included one isoform of GRX; two isoforms each of cyclophilin, Cu/ZnSOD, osmotin (OSM), peroxiredoxin (PRX), protein disulfide isomerase-like protein (PDIL), cobalamin-independent methionine synthase (CIMS) and one isoform of ferredoxin (FRX) (Figures 5.7A, B; Tables 5.7A, B).

Expression of GRX was found to be relatively high and constant between the 8 and 12 hour timepoints, but fell sharply at 48 hours. This expression profile was also observed for Cu-binding GRX, as described in (*Section 4.3.1.1 – Shoot Proteins*). The function of GRX has been discussed previously in reference to its expression in WT and mutant root tissues. As was the

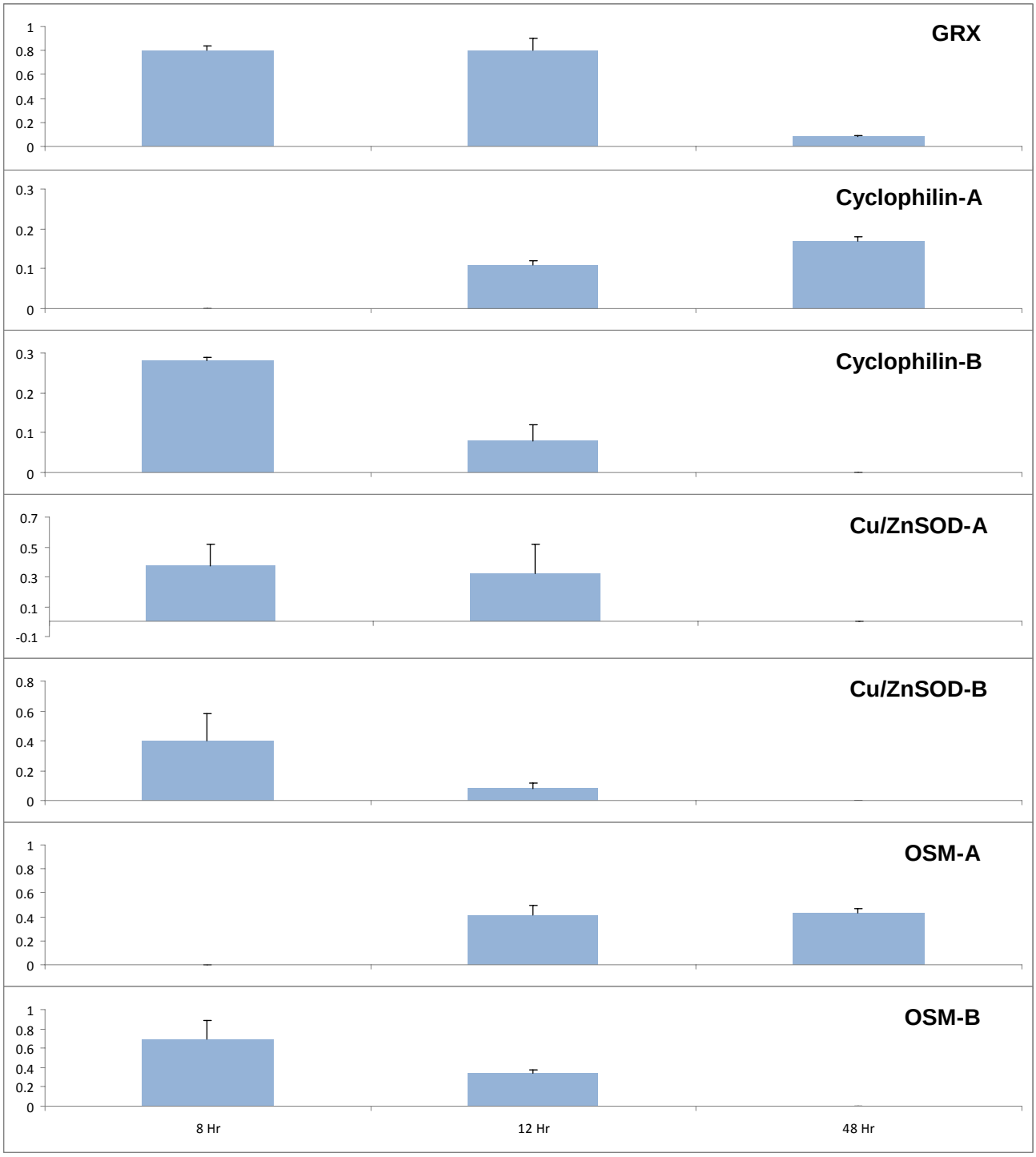

Figure 5.7A: Expression profiles of GRX, cyclophilin, Cu/ZnSOD and OSM in mutant *A. thaliana* shoots during Cu exposure. (- mutant) Data presented are the means of duplicate gels. Error bars represent + standard deviation.

Table 5.7A: MS data for proteins identified in Figure 5.7A. Threshold = the score over which an identified protein is considered a non-random event; MW = molecular weight; pI = isoelectric point; Score = individual score for the protein identified; # peptides = the number of unique peptides identified in the protein sequence; % Cov. = percentage of the total identified protein sequence represented by the identified peptides.

Protein Name	Genotype	Timepoint	Accession #	Threshold	MW	pI	Score	# peptides	% Cov.
GRX	**mutant**	**8 hour**		37			136	2	24
		12 hour	gi|15242674	36	11920	6.71	201	4	39
		48 hour		38			67	1	12
Cyclophilin - A	**mutant**	**12 hour**	gi|11762200	36	28532	8.96	125	3	11
		48 hour		37			96	2	8
Cyclophilin - B	**mutant**	**8 hour**	gi|11762200	37	28532	8.96	153	3	11
		12 hour		36			125	3	11
Cu-ZnSOD - A	**mutant**	**8 hour**	gi|3273753	37	22332	6.28	92	2	12
		12 hour		37			76	2	16
Cu-ZnSOD - B	**mutant**	**8 hour**	gi|3273753	36	22332	6.28	242	5	23
		12 hour		36			205	4	23
OSM - A	**mutant**	**12 hour**	gi|15226956	37	27912	7.61	150	3	12
		48 hour		37			57	1	4
OSM - B	**mutant**	**8 hour**	gi|15226956	37	27912	7.61	269	5	22
		12 hour		39			63	1	4

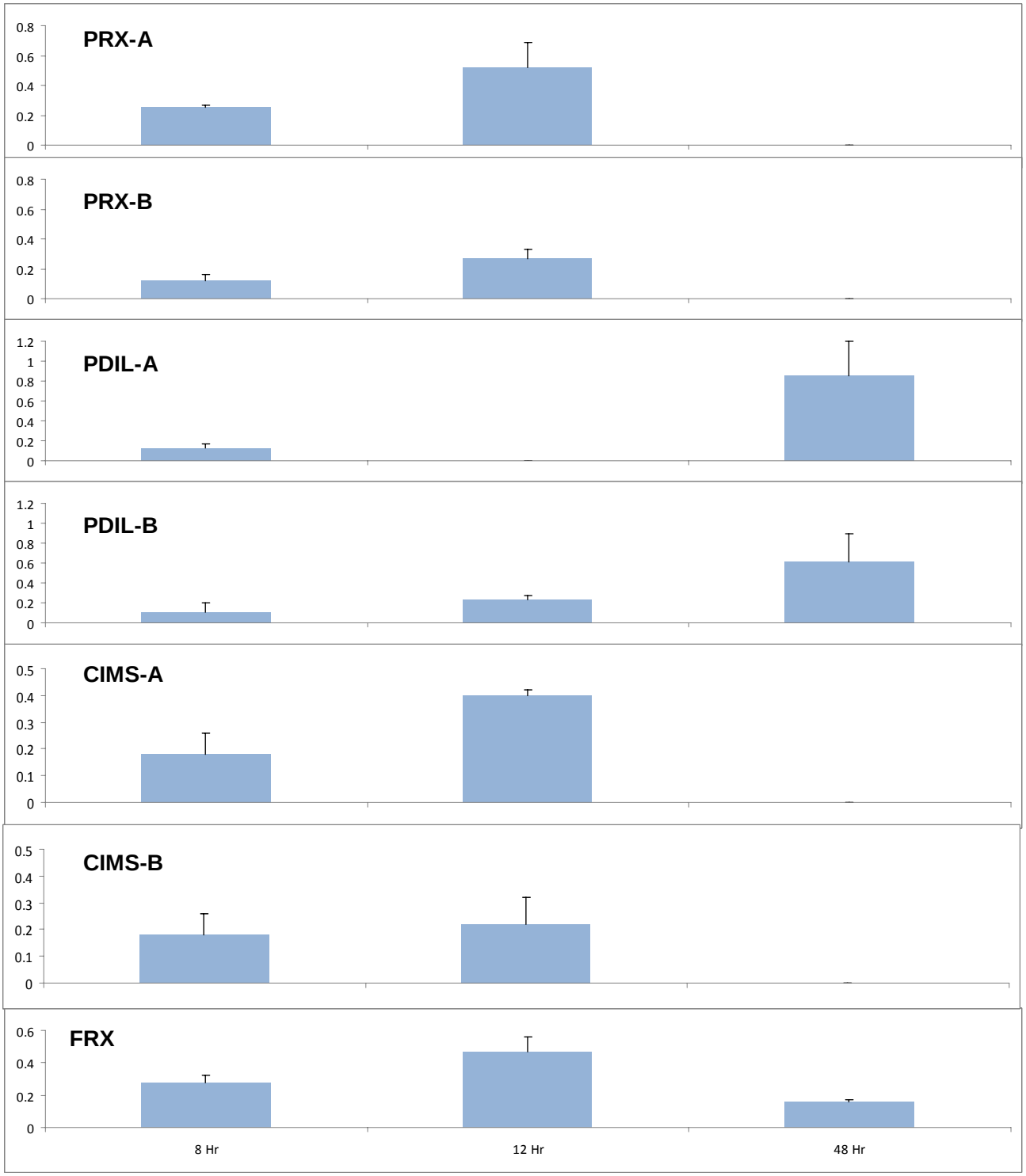

Figure 5.7B: Expression profiles of PRX, PDIL, CIMS and FRX in mutant *A. thaliana* shoots during Cu exposure. (- mutant) Data presented are the means of duplicate gels. Error bars represent + standard deviation.

