DISSERTATION

COPPER TRAFFIC IN PLANTS: ROLES FOR NEWLY ISOLATED CHLOROPLAST PROTEINS

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY JASON BURKHEAD ENTITLED: COPPER HOMEOSTASIS IN PLANTS: ROLES FOR NEWLY ISOLATED CHLOROPLAST PROTEINS BE ACCEPTED AS FULFILLING IN PART THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

COPPER TRAFFIC IN PLANTS: ROLES FOR NEWLY ISOLATED CHLOROPLAST PROTEINS

Plant cells must acquire copper for photosynthesis as well as other cellular functions. As the site of photosynthesis, chloroplasts must obtain copper from the cytosol and incorporate it into the photosynthetic machinery and other enzymes while avoiding the inherent toxicity of copper ions. Four genes coding for putative copper homeostasis proteins were identified in *Arabidopsis*. *AtCUTA*, a homologue of *Escherichia coli CUTA*, was characterized as coding for a copper binding protein that is upregulated during senescence as well as in plants deficient in chloroplast copper transport. We hypothesize that involved in chloroplast copper homeostasis. Data indicate that *AtCUTA* expression is likely posttranslationally regulated. *CpCOPZ* encodes a predicted protein with a conserved copper chaperone-like metal binding domain at the C-terminus. A fusion protein of the predicted CpCOPZ chloroplast transit peptide and a GFP (Green Fluorescent Protein) passenger protein was localized to chloroplasts. *CpCCS* is a homologue of yeast *Lys7*, a copper chaperone for copper-zinc superoxide dismutase. A cpCCS:GFP fusion protein was localized to chloroplasts indicating that the protein is likely the specific chaperone to deliver copper to the

chloroplastic Cu-Zn superoxide dismutase (CSD2). *ATX2* encodes a predicted homologue of *ATX1*, which delivers copper to the secretory pathway. A fusion protein of the predicted chloroplast transit peptide of *ATX2* and GFP was localized to the cytosol, indicating that the protein functions in the cytosol.

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Chapter 1

Introduction

This work was performed to gain insight into mechanisms of copper homeostasis in plant chloroplasts. The topics in this Introduction chapter are presented to provide background information for the research presented in the following chapters.

Essential Copper

The transition metal copper is essential to life as a cofactor in numerous biochemical reactions (Linder, 1991). The utility of copper in biochemical reactions is due to its ability to cycle between oxidized Cu(II) and reduced Cu(I) states. It is thought that copper did not become available to living organisms prior to the appearance of cyanobacteria and dioxygen-generating photosynthesis (Harrison and Hoare, 1980). The oxidizing atmosphere could liberate copper from insoluble sulfide salts, yielding the soluble Cu(II) form. Organisms in the early oxidizing atmosphere would initially require copper detoxification systems. The reactivity of copper would cause unintended redox reactions and create hydroxyl radicals, resulting in oxidative damage to biological molecules. Once detoxification systems were in place, copper could have been incorporated into biomolecules to participate in directed chemical reactions. Copper is now necessary to life as it functions as an enzyme cofactor

involved in redox reactions and in electron transfer. An extensive listing of copper proteins and function can be found at http://metallo.scripps.edu/PROMISE/CUMAIN.html#function.

Due to copper redox activity, copper in cells must always be sequestered to prevent toxicity. In the model organism yeast, it has been shown that there is no intracellular free copper (Rae *et al.*, 1999). Therefore, copper must be excluded from the cell until it is needed, taken up by specific transporters, transported in a complex with another molecule, and accurately delivered to its target locations.

Primary Copper trafficking proteins

Cells can transport copper across membranes through ATP-dependent transport as in P-type ATPases such as the human *ATP7A* protein (Voskoboinik *et al.*, 1998). Transport can also be ATP-independent as in the CTR family of transporters (Lee *et al.*, 2002). Copper chaperones are small, soluble molecules that route copper to specific targets in the cell and prevent reaction of copper in the cytoplasm while in transit (Harrison *et al.*, 1999). It is thought that copper chaperones are target-specific, delivering copper in direct protein-protein interactions (O'Halloran and Culotta, 2000). The N-terminal domains of P-type ATPases share one or more consensus CXXC metal binding motifs with copper chaperones and are predicted to have similar secondary and tertiary strucuture (Arnesano *et al.*, 2002). The Metallothioneins are small peptides induced under copper stress that coordinate copper and other metals by cysteine residues (Mercer *et al.*, 1981; Molnar *et al.*, 1995). In contrast to copper chaperones, which deliver to specific targets, metallothioneins are thought to sequester excess copper.