Table 5.7B: MS data for proteins identified in Figure 5.7B. Threshold = the score over which an identified protein is considered a non-random event; MW = molecular weight; pI = isoelectric point; Score = individual score for the protein identified; # peptides = the number of unique peptides identified in the protein sequence; % Cov. = percentage of the total identified protein sequence represented by the identified peptides.

Protein Name	Genotype	Timepoint	Accession #	Threshold	MW	pI	Score	# peptides	% Cov.
PRX - A	**mutant**	**8 hour**	gi|8778521	36	54037	6.96	739	13	39
		12 hour		38			949	15	32
PRX - B	**mutant**	**8 hour**	gi|8778521	37	54037	6.96	347	9	24
		12 hour		38			557	11	25
PDIL - A	**mutant**	**8 hour**	gi|15226610	37	39815	5.8	407	9	26
		48 hour		38			324	7	18
PDIL - B	**mutant**	**8 hour**		36			113	4	11
		12 hour	gi|15226610	38	39815	5.8	233	4	11
		48 hour		38			66	1	2
CIMS - A	**mutant**	**8 hour**	gi|15238686	37	84646	6.09	71	3	4
		12 hour		39			736	18	19
CIMS - B	**mutant**	**8 hour**	gi|15238686	37	84646	6.09	71	3	4
		12 hour		38			660	15	18
FRX	**mutant**	**8 hour**		37			492	11	33
		12 hour	gi|8778996	38	39147	8.65	549	12	27
		48 hour		37			62	2	5

case in the Cu proteome survey, the observed expression pattern of GRX here is suggestive of constitutive expression as a further modification of the plant to the metal accumulation phenotype.

Two isoforms of cyclophilin met the criteria for expression analysis in mutant shoots. However, these two isoforms showed opposite trends, one increasing and the other decreasing over time. One possible explanation for these differing profiles is that they arise from different post-transcriptional and/or post-translational modifications of cyclophilin and could represent the conversion of one ioform to the other, which could cause a change in pI and/or molecular weight over time. Alternatively, combination of the two expression profiles suggests a cyclic trend for cyclophilin, much like that observed for PR in WT shoots. In contrast, the expression pattern of cyclophilin isolated from mutant shoots using Cu(II)-IMAC (*Section 4.3.1.1 – Shoot Proteins*) showed an increase from 8 to 12 hours with no sign of induction at 48 hours, possibly because the global and Cu proteomic procedures target different cyclophilin isoforms. These proteins are believed to be involved in stress response functions such as signaling (Yurchenko *et al.,* 2002), complex stabilization (Leverson & Ness, 1998) and apoptosis (Lin & Lechleiter, 2002) as they are involved in a rate limiting step of protein folding (Brandts *et al.,* 1975). Cyclophilins can be induced by both biotic and abiotic stresses (Marivet *et al.,* 1992, 1994, 1995; Chou & Gasser, 1997; Kong *et al.,* 2001; Sharma & Singh, 2003). The rapid induction of cyclophilin isoforms in mutant shoots 8 hours after exposure to Cu is consistent with these previous reports, suggesting a role for these proteins in metal accumulation.

A pattern of decreasing expression over time was observed for both isoforms of Cu/ZnSOD that met the expression profile criteria in mutant shoots. The Cu/ZnSOD expression profile determined using Cu(II)-IMAC (*Chapter 4*) also demonstrated induction in response to Cu exposure, falling to undetectable levels by 48 hours. SODs are one of the two main scavengers that function to detoxify ROS (Beyer & Fridovich, 1987). Specifically, SODs convert superoxide to either molecular oxygen or H_2O_2 (Kliebenstein *et al.,* 1998). Cu/ZnSODs are found in the chloroplast, the cytosol and in extracellular spaces (Alscher *et al.,* 2002). Given the key role played by SODs in cellular redox homeostasis, it is somewhat surprising that the high expression levels observed at 8 hours are not sustained over a longer time period. However, the trends observed using global and Cu proteome are consistent, and most likely reflect the toxic effects of Cu accumulation on protein biosynthesis at later timepoints.

Two isoforms of osmotin qualified for expression analysis in the mutant shoots. One showed strong induction at 8 hours but decreased to undetectable levels by 48 hours, while the other showed a similar level of induction, but at 12 hours instead of 8, and remained at similar levels until 48 hours. This apparent discrepancy could again be due to distribution of osmotin between two (modified) isoforms since, on aggregate, the overall expression or OSM remained uniformly high from 8 hours onwards. OSM is a vacuolar protein (Melchers *et al.,* 1993) and a member of the thaumatin-like family of proteins (Capelli *et al.,* 1997). OSM is believed to participate in SAR although its exact molecular function is unknown (Hu & Reddy, 1997). As discussed previously, OSM contains a transmembrane domain and seven putative disulfide bonds (*Section 4.3.2.2 – Root Proteins*). These attributes led to earlier speculation (*Chapter 4 – Section 4.3.1.2 Root Proteins*), that OSM may function as a previously undocumented chaperone for delivery of excess metal ions to the vacuole for sequestration. The combination of the profiles for the two isoforms shows an apparent maximum in OSM expression at 12 hours, a trend consistent with its potential role as a metal chaperone.

The two PRX isoforms included in this analysis both showed increasing expression from 8 to 12 hours but were undetectable by 48 hours. Peroxiredoxin is a type of peroxidase that is localized to the chloroplast (Baier & Dietz, 1997) and functions to reduce H_2O_2 and alkyl peroxides to water and alcohols respectively (Bréhélin *et al.,* 2003). It has been shown that PRX participates as an antioxidant, a redox sensor, and in signaling pathways (Dietz *et al.,* 2006). Evidence also demonstrates that the biosynthesis and activity of PRX proteins increases under conditions of stress (Foyer *et al.,* 1994; Karpinski *et al.,* 1997; Kliebenstein *et al.,* 1998; Baier *et al.,* 2000; Rossel *et al.,* 2002; Horling *et al.,* 2003; Mittler *et al.,* 2004). Unlike other peroxidases, PRXs do not contain metal co-factors (Bréhélin *et al.,* 2003), and so the fact that PRX was not detected during Cu proteome analysis again highlights the importance of using both the global and targeted approaches. As internal metal concentrations are increasing in the shoots from 0 to 48 hours, it can also be expected that the concentration of oxygen radicals such as peroxide are also increasing. This explains the consequent increase in PRX. The lack of detectable protein at 48 hours could be due either to Cu toxicity or to the depletion of synthesized protein to below detectable levels.

Expression of PDIL isoforms increased from relatively low-level induction at 8 hours to levels at least 3 times higher at 48 hours. Localized to the endoplasmic reticulum (ER), PDIL is

a member of the thioredoxin family of proteins (Houston *et al.,* 2005) and functions in cellular redox homeostasis by catalyzing the formation, isomerization and reduction/oxidation of disulfide bonds (Wilkinson & Gilbert, 2004). PDIL also plays an important role in protein secretion and storage (Houston *et al.,* 2005). The profile of induction demonstrated by PDIL could be expected as a response to the increasing levels of ROS that likely accompany the increase in Cu ions. Although it was previously speculated that, in general, the apparent repression and/or degradation of proteins at 48 hours is likely due to Cu toxicity, the expression of PDIL increases 4-fold during this same period. Because PDIL is localized in the ER, it may remain segregated from free Cu ions so that the detrimental effects of Cu on protein integrity are avoided. In any event, the increasing levels of PDIL observed in mutant shoots in response to Cu exposure strongly suggests a role for this protein in metal accumulation in harverstable tissues.

The two isoforms of CIMS that met the criteria for expression profiling both showed increased expression in mutant shoots between 8 and 12 hours exposure, but fell to below detectable limits by 48 hours. CIMS, a cytosolic protein, catalyzes the conversion of homocysteine to methionine, and therefore plays a key role in methionine synthesis (Eichel *et al.,* 1995) and sulfur metabolism. As many of the proteins involved in either metal chelation or ROS scavenging contain sulfur, and are induced by increasing metal and/or ROS concentrations, a concomitant increase in the expression of proteins involved in sulfur metabolism is to be expected. Furthermore, the apparently high susceptibility of CIMS to Cu toxicity after 48 hours of exposure is consistent with high cytoplasmic concentrations of free Cu and the fact that CIMS is a cytosolic protein.

The expression pattern of FRX identified in mutant shoots demonstrates a cyclic profile with expression increasing from 8 to 12 hours and subsequently decreasing at 48 hours. FRX functions as an electron donor and, although known mostly for its photosynthetic role, has also been shown to participate in other essential cellular processes such as nitrogen and sulfur assimilation, amino acid biosynthesis and redox regulation (Hanke *et al.,* 2004). The increase in expression from 8 to 12 hours is therefore to be expected, since increasing concentrations of both Cu and ROS are likely to induce expression of defense or stress response proteins, some of which may depend on FRX for reduction and/or activation.

In making comparisons between the expression patterns observed in the global and Cu proteomes it is important to note that, in most cases, the semi-quantitative profiles obtained from

global proteome analysis represent the expression patterns of individual isoforms of a protein. In contrast, the qualitative expression patterns determined by studying the Cu proteome represent all isoforms of a particular protein that interact with the immobilized Cu ions on an IMAC column. Some discrepancies between global (semi-quantitative) and Cu (qualitative) proteomes may therefore occur, as discussed above. Furthermore, in addition to affecting pI and/or molecular weight, differences in post-transcriptional or post-translational modification could impact the Cu-affinity of a protein.

5.2.4 Expression Patterns of Ubiquitous Proteins

A number of important storage and stress/defense-related proteins were present in high abundance in one or more plant tissue extracts. The proteins discussed below were either numerous in at least one tissue/timepoint or were widely distributed throughout the gel(s). The ubiquitous nature of certain proteins, both in terms of the number and distribution of isoforms detected on 2DE gels, is exemplified by the results obtained for differentially phosphorylated cruciferin isoforms/splice variants extracted from *A. thaliana* seeds by Wan *et al.* (2007). Any interpretation placed on the semi-quantitative expression pattern for an individual isoform of such a protein would be of limited value. Instead, the total number of isoforms of the important ubiquitous proteins glutathione-*S*-transferase (GST), peroxidase (POX), catalase (CAT) and vegetative storage protein (VSP) identified in each tissue/timepoint has been used as a qualitative indication of their general expression (Figure 5.8).

For both genotypes, the number of identified GST isoforms was much higher in shoots than in roots, for which the number of isoforms was similar between genotypes and at all timepoints. While the number of isoforms identified in WT and mutant shoots was similar at both 12 and 48 hours, a marked difference was observed at 8 hours, levels being initially higher than at 12 hours in the mutant and lower in the WT. Known to be induced in response to stressors such as ROS, heat, heavy metals and infection (Marrs, 1996), GSTs function in cellular detoxification in the cytosol (Wagner *et al.,* 2002). The pattern observed in the WT shoots suggests induction of GST in response to Cu, and is similar to the Cu proteome expression pattern observed for GST. Earlier work (*Chapter 4*) led to the speculation that the mutant phenotype may employ constitutive expression of certain proteins, such as those involved in

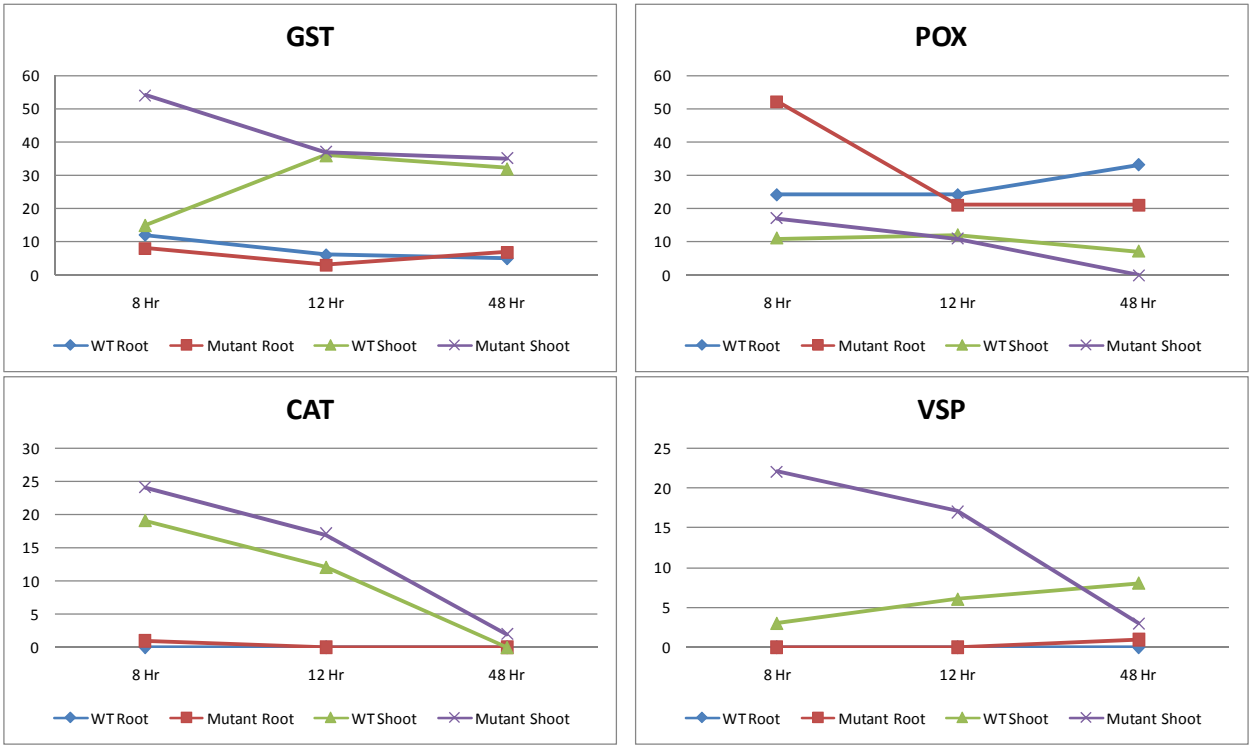

Figure 5.8: Total number of isoforms of GST, POX, CAT and VSP identified in the roots and shoots of WT and mutant *A. thaliana* plants during exposure to Cu.

defense or stress reactions, as a preparative measure for metal exposure. A drop from the elevated number of GST isoforms observed in the mutant shoots at 8 hours to WT levels at 12 and 48 hours suggests a return by the mutant to the WT control of this protein following an initial preparative state for Cu exposure.

The numbers of POX isoforms were lower in the shoots of both genotypes than in the roots. Overall, the numbers of shoot isoforms decreased over time with a slightly more pronounced decrease in the mutant. This pattern was also broadly observed in the Cu proteome. The number of isoforms also decreased over time in the mutant roots. At 8 hours, more than 50 isoforms were identified, whereas half that number was observed at 12 and 48 hours. In contrast, the number of isoforms identified in WT roots increased over time. Peroxidase is important in the detoxification of ROS, acting as a scavenger of H_2O_2 (Kvaratskhelia *et al.,* 1999). Induced in response to stress, POXs are located in vacuoles and extracellular spaces (NCBI; CDD: 29388). The higher number of POX isoforms in mutant roots and shoots when compared to the WT at 8 hours could be an indication of constitutively elevated numbers of defense related proteins associated with the metal accumulating mutant phenotype. Given the role of POX as an antioxidant, the number of isoforms identified in mutant roots could have been expected to have either increased or remained high at 12 hours. That such a trend is observed in WT but not in mutant roots suggests that the decreasing number of isoforms identified in the mutant over time cannot be attributed to Cu inhibition, but that the initially high number found in the mutant may again be indicative of a rapid response that obviates the need for sustained production of POX over time.

The numbers of catalase isoforms identified in the roots of both genotypes were relatively low. In the shoots, however, the numbers of isoforms were much higher at 8 hours and decreased at similar rates in both genotypes to very low levels at 48 hours. Although the numbers of isoforms were slightly higher in roots and shoots of the mutant when compared to the WT, the numbers are quite similar. This suggests that, in the case of catalase, expression in the mutant phenotype remains largely unchanged compared with the WT. Catalase, an important antioxidant, is mainly found in vascular tissues and leaves (McClung, 1997) and catalyzes the conversion of H_2O_2 to water (Dat *et al.,* 2000). Catalases have long been linked with the production of oxygen radicals that arise as a consequence of photorespiration. Catalases are highly sensitive to environmental stress (Matters *et al.,* 1986; Willekens *et al.,* 1994; Scandalios

et al., 1997) and consequently, it has been suggested that early degradation of catalase serves as a signal for antioxidant defense mechanisms (Feierabend, 1996). This is certainly supported by these results in that the number of isoforms of catalase in the leaves decreases rapidly upon uptake of Cu by the plant.

The number of isoforms of VSP was highly divergent between mutant and WT shoots. Very few VSPs were found in the roots of either genotype. In shoots, at the 8 hour timepoint, VSP isoforms in the mutant were 4-times as numerous as in the WT. Subsequently, the number of isoforms decreased rapidly in the mutant whereas the number of isoforms increased slightly in the WT. This trend continued until the WT isoforms outnumbered those in the mutant at 48 hours. A similar trend in VSP expression was observed when using the targeted Cu proteome approach (*Chapter 4 – Section 4.3.1.2 Root Proteins*). Carbon and nitrogen stores are housed in the vacuoles by VSPs for later use in growth and development (Utsugi *et al.,* 1998). Induction of VSP genes has been shown in response to environmental stress, and there is speculation that these proteins serve as a nitrogen source for the biosynthesis of molecules involved in plant defense or repair (Utsugi *et al*., 1998). WT shoot expression of VSP appears to be induced by Cu exposure. In accordance with the elevated metal loads expected of the mutant phenotype, it is possible that such plants tend to maintain relatively high levels of VSPs (at least in their roots) in readiness of the requirement for rapid synthesis of defense products. VSPs may also possess some metal-chelating ability and, in a circumstance of excess free metal ions, could serve as an alternate sequestration pathway. As with POX, the decreased expression in the mutant shoot cannot be attributed to Cu sensitivity, since the number of VSP isoforms increases in the WT over time.

In general, the qualitative analysis of expression based on numbers of identified isoforms (detailed above) supports the qualitative expression data obtained by targeted analysis of the Cu proteome (*Chapter 4*). This is encouraging, since both interpretations are based on the total isoform populations of individual protein species and should, therefore, give comparable results.

5.4 Conclusion

The mechanism of metal accumulation is necessarily complex as it involves the co-ordination of compounds involved in tolerance, uptake, translocation, chelation and sequestration

of metal ions. Compounding this complexity is the production and control of proteins and other biomolecules involved in stress responses. The large-scale proteomic approach and the different interpretations of protein expression undertaken here have allowed insights into some of the mechanisms involved in metal accumulation by plants. Clearly, it is a complicated phenomenon. Perhaps the most significant results from this large study are the many potential leads left to explore. The expression profiles of a few individual proteins that may prove to be more involved in metal accumulation than previously assumed have been identified and partially characterized. Additionally, the importance of antioxidants and antioxidant-mediating proteins has been demonstrated.

While it was hoped to identify proteins involved specifically in the transport and chelation of metal ions, the methods used were not optimized for isolating certain of these compounds. For example, many known chelators are small, thiol-containing proteins and are consequently sensitive to oxidation. Methodologies involving the isolation of low molecular weight protein fractions, thiol-affinity and the use of reducing conditions are necessary for their isolation. Many of the transporters responsible for the uptake of metals into the roots or across the membranes of sequestration bodies are non-soluble membrane proteins. The targeted isolation of membrane proteins would be necessary for their study.

The results of this study indicate that the mechanisms of metal tolerance and response to oxidative stress may be as important as the proteins involved specifically in metal transport and accumulation in the development of hyperaccumulating plants suitable for phytoremediation.

6.0 GENERAL DISCUSSION

6.1 Mechanistic Insights

The preceding chapters describe the differential proteomic responses of two *A. thaliana* genotypes to copper exposure in both root and shoot tissues over time. In Chapter 3, the global responses of each genotype at the proteome level were investigated by examining the contributions of different functional categories to the overall complement of differentially expressed proteins for each tissue and timepoint. Copper accumulation and its effect on biomass production in the two genotypes were also studied. Chapter 4 described the targeted isolation and identification of copper-binding proteins and the differential expression of key proteins within this sub-proteome. Finally, in Chapter 5, the differential expression of protein isoforms identified from the global proteome that are involved in plant defense/stress reactions and/or the transport and storage of compounds was explored. Interpretation of these results has provided insights into protein-mediated mechanisms of metal tolerance and accumulation in plants.

The use of closely related *A. thaliana* genotypes, which are documented as having contrasting responses to elevated metal concentrations (Delhaize, 1996), has allowed for the examination of differential proteome control in plants with nearly identical genetic backgrounds. As only one genetic locus differs between the Columbia ecotype and the frd3-3 mutant, the genotype-specific protein responses must arise from differential regulation within the two genotypes, a hypothesis that has also been suggested by Cobbett (2003). While a strategy for dealing with elevated metal concentrations is necessary for plants experiencing heavy metal exposure, mechanisms for countering associated increases in ROS concentrations may also be a requirement. Baseline ROS levels are a necessary consequence of aerobic metabolism (Puntarulo *et al.,* 1988; Volkov *et al.,* 2006) and are required by plants, since certain oxygen radicals function in signal transduction pathways (Karpinski *et al.,* 1999; Orozco-Cardenas *et al.,* 2001; Mullineaux & Karpinski, 2002). For these two reasons, under non-stress conditions, homeostatic redox levels are tightly maintained. If this balance is not maintained, damage to nucleic acids, lipids, and proteins can readily occur (Bowler *et al.*, 1992; Chen *et al.*, 1993; Asada, 1999; Finkel & Holbrook, 2000; Moller, 2001). The discussion of the metal accumulation mechanism is therefore impossible without also considering antioxidant regulation. Indeed, antioxidants

(GRX, TRX, FRX, PRX, POX, CAT, and SOD) represented a significant proportion of the proteins discussed in Chapters 3-5.

The *frd3* mutation causes constitutive ferric chelate reductase activity that results in the transportation into the roots, and translocation to the shoots, of metal concentrations above those required for normal metabolism. Since the mutant is expected to be challenged with elevated metal concentrations, it is conceivable that the mutant phenotype places the plant into a "state-of-readiness" whereby the protein complement in the plant is perturbed from the wildtype (WT) condition. This altered protein complement likely consists of elevated numbers of metal- and ROS-responsive proteins. In examining the distribution of differentially expressed proteins between functional categories, no significant differences were found to exist between the shoots of mutant and WT plants. In contrast, the proportion of defense related proteins was considerably higher at after 8 hours of copper exposure in mutant than in WT roots, whereas proteins involved in transport and storage were at all times higher in the WT. These differences support the hypothesis of altered protein complements between the mutant and the WT. Differences in the protein complements of the roots and not the shoots could be expected, since roots are the first point of contact with metals in the growth medium. A greater proportion of defense-related proteins also suggests that the mutant is "pre-conditioned" to tolerate elevated metal concentrations in the growth medium.

Despite lower root and shoot biomass production, WT plants had higher *in situ* shoot copper concentrations. This, in conjunction with a significantly lower proportion of transport and storage proteins, suggests that in mutant roots the rates of uptake and translocation of metal ions are lower compared to WT. A more controlled uptake of metal by the mutant, and the elevated levels of defense proteins would allow for a quick response and could in turn prevent, or at least temper, any deleterious effects caused by increasing copper concentrations. A lower level of damage can be inferred by the greater biomass production in the mutant, which implies that the elevated copper concentrations have less of an adverse effect than in the WT.

The expression levels of some identified proteins indicated that the mutant plant exists in an initial "state-of-readiness" with higher than WT levels of many defense-related proteins. In contrast, many expression profiles in the WT showed patterns consistent with protein induction. This is in keeping with previous studies that report induction of many of the defense-related proteins identified in this study in response to both abiotic and biotic stressors. Usually, elevated

internal metal concentrations cause an oxidative burst in plants, the associated increase in ROS generally being followed by an induction of antioxidants. Induction was not observed to the same extent in the mutant. If the mutant is indeed prepared for elevated metal concentrations via constitutively high expression of some proteins, there may not be the same requirement in the mutant for an oxidative burst of the kind observed in the WT. As the mutant is already prepared for stress with an elevated complement of defense-related compounds, the level of stress required to cause induction may be higher than in the WT. In essence, the mutant phenotype may result in the re-setting of threshold levels for oxidative burst and/or antioxidant synthesis or activation.

6.2 Implications for the Development of Enhanced Phytoremediation Species

As discussed earlier (Chapter 1 – Introduction) most natural hyperaccumulator species are impractical for large-scale remediation efforts. This is due mainly to their small biomass, which renders the use of common agronomic practices infeasible. Increasing the biomass of existing hyperaccumulating species has generally been unsuccessful, with one or two exceptions (Brewer *et al.,* 1999; Gleba *et al.,* 1999). However, genetic engineering (GE) has the potential to overcome this challenge. Most GE efforts to enhance phytoremediation have been directed at changing the expression of key genes thought to be involved in some aspect of the metal accumulation mechanism, either by inducing expression to constitutive levels or by decreasing expression through the use of antisense or RNA*i* (RNA interference) technologies.

For example, a study by Dhankar *et al.* (2006) in which the expression of arsenate reductase in *A. thaliana* plants was silenced, showed similar *in vitro* tolerance of arsenate and arsenite concentrations to that of WT plants as well as increased translocation of total arsenic to the shoots over the WT levels. While this example certainly suggests the possibility of enhanced phytoremediation of arsenic by altering the expression of only one key gene, it should be noted that these are *in vitro* results and therefore may not translate to greenhouse or field-scale implementations.

If however, the altered expression of key genes can in fact result in a significant change in the metal tolerance and/or accumulation of a previous non-accumulator, then, from the results presented in this thesis, those genes encoding proteins with higher than WT initial expression levels (*e.g.* GAPC, TRX, GRX, VSP, OSM, GST, Unknown 1), may prove to be good candidates

for enhancing metal accumulation. Additional results presented in this thesis suggest that one of the main differences between the heavy metal exposure strategies of the mutant and the WT is the difference in transport and storage proteins in the root. Therefore another possibility for GE efforts may be the knockdown or knockout of key transport and storage genes, thereby moderating uptake and subsequent translocation of metal ions to shoots and allowing endogenous detoxification mechanisms to mediate the increased concentrations.

While the silencing of arsenate reductase shows promise for enhancing phytoremediation of arsenic (Dhankar *et al.,* 2006), there are also examples where the alteration of gene expression is ineffective. The production of transgenic *A. thaliana* plants with altered levels (3-200% those of WT) of glutathione (GSH) by constitutive promotion of γ-glutamyl-cysteine sythetase in both sense and antisense orientations is one such example (Xiang *et al.,* 2001). While the antisense lines, with lower GSH levels than WT, showed increased sensitivity to cadmium, the sense lines, with higher than WT GSH levels, showed no change in cadmium sensitivity. Another well-known example is the engineering of higher expression levels of phytochelatin synthase, the enzyme catalyzing the synthesis of phytochelatin, in *A. thaliana* for increased cadmium tolerance and accumulation. In this example, constitutive expression of the gene encoding a protein thought to play a significant role as a metal chaperone in the metal accumulation process (Vatamaniuk *et al.,* 1999) actually resulted in increased sensitivity to, and decreased accumulation of, cadmium (Lee *et al.,* 2003a,b).

The elevated internal metal concentrations inherent in metal (hyper)accumulation are also likely to result in an increase in reactive oxygen species (ROS) and/or antioxidants. The results of this research strongly imply that an altered sensing of ROS levels or a re-setting of ROS-tolerance thresholds is indeed an important aspect of metal accumulation. One possible strategy for the development of enhanced hyperaccumulation species therefore might be the alteration of endogenous levels of certain important antioxidants such as catalase, superoxide dismutase, glutathione-*S*-transferase, and/or peroxidase. As an example of this strategy, Sunkar *et al.* (2003) overexpressed a gene encoding an aldehyde dehydrogenase (*ALDH3*) in *A. thaliana* which resulted in transgenic lines showing increased tolerance to stress-inducing factors such as dehydration, heavy meal exposure and ROS exposure. While no information was reported about altered metal accumulation, an obligatory aspect of hyperaccumulation is tolerance to heavy metal exposure.

The success of altering the expression of one or a few genes, whether they are involved in the uptake, translocation or sequestration of metals or involved in ROS tolerance/detoxification, to create a hyperaccumulator of sufficient biomass for use in field-scale phytoremediation efforts remains unclear. As has been discussed throughout the body of this thesis, the hyperaccumulation mechanism is necessarily complex and appears to involve metal accumulation, metal tolerance and altered sensing of stress compounds. It is therefore unlikely that one or a small set of genes can account for the hyperaccumulation phenomenon and that the differential regulation of existing mechanisms in the plant is likely the cause (Cobbett, 2003). Because of this likelihood, instead of altering the expression of "metal tolerance and accumulation" genes and thereby proteins, perhaps more focus should be directed to altering the regulation of the existing mechanisms by altering the expression of the regulatory elements that are responsible for the setting of internal thresholds. However, effective implementation of this approach will ultimately require a more complete description of the entire metal accumulation mechanism within the plant.

7.0 CONCLUDING REMARKS

7.1 Conclusions

The objectives of this research were to characterize and identify changes in the global and copper-binding proteomes of two *A. thaliana* genotypes during exposure to copper, thereby gaining insights into the mechanisms of metal (hyper)accumulation in plants. Based on the results obtained during this research, the following conclusions can be drawn:

1. Copper exposure caused a general upregulation of protein expression in the metal-accumulating (frd3-3 mutant) and non-accumulating (Columbia WT) *A. thaliana* plants under investigation.
2. Prolonged exposure to 30 μM copper caused an overall decrease in protein abundance, which can be attributed to a general inhibition of protein synthesis due to copper toxicity.
3. The differential expression of individual protein spots changed over the 48 hour period of investigation.
4. There was no discernable difference in distribution between functional categories for all differentially expressed proteins, identified in WT and mutant (frd3-3) shoots.
5. Among all differentially expressed proteins, frd3-3 roots contained a greater proportion of proteins in the defense/stress related and sulfur metabolism categories than did WT roots.
6. Among all differentially expressed proteins, WT roots had a greater proportion of proteins in the transport and storage category than did frd3-3 roots.
7. The distribution over time between functional categories of proteins that were induced following copper exposure was similar for frd3-3 and WT shoots.
8. The differential control of defense/stress related proteins was different between frd3-3 and WT roots.
9. The differential control of transport and storage proteins was also different between frd3-3 and WT roots.

10. The lower *in situ* copper concentrations and the smaller proportion of total root proteins devoted to transport and storage suggest that the mutant, frd3-3, regulates the transport of copper to aerial tissues as a tolerance mechanism.
11. The expression pattern of defense/stress related proteins in the frd3-3 roots is generally one of constitutive expression rather than induction, effectively creating a "state-of-readiness" towards metal exposure in the frd3-3 plant.
12. The detoxification of reactive oxygen species is clearly an important aspect of the plant response to copper exposure.
13. Suppression of the oxidative burst that usually follows exposure to heavy metals appears to be part of the frd3-3 phenotype of metal accumulation.

7.2 Future Research

The results of this research have allowed for partial characterization of the proteomic and mechanistic responses of plants to metal exposure and accumulation. Additional experiments that would help to clarify and further characterize these mechanisms include:

- A proteomic comparison of metal-accumulating and non-accumulating plants during exposure to lower, sub-lethal concentrations of copper (*e.g.* 1 to 10 μM).
- The use of closely related hyperaccumulating genotypes such as *A. halleri* or the *A. thaliana HMA* mutants.
- The measurement of ROS in tissues at each timepoint during exposure.

Additionally, several plant proteins identified as being induced in response to copper exposure have not yet been fully characterized. Further characterization of these proteins with respect to metal accumulation or ROS detoxification would be very useful. Examples include:

- LP1 (lipid transfer protein)
- Lipid associated protein
- ML-proteins (MD-2- related lipid recognition domain-containing protein)
- UCC2 (Uclacyanin 2)
- DREPP (Developmentally-regulated plasma-membrane polypeptides) proteins

- Osmotin
- Unknown 1 (At5g19860)
- Unknown 2 (At4g10860)

Finally, adaptations to existing methodology could improve results and broaden the scope of this research. Such refinements might include:

- Techniques for the isolation of small (< 10 kD) proteins and peptides.
- Techniques for the isolation of oxygen-sensitive, thiol-containing proteins (eschewed here in favor of a more inclusive approach to proteome analysis).
- Optimization of desalting/precipitation techniques to improve streaking on 2DE gels.
- The use of larger gels to increase the resolution of individual proteins spots.
- Improved techniques for the isolation of insoluble, membrane-bound proteins.

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Appendix 1

MS data of co-localized protein spots excised from duplicate gels demonstrating reproducibility of protein identification and scores between duplicates. Spot = 1 of 2 spots excised from each gel to demonstrate reproducibility; Thr. = score above which a protein identification is considered a non-random event; Acc. # = Accession Number; Peptides = unique peptide sequences identified through MS/MS; Score = individual protein score; % Cov. = percentage of total protein sequence accounted for by the identified peptides. WT = wildtype; M = mutant; HMW = high molecuare wight gels; LMW = low molecular weight gels; Blue text indicates data generated from the original set of analysed gels (*i.e.* Figures 3.7-3.14) Green text indicates data generated from the dulplicate gels.

	Spot	Thr.	Acc. #	Protein ID	Peptides	Score	% Cov.
Shoot							
WT HMW Gel							
8 Hr	**1**		gi|430947	PSI type III chlorophyll a/b-binding protein	R.FAMLGAAGAIAPEILGK.A + Oxidation (M)	89	6
			gi|15225120	unknown protein	K.ELVNSQK.D	73	7
		30			R.IPGTVAPLPGSVAK.L		
			gi|30678347	CA	K.AFDPVETIK.Q	58	7
					R.NIANMVPPFDK.V + Oxidation (M)		
			gi|8778432	GST	R.ALLTLEEK.S	41	1
			gi|15225120	unknown protein [Arabidopsis thaliana]	K.SEITGVIK.G	111	14
					R.IPGTVAPLPGSVAK.L		
					K.FPGIDQITVGENFSPAR.A		
			gi|430947	PSI type III chlorophyll a/b-binding protein	K.QYFLGLEK.G	99	17
		30			R.FAMLGAAGAIAPEILGK.A + Oxidation (M)		
					K.GLAGSGNPAYPGGPFFNPLGFGK.D		
			gi|8778432	GST	K.YPDPPLK.T	66	5
					R.ALLTLEEK.S		
					R.VSAVDLSLAPK.L		
			gi|30678347	CA	K.AFDPVETIK.Q	62	19
					R.NIANMVPPFDK.V + Oxidation (M)		
					K.YGGVGAAIEYAVLHLK.V		
					R.EAVNVSLANLLTYPFVR.E		
	2		gi|15242466	PMDH2	R.IQNGGTEVVEAK.A	108	7
		30			K.AGAGSATLSMAYAAAK.F + Oxidation (M)		
			gi|15242466	PMDH2	K.LLGVTTLDVAR.A	144	11
		30			R.IQNGGTEVVEAK.A		
					K.AGAGSATLSMAYAAAK.F + Oxidation (M)		

	Spot	Thr.	Acc. #	Protein ID	Peptides	Score	% Cov.
12 Hr	1		gi|8778432	GST	K.YPDPPLK.T	343	22
					R.ALLTLEEK.S		
					R.VSAVDLSLAPK.L		
		30			K.SHDGPFIAGER.V		
					K.TLFSLDSFEK.T		
					K.LYHLQVALGHFK.S		
					K.AAVGAPDHLGDCPFSQR.A		
					K.TPAEFASVGSNIFGTFGTFLK.S		
			gi|8778432	GST	K.YPDPPLK.T	351	22
					R.ALLTLEEK.S		
					R.VSAVDLSLAPK.L		
		30			K.SHDGPFIAGER.V		
					K.TLFSLDSFEK.T		
					K.LYHLQVALGHFK.S		
					K.AAVGAPDHLGDCPFSQR.A		
					K.TPAEFASVGSNIFGTFGTFLK.S		
	2	30	gi|15237059	AtRABE	K.VGETVDLVGLR.E	57	2
		30	gi|15237059	AtRABE1b	K.VGETVDLVGLR.E	83	2

	Spot	Thr.	Acc. #	Protein ID	Peptides	Score	% Cov.
48 Hr	1		gi|15231805	carboxylic ester hydrolase	K.LCEYQR.S	547	26
					K.LLYSLGASK.F		
					R.SYFFFDGR.H		
					K.IGPMLNEFAK.I + Oxidation (M)		
					R.ELIVYPTGETMR.E + Oxidation (M)		
		40			K.TGNECYELLNDLAK.Q		
					K.ANPNADASAQQAFVTNVINR.L		
					K.AQEEMAHLLYGADPDVVQPMTVR.E + 2 Oxidation (M)		
			gi|4587541	Lipase/Acylhydrolase	K.LCEYQR.S	56	4
					K.IGPMLNEMAR.N + 2 Oxidation (M)		
			gi|15231805	carboxylesterase	K.LCEYQR.S	399	34
					R.SYFFFDGR.H		
					K.IGPMLNEFAK.I + Oxidation (M)		
					R.ELIVYPTGETMR.E + Oxidation (M)		
		29			K.TGNECYELLNDLAK.Q		
					K.FVVQLLAPLGCLPIVR.Q		
					K.ANPNADASAQQAFVTNVINR.L		
					K.AQEEMAHLLYGADPDVVQPMTVR.E + 2 Oxidation (M)		
					R.GVSFAVADASILGAPVESMTLNQQVVK.F + Oxidation (M)		
	2		gi|15222166	PSBP-1	K.EIEYPGQVLR.F	267	13
					K.HQLITATVNGGK.L		
		30			K.TNTDFLPYNGDGFK.V		
			gi|8778432	GST	R.ALLTLEEK.S	78	4
					R.VSAVDLSLAPK.L		
			gi|15222166	PSBP-1	K.EIEYPGQVLR.F	232	21
					K.HQLITATVNGGK.L		
					K.TNTDFLPYNGDGFK.V		
					K.SITDYGSPEEFLSQVNYLLGK.Q		
			gi|8778432	GST	K.YPDPPLK.T	131	17
		30			R.ALLTLEEK.S		
					R.VSAVDLSLAPK.L		
					K.SHDGPFIAGER.V		
					K.TLFSLDSFEK.T		
					K.LYHLQVALGHFK.S		
					K.AAVGAPDHLGDCPFSQR.A		

	Spot	Thr.	Acc. #	Protein ID	Peptides	Score	% Cov.
WT LMW Gel							
8 Hr	**1**		gi|15242045	CPN20	K.YAGNDFK.G	404	50
					K.DGSNYIALR.A		
					K.YTSIKPLGDR.V		
					K.TAGGLLLTETTK.E		
					K.YAGTEVEFNDVK.H		
		30			K.IDITVPTGAQIIYSK.Y		
					K.ITPLPVSTGSTVLYSK.Y		
					K.EKPSIGTVIAVGPGSLDEEGK.I		
					K.TLGGILLPSTAQSKPQGGEVVAVGEGR.		
			gi|13265501	AT3g54890	K.YPGGAFDPLGYSK.D	69	5
			gi|12322730	EF-P	R.LNESDMGEK.T + Oxidation (M)	30	10
					R.NYVNGSTVER.T		
			gi|15242045	CPN20	K.YAGNDFK.G	237	49
					K.DLKPLNDR.V		
					K.DGSNYIALR.A		
					K.TAGGLLLTETTK.E		
					K.YAGTEVEFNDVK.H		
					K.EDDIVGILETEDIK.D		
		29			K.ITPLPVSTGSTVLYSK.Y		
					K.EKPSIGTVIAVGPGSLDEEGK.I		
					K.TLGGILLPSTAQSKPQGGEVVAVGEGR.T		
			gi|13265501	AT3g54890	K.ESELIHCR.W	87	8
					K.YPGGAFDPLGYSK.D		
			gi|12322730	EF-P	R.NYVNGSTVER.T	42	17
					K.VIDFDLPITVK.L		
					R.AGISVEEANIYK.E		
	2		gi|1755154	GLP	K.GPQSPSGYSCK.N	154	16
					K.AAVTPAFAPAYAGINGLGVSLAR.L		
		30	gi|15239652	FQR1	K.GAASVEGVEAK.L	73	9
					R.FGMMAAQFK.A + 2 Oxidation (M)		
			gi|16209712	At1g12410/F5O11_7	R.FAMPLSR.I + Oxidation (M)	65	6
					R.GQADDIQNEAK.E		
			gi|1755154	GLP	K.GPQSPSGYSCK.N	233	25
					R.GDSMVFPQGLLHFQLNSGK.G + Oxidation (M)		
					K.AAVTPAFAPAYAGINGLGVSLAR.L		
		30	gi|15239652	FQR1	K.GAASVEGVEAK.L	114	13
					K.GGSPYGAGTFAGDGSR.Q		
			gi|16209712	At1g12410/F5O11_7	R.FAMPLSR.I + Oxidation (M)	98	10
					R.IALQSPAGAAR.G		
					R.GQADDIQNEAK.E		

	Spot	Thr.	Acc. #	Protein ID	Peptides	Score	% Cov.
12 Hr	1		gi|30678347	CA	K.GGYYDFVK.G	252	33
					K.AFDPVETIK.Q		
					R.NIANMVPPFDK.V + Oxidation (M)		
					K.YETNPALYGELAK.G		
					K.VENIVVIGHSACGGIK.G		
					K.VISELGDSAFEDQCGR.C		
					R.VCPSHVLDFQPGDAFVVR.N		
			gi|2554769	GST	R.VLIALHEK.N	182	22
					K.VLDVYEAR.L		
					K.LAFEQIFK.S		
		30			R.VNEWVAEITK.R		
					R.YENQGTNLLQTDSK.N		
			gi|1944432	Rubisco	R.AVYECLR.G	158	13
					K.SQAETGEIK.G		
					K.DTDILAAFR.V		
					R.DLAVEGNEIIR.-		
					R.ESTLGFVDLLR.D		
					R.FLFCAEAIYK.S		
			gi|15237059	AtRABE1b	K.VGETVDLVGLR.E	78	2
			gi|1279212	MSR	R.IVTEILPATK.F	53	3
			gi|1668706	atran2	K.LVIVGDGGTGK.T	33	4
			gi|30678347	CA	K.GGYYDFVK.G	195	38
					K.AFDPVETIK.Q		
					K.YMVFACSDSR.V + Oxidation (M)		
					R.NIANMVPPFDK.V + Oxidation (M)		
					K.VENIVVIGHSACGGIK.G		
					K.YGGVGAAIEYAVLHLK.V		
					K.VISELGDSAFEDQCGR.C		
					R.VCPSHVLDFQPGDAFVVR.N		
			gi|2554769	GST	K.LFTERPR.V	176	40
					R.VLIALHEK.N		
					K.VLDVYEAR.L		
					K.LAFEQIFK.S		
		30			K.VFGHPASIATR.R		
					R.VNEWVAEITK.R		
					R.NPFGQVPAFEDGDLK.L		
					K.SIYGLTTDEAVVAEEEAK.L		
			gi|1944432	Rubisco	K.DTDILAAFR.V	138	15
					R.VALEACVQAR.N		
					R.DLAVEGNEIIR.-		
					R.ESTLGFVDLLR.D		
					K.LTYYTPEYETK.D		
					R.LSGGDHIHAGTVVGK.L		
			gi|15237059	AtRABE1b	K.VGETVDLVGLR.E	83	2
			gi|1668706	atran2	K.LVIVGDGGTGK.T	41	4
			gi|1279212	MSR	R.HDPTTLNR.Q	38	6
					R.IVTEILPATK.F		

	Spot	Thr.	Acc. #	Protein ID	Peptides	Score	% Cov.
	2		gi|1944432	Rubisco	R.VALEACVQAR.N	179	9
					R.DLAVEGNEIIR.-		
		30			R.ESTLGFVDLLR.D		
					R.FLFCAEAIYK.S		
			gi|15224582	GST	K.IPVLVDGDYK.I	48	4
			gi|1944432	Rubisco	R.VALEACVQAR.N	147	10
					R.DLAVEGNEIIR.-		
					R.ESTLGFVDLLR.D		
		30			R.LSGGDHIHAGTVVGK.L		
			gi|15224582	GST	K.IPVLVDGDYK.I	51	17
					M.VLTIYAPLFASSK.R		
					K.LAEVLDVYEAQLSK.N		
48 Hr	1		gi|438449	CA	R.NIANMVPPFDK.V + Oxidation (M)	50	4
		31	gi|7769871	F12M16.14	R.TQDGGTEVVEAK.A	45	3
			gi|7769871	F12M16.14	K.EGLEALKPELK.S	68	14
					R.TQDGGTEVVEAK.A		
					R.DDLFNINAGIVK.N		
		31			K.ALEGADLVIIPAGVPR.K		
			gi|30678347	CA	R.NIANMVPPFDK.V + Oxidation (M)	40	16
					K.YGGVGAAIEYAVLHLK.V		
					R.VCPSHVLDFQPGDAFVVR.N		
	2		gi|15219257	PAB1	R.VLTPAEIDDYLAEVE.-	40	15
		30			K.LVQIEHALTAVGSGQTSLGIK.A		
			gi|15219257	PAB1	K.EGFEGEISSK.N	59	15
		32			K.ASNGVVIATEK.K		
					R.VLTPAEIDDYLAEVE.-		

	Spot	Thr.	Acc. #	Protein ID	Peptides	Score	% Cov.
M HMW Gel							
8 Hr	**1**		gi|15228407	MnSOD	K.NLAPSSEGGGEPPK.G	197	20
		29			K.LVVDTTANQDPLVTK.G		
					K.GSLGSAIDAHFGSLEGLVK.K		
			gi|15228407	MnSOD	K.YASEVYEK.E	102	19
		30			K.NVRPEYLK.N		
					K.NLAPSSEGGGEPPK.G		
					K.LVVDTTANQDPLVTK.G		
	2		gi|15232559	GR	K.LIVCANTNK.V	103	14
					R.DFVGEQMSLR.G + Oxidation (M)		
					K.TLFQNEPTKPDYR.A		
		30			R.INLTPVALMEGGALAK.T + Oxidation (M)		
					R.HYDFDLFTIGAGSGGVR.A		
					K.EFAIDSDAALDLPSKPK.K		
			gi|15239835	unknown protein [Arabidopsis thaliana]	R.LATGEPLR.L	35	1
			gi|15239835	unknown protein [Arabidopsis thaliana]	R.LATGEPLR.L	40	1
		30	gi|15232559	GR	R.INLTPVALMEGGALAK.T + Oxidation (M)	32	2
12 Hr	**1**	29	gi|4803941	putative major intrinsic (channel) protein	K.LIYSINTK.C	45	3
		31	gi|4803941	putative major intrinsic (channel) protein	K.LIYSINTK.C	33	3
	2		gi|8778432	GST	R.ALLTLEEK.S	68	6
		30			R.VSAVDLSLAPK.L		
					K.TLFSLDSFEK.T		
			gi|8778432	GST	R.ALLTLEEK.S	66	6
		30			R.VSAVDLSLAPK.L		
					K.TLFSLDSFEK.T		

	Spot	Thr.	Acc. #	Protein ID	Peptides	Score	% Cov.
48 Hr	**1**		gi|15231805	carboxylesterase	K.LCEYQR.S	104	11
					K.LLYSLGASK.F		
		31			R.SYFFFDGR.H		
					K.IGPMLNEFAK.I + Oxidation (M)		
					R.ELIVYPTGETMR.E + Oxidation (M)		
			gi|4803941	putative major intrinsic (channel) protein	K.LIYSINTK.C	40	3
			gi|15231805	carboxylesterase	K.LLYSLGASK.F	134	23
					K.IGPMLNEFAK.I + Oxidation (M)		
					R.ELIVYPTGETMR.E + Oxidation (M)		
		31			K.TGNECYELLNDLAK.Q		
					K.ANPNADASAQQAFVTNVINR.L		
					R.GVSFAVADASILGAPVESMTLNQQVVK.F + Oxidation (M)		
			gi|4803941	putative major intrinsic (channel) protein	K.LIYSINTK.C	35	3
	2		gi|15235745	SHM1	R.MGTPALTSR.G + Oxidation (M)	97	9
					K.FAQTLMER.G + Oxidation (M)		
					K.LIVAGASAYAR.L		
		30			K.EVLYDFEDK.I		
					K.AYQEQVLSNSAK.F		
			gi|16215	catalase	R.LNVRPSI.-	50	5
					K.SLLEEDAIR.L		
					R.APGVQTPVIVR.F		
			gi|15235745	SHM1	K.VAEYFDK.A	178	22
					K.SATLFRPK.L		
					R.MGTPALTSR.G + Oxidation (M)		
					K.YSEGYPGAR.Y		
					R.GFVEEDFAK.V		
					K.LIVAGASAYAR.L		
					K.EVLYDFEDK.I		
					K.VLEAVHIASNK.N		
		30			K.AYQEQVLSNSAK.F		
					K.NTVPGDVSAMVPGGIR.M + Oxidation (M)		
					R.LDESTGYIDYDQMEK.S + Oxidation (M)		
			gi|1246399	catalase	R.LNVRPSI.-	73	12
					R.FSTVIHER.G		
					K.SLLEEDAIR.V		
					R.APGVQTPVIVR.F		
					R.LGPNYLQLPVNAPK.C		
					R.GPILLEDYHLVEK.L		

	Spot	Thr.	Acc. #	Protein ID	Peptides	Score	% Cov.
M LMW Gel							
8 Hr	**1**		gi|11762200	AT3g62030	R.TLESQETR.A	110	15
					K.FEDENFTLK.H		
					R.IVMGLFGEVVPK.T + Oxidation (M)		
					K.HVVFGQVIEGMK.L + Oxidation (M)		
			gi|3273753	Cu-ZnSOD	R.LACGVIGLTPL.-	67	12
		30			K.GGHELSLTTGNAGGR.L		
			gi|1944432	Rubisco	K.DTDILAAFR.V	61	4
					K.LTYYTPEYETK.D		
			gi|22571	33 kDa oxygen-evolving protein	K.FLVPSYR.G	45	7
					R.VPFLFTVK.Q		
					R.LTYDEIQSK.T		
			gi|11762200	AT3g62030	R.TLESQETR.A	97	11
					K.FEDENFTLK.H		
					R.IVMGLFGEVVPK.T + Oxidation (M)		
			gi|1944432	Rubisco	K.DTDILAAFR.V	85	7
		30			R.DLAVEGNEIIR.-		
					K.LTYYTPEYETK.D		
			gi|3273753	Cu-ZnSOD	K.GGHELSLTTGNAGGR.L	40	6
			gi|22571	33 kDa oxygen-evolving protein	R.VPFLFTVK.Q	39	2
	2		gi|30678347	CA	K.GGYYDFVK.G	299	43
					K.AFDPVETIK.Q		
					K.YMVFACSDSR.V + Oxidation (M)		
					R.NIANMVPPFDK.V + Oxidation (M)		
					K.YETNPALYGELAK.G		
					K.YGGVGAAIEYAVLHLK.V		
		30			K.VISELGDSAFEDQCGR.C		
					R.EAVNVSLANLLTYPFVR.E		
					R.VCPSHVLDFQPGDAFVVR.N		
			gi|15235029	LHCB5	K.DPEQGALLK.V	38	6
					R.IFLPDGLLDR.S		
			gi|16348	inorganic pyrophosphatase	R.NPNVTLNER.N	35	3
			gi|1944432	Rubisco	R.DLAVEGNEIIR.-	34	2
			gi|30678347	CA	K.GGYYDFVK.G	261	39
					K.AFDPVETIK.Q		
					R.NIANMVPPFDK.V + Oxidation (M)		
					K.YETNPALYGELAK.G		
					K.VENIVVIGHSACGGIK.G		
		29			K.YGGVGAAIEYAVLHLK.V		
					K.VISELGDSAFEDQCGR.C		
					R.VCPSHVLDFQPGDAFVVR.N		
			gi|15235029	LHCB5	R.IFLPDGLLDR.S	45	3
			gi|16348	inorganic pyrophosphatase	R.NPNVTLNER.N	40	9
					R.VLYSSIVYPHNYGFIPR.T		
			gi|1944432	Rubisco	R.DLAVEGNEIIR.-	38	2

	Spot	Thr.	Acc. #	Protein ID	Peptides	Score	% Cov.
12 Hr	**1**		gi|1272406	immunophilin	K.LGYGDNGSPPK.I K.SGDVTELQIGVK.Y K.LTDGTVFDSSFER.G K.IPGGATLIFDTELVAVNGEPSSEAK.S	202	41
		30	gi|15233587	PSBQ	K.LFDTIDNLDYAAK.K R.FYLQPLPPTEAAAR.A	68	12
			gi|15237018	NDPK3	K.LIGATDPQK.S R.GLISEIISR.F	50	7
			gi|1272406	immunophilin	K.LGYGDNGSPPK.I K.SGDVTELQIGVK.Y	189	24
		32			K.LTDGTVFDSSFER.G		
			gi|15237018	NDPK3	R.GLISEIISR.F	56	3
			gi|15234637	PSBQ	K.LFQTIDNLDYAAR.S	48	5
	2	29	gi|9843639	Rieske FeS protein	K.GDPTYLVVENDK.T R.GPAPLSLALAHADIDEAGK.V	179	13
		30	gi|9843639	Rieske FeS protein	K.GDPTYLVVENDK.T R.GPAPLSLALAHADIDEAGK.V	182	13
48 Hr	**1**	30	gi|18412149	UBC13A	R.ICLDILK.D K.SNEAEAVDTAK.E	105	11
		30	gi|18394416	UBC13B	R.ICLDILK.D K.LELFLPEEYPMAAPK.V + Oxidation (M) R.LLSEPAPGISASPSEENMR.Y + Oxidation (M)	91	26
	2		gi|15237998	ATP synthase delta' chain, mitochondrial	K.GLAEFQQK.L K.LTVNFVLPYTSELTGK.E	50	11
			gi|1498198	2-Cys peroxiredoxin bas1	R.GLFIIDK.E K.SGGLGDLNYPLISDVTK.S	48	9
		29	gi|4741960	Lhcb6	R.FFDPLGLAGK.N R.DGVYEPDFEK.L K.TAENFANYTGDQGYPGGR.F	44	14
			gi|22571	33 kDa oxygen-evolving protein	K.FLVPSYR.G R.VPFLFTVK.Q	32	4
			gi|15237998	ATP synthase delta' chain, mitochondrial	K.GLAEFQQK.L K.LASATTDLEK.A	47	8
		30	gi|1498198	2-Cys peroxiredoxin bas1	K.SFGVLIHDQGIALR.G K.EGVIQHSTINNLGIGR.S	42	11
			gi|4741960	Lhcb6	R.FFDPLGLAGK.N	36	3
			gi|22571	33 kDa oxygen-evolving protein	R.VPFLFTVK.Q	31	2

	Spot	Thr.	Acc. #	Protein ID	Peptides	Score	% Cov.
Root WT HMW Gel							
8 Hr	**1**	31	gi|15241583	GLP	K.AFQVDPR.V R.VVMDLQTK.F + Oxidation (M)	75	6
		31	gi|15241583	GLP	K.AFQVDPR.V R.VVMDLQTK.F + Oxidation (M) R.IDYAVDGQNPPHTHPR.A K.VLNEGDVFVFPEGLIHFQANIGK.A	70	24
	2	31	gi|15236606	peroxidase	K.YYVNLK.E R.TPTLFDNK.Y K.DAFGNANSAR.G R.MSSLSPLTGK.Q + Oxidation (M) K.NQCQFIMDR.L + Oxidation (M) K.GLIQSDQELFSSPDASDTLPLVR.E	209	19
		31	gi|1402908	peroxidase	K.YYVNLK.E R.GFPVIDR.M R.TPTVFDNK.Y R.DTIVNELR.S K.AAVETACPR.T K.DAAPNANSAR.G K.NQCQFIMDR.L + Oxidation (M) K.FFNAFVEAMNR.M + Oxidation (M) R.MGNITPLTGTQGQIR.Q + Oxidation (M) K.ASFQNVGLDRPSDLVALSGGHTFGK.N	200	30
12 Hr	**1**	31	gi|15241583	GLP	K.AFQVDPR.V R.VVMDLQTK.F + Oxidation (M) R.IDYAVDGQNPPHTHPR.A	68	13
		30	gi|15241589	GLP	R.IDYGINGQNPPHTHPR.A	66	7
	2	30	gi|5002232	NADPH:quinone oxidoreductase	R.VAALSGSLR.K K.FDAEGNLVDEVTK.E	131	11
			gi|15241583	GLP	R.VVMDLQTK.F + Oxidation (M)	65	3
		32	gi|5002232	NADPH:quinone oxidoreductase	R.VAALSGSLR.K K.FDAEGNLVDEVTK.E	139	11
			gi|15241583	GLP	R.VVMDLQTK.F + Oxidation (M)	48	3

	Spot	Thr.	Acc. #	Protein ID	Peptides	Score	% Cov.
48 Hr	**1**	28	gi|8778432	GST	R.VSAVDLSLAPK.L	54	2
		29	gi|8778432	GST	R.VSAVDLSLAPK.L	47	2
	2		gi|1402908	peroxidase	R.TPTVFDNK.Y R.DTIVNELR.S K.AAVETACPR.T K.DAAPNANSAR.G K.NQCQFIMDR.L + Oxidation (M) R.MGNITPLTGTQGQIR.Q + Oxidation (M) R.DSLQAFFALANTNLPAPFFTLPQLK.A	255	23
		38					
			gi|1402908	peroxidase	K.YYVNLK.E R.TPTVFDNK.Y R.DTIVNELR.S K.AAVETACPR.T K.DAAPNANSAR.G K.NQCQFIMDR.L + Oxidation (M) K.FFNAFVEAMNR.M + Oxidation (M) R.MGNITPLTGTQGQIR.Q + Oxidation (M)	311	21
		29					
WT LMW Gel							
8 Hr	**1**		gi|1402908	peroxidase	R.GFPVIDR.M R.DTIVNELR.S K.AAVETACPR.T	52	6
		31					
		30	gi|1402908	peroxidase	R.DTIVNELR.S	50	2
	2	31	gi|15241583	GLP	R.VVMDLQTK.F + Oxidation (M)	65	3
		31	gi|15241583	GLP	R.VVMDLQTK.F + Oxidation (M)	52	3

	Spot	Thr.	Acc. #	Protein ID	Peptides	Score	% Cov.
12 Hr	**1**		gi|15224582	GST	R.SQGPDLLGK.T	175	26
					K.IPVLVDGDYK.I		
					R.QPEYLAIQPFGK.I		
		31			K.GVSFETVNVDLMK.G + Oxidation (M)		
					K.LAEVLDVYEAQLSK.N		
			gi|15241583	GLP	K.AFQVDPR.V	51	6
					R.VVMDLQTK.F + Oxidation (M)		
			gi|15224582	GST	K.IPVLVDGDYK.I	210	23
					M.VLTIYAPLFASSK.R		
		31			K.GVSFETVNVDLMK.G + Oxidation (M)		
					K.LAEVLDVYEAQLSK.N		
			gi|15241583	GLP	R.VVMDLQTK.F + Oxidation (M)	33	3
	2		gi|15234781	cyclophillin	R.IVMELYTDK.T	155	34
					K.VYFDMTIDGQPAGR.I + Oxidation (M)		
		31			K.HVVFGQVVEGLDVVK.A		
					K.VGSSSGKPTKPVVVADCGQLS.-		
			gi|1402908	peroxidase	R.DTIVNELR.S	67	4
					K.AAVETACPR.T		
			gi|2443757	cyclophilin	K.FEDENFER.K	115	18
		31			K.IVMELYTDK.T + Oxidation (M)		
					K.HVVFGQVVEGLDVVK.A		
			gi|1402908	peroxidase	R.DTIVNELR.S	32	2
48 Hr	**1**	37	gi|15241583	NR	R.VVMDLQTK.F + Oxidation (M)	43	3
			gi|145358636	NR	R.VVMDLQTK.F + Oxidation (M)	78	27
		31			R.IDYAVNGQNPPHTHPR.A		
					K.APAVAFAALSSQNPGVITIANTVFGANPAINPTILAK.A		
	2	36	gi|15241589	NR	R.IDYGINGQNPPHTHPR.A	61	7
			gi|145358636	NR	R.VVMDLQTK.F	78	27
		31			R.IDYAVNGQNPPHTHPR.A		
					K.APAVAFAALSSQNPGVITIANTVFGANPAINPTILAK.A		

	Spot	Thr.	Acc. #	Protein ID	Peptides	Score	% Cov.
M HMW Gel							
8 Hr	**1**		gi|15220463	peroxidase	R.DATPNLTVR.G	39	2
			gi|15238030	peroxidase	R.DSVALAGGPSYSIPTGR.R	49	5
	2	38	gi|497788	GST	R.VLIALHEK.N K.VPAFEDGDFK.I	64	8
		30	gi|497788	GST	R.VLIALHEK.N K.VPAFEDGDFK.I	44	8
12 Hr	**1**	31	gi|15219721	MDH	K.SQAAALEK.H R.ALGQISER.L K.EFAPSIPEK.N K.MELIDAAFPLLK.G + Oxidation (M)	121	11
		32	gi|15219721	MDH	R.ALGQISER.L K.MELIDAAFPLLK.G + Oxidation (M) K.VLVVANPANTNALILK.E R.VLVTGAAGQIGYALVPMIAR.G + Oxidation (M)	101	16
	2	31	gi|1402908	peroxidase	R.TPTVFDNK.Y K.DAAPNANSAR.G K.NQCQFIMDR.L + Oxidation (M)	68	7
		31	gi|15236606	peroxidase	K.FFDAFAK.A R.TPTLFDNK.Y K.DAFGNANSAR.G R.MSSLSPLTGK.Q + Oxidation (M) K.NQCQFIMDR.L + Oxidation (M) R.ASDLVALSGGHTFGK.N K.TCPQVFDIATTTIVNALR.S K.GLIQSDQELFSSPDASDTLPLVR.E	63	28

	Spot	Thr.	Acc. #	Protein ID	Peptides	Score	% Cov.
48 Hr	**1**		gi|15239652	FQR1	K.GAASVEGVEAK.L R.FGMMAAQFK.A + 2 Oxidation (M) K.AFLDATGGLWR.A K.GGSPYGAGTFAGDGSR.Q K.VYIVYYSMYGHVEK.L + Oxidation (M)	244	29
		30	gi|20197312	GST	K.VLFDSRPK.V K.VLDVYEAR.L R.AITQYLAEEYSEK.G	110	13
			gi|1755152	GLP	R.TDDTTVQNLK.S K.VLNAGEAFVIPR.G	100	10
			gi|15239652	FQR1	K.GAASVEGVEAK.L R.FGMMAAQFK.A K.AFLDATGGLWR.A K.GGSPYGAGTFAGDGSR.Q K.VYIVYYSMYGHVEK.L + Oxidation (M) K.LWQVPETLHEEALSK.M R.QPTELELQQAFHQGQYIASITK.K K.SESPIITPNELAEADGFVFGFPTR.F	262	59
		32	gi|1755152	GLP	R.TDDTTVQNLK.S K.VLNAGEAFVIPR.G	137	10
			gi|20197312	GST	K.VLFDSRPK.V K.DLQFELIPVDMR.A + Oxidation (M) R.AITQYLAEEYSEK.G K.GMFGMTTDPAAVQELEGK.L + 2 Oxidation (M)	87	23
	2		gi|15229095	peroxidase	K.YYVNLK.E R.GFPVIDR.M R.TPTVFDNK.Y R.ETIVNELR.S K.DAFGNANSAR.G R.SALVDFDLR.T	82	13
		29	gi|15229095	peroxidase	K.YYVNLK.E R.TPTVFDNK.Y R.ETIVNELR.S K.DAFGNANSAR.G R.SALVDFDLR.T	85	11
		31					

	Spot	Thr.	Acc. #	Protein ID	Peptides	Score	% Cov.
M LMW Gel							
8 Hr	**1**	30	gi|4098968	GLP	R.AFQMDVNAVR.N + Oxidation (M) IDYAPNGQNPPHTHPR.A	61	11
		30	gi|4098968	GLP	R.AFQMDVNAVR.N + Oxidation (M)	51	4
	2	30	gi|15231176	ATPQ	K.VLVTDEAR.R K.EIADVQEISK.K K.EAYDSIEIPK.Y R.AFDEVNTQLQTK.F	104	23
			gi|15226197	leucine-rich repeat transmembrane protein kinase, putative	R.VVSLSIPR.K	31	1
		32	gi|15231176	ATPQ	K.GIGAGIVDK.Y K.VLVTDEAR.R K.VTPEYKPK.F K.FDALLVELK.E K.EIADVQEISK.K K.EAYDSIEIPK.Y R.AFDEVNTQLQTK.F	112	39
			gi|15226197	leucine-rich repeat transmembrane protein kinase, putative	R.VVSLSIPR.K	35	1
12 Hr	**1**	30	gi|15234781	cyclophillin	R.IVMELYTDK.T + Oxidation (M) K.VYFDMTIDGQPAGR.I + Oxidation (M) K.VGSSSGKPTKPVVVADCGQLS.-	124	25
		29	gi|15234781	cyclophillin	K.FEDENFER.K K.VYFDMTIDGQPAGR.I + Oxidation (M) K.HVVFGQVVEGLDVVK.A	136	21
	2	37	gi|15227259	ROC3; peptidyl-prolyl cis-trans isomerase	K.VYFDMTVGGK.S + Oxidation (M) K.HVVFGQVVEGLNVVR.D R.IVMELYADTTPETAENFR.A + Oxidation (M)	157	24
		29	gi|15227259	cyclophillin	K.HVVFGQVVEGLNVVR.D R.IVMELYADTTPETAENFR.A + Oxidation (M)	64	19
48 Hr	**1**		gi|2760606	peroxidase	K.GNDVDLSIYK.G R.FAPTTSPLSIEK.D	64	13
			gi|2760606	peroxidase	R.FAPTTSPLSIEK.D	40	7
	2	32	gi|15238030	peroxidase	R.FGVTPTVTAALLR.M	63	4
		30	gi|15238030	peroxidase	R.FGVTPTVTAALLR.M	50	4

Appendix 2

Functionally categorized proteins identified in spots isolated from WT shoot gels.

http://homepage.usask.ca/~cas136/appendices.html

Appendix 3

Functionally categorized proteins identified in spots isolated from mutant shoot gels.

http://homepage.usask.ca/~cas136/appendices.html

Appendix 4

Functionally categorized proteins identified in spots isolated from WT root gels.

http://homepage.usask.ca/~cas136/appendices.html

Appendix 5

Functionally categorized proteins identified in spots isolated from mutant root gels.

http://homepage.usask.ca/~cas136/appendices.html

Appendix 6

Clustal W amino acid pair distances for Cu/ZnSOD (gi|3273753) and MnSOD (At3g10920; gi|3273751).

Percent Identity

Divergence	1	2		
1		13.0	1	Cu_ZnSOD
2	380.0		2	MnSOD
	1	2		

Appendix 7

Plot of Mean MASCOT™ Scores as a function of BSA concentration showing logarithmic relationship and high correlation factor.