Copper in the model bacteria *Enterococcus hirae* and *Escherichia coli*

The basic mechanisms of copper homeostasis have been most completely characterized in *Enterococcus hirae* (for an extensive review, see Solioz and Stoyanov (2003). Similar primary components (transporters, chaperones, and regulators) are present in other organisms, including Eukaryotes; therefore, a discussion of copper in *E. hirae* is warranted. A schematic diagram of copper regulation in *E. hirae* (Solioz and Stoyanov, 2003) is shown in Figure 1. The *E. hirae* genome encodes a copper (*cop*) operon containing four genes including the P-type ATPases copA and copB (Odermatt *et al.*, 1993), the chaperone CopZ (Cobine *et al.*, 1999), and the repressor CopY (Strausak and Solioz, 1997). The copper-transporting ATPases CopAp and CopBp were first cloned in a screen for potassium-transporting ATPases and were the first reported copper-transporting ATPases (Odermatt *et al.*, 1992). It is thought that CopAp functions in copper uptake, as *ΔcopA* strains of *E. hirae* show reduced growth on media with limited copper supply and resistance to Ag(I) in growth media, which is considered analogous to Cu(I) (Odermatt *et al.*, 1994). For CopAp to bring copper into cells, either its direction of copper transport relative to ATP binding site or its orientation in the membrane would have to be opposite of the transport direction or orientation thought to hold for most copper ATPases. In CopBp, which has been shown to export copper from the cytosol, the heavy metal binding site (CxxC) and ATP-binding domain are on the cytosolic side of the membrane (Solioz and Odermatt, 1995). *CopZ* encodes a copper chaperone that delivers copper to the CopY repressor (Cobine *et al.*, 1999). The physical structure of CopZp has been solved (1CPZ in PDB database, (Herrmann *et al.*, 1999)) consisting

of four β-sheets and two α-helical domains in a β-α-β-β-α-β conformation with the CxxC metal binding motif in a loop between the first and second domains. It is thought that CopZp may also deliver copper to CopBp for export under conditions of copper excess (Solioz and Stoyanov, 2003). This arrangement is conserved in many other copper chaperones (Arnesano *et al.*, 2003).

CopYp is a two-part protein with an N-terminal DNA-binding domain and a cysteine-rich C-terminal domain that shares characteristics with eukaryotic copper-responsive elements (Dobi *et al.*, 1995; Wittman and Wong, 1988; Zhou and Thiele, 1991). CopYp binds DNA upstream of *CopY* through a complex with Zn(II) and is displaced by binding of two Cu(I) ions from CopZp (Cobine *et al.*, 1999). Therefore, CopYp regulates the *cop* operon in *E. hirae* (Odermatt and Solioz, 1995) through interaction with CopZp.

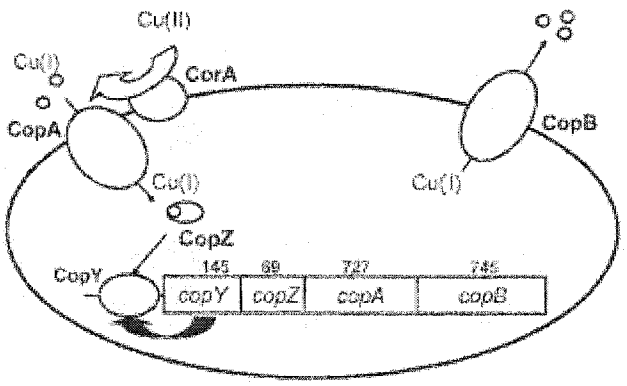

Figure 1. Schematic model of the *cop* operon and its gene products in *E. hirae*. Cu(II) is reduced to Cu(I) by an extracellular reductase (CorAp) for import by CopAp. CopZp acquires Cu(I) inside the cell and delivers it to CopY. Cu-CopY releases the upstream (promoter region) and allows transcription of the operon.

Despite broad conservation of homologues to CopZp and both *E. hirae* copper-transporting P-type ATPases, it is reported that homologues to CopY are only found in gram-positive bacteria (Solioz and Stoyanov, 2003). In contrast to *E. hirae*, *Escherichia coli* must maintain copper homeostasis in the periplasm in addition to the cytoplasm of the cell. Two chromosomally encoded copper-responsive regulatory

mechanisms have been identified in *E. coli* (*cus*: Cu-sensing and *cue*: Cu-efflux). The Cu-sensing (*cus*) locus encodes *cusR* and *cusS* as a two-component signal transduction system (*R*egulation and *S*ensing) (Munson *et al.*, 2000). The *cusCFBA* genes are regulated by *cusR* and *cusS* (Franke *et al.*, 2003; Munson *et al.*, 2000). The proteins are thought to function in periplasmic and cytoplasmic copper efflux (Rensing *et al.*, 2003) with the proteins of *cusCBA* forming a channel through the outer membrane. Copper may be delivered by the copper binding cusF protein, which is localized to the periplasm (Franke *et al.*, 2003).

The *cue* system encodes the regulatory protein cueR, controlling expression of *copA* and *cueO* (Outten *et al.*, 2000). *CopA* encodes a copper P-type ATPase functioning to remove copper from the cytoplasm (Fan *et al.*, 2000). *CueO* encodes a multicopper oxidase in the periplasm (Outten *et al.*, 2001). In addition to chromosomally encoded copper homeostasis systems, *E. coli* gains additional copper resistance from the plasmid-encoded *pco* system containing seven genes *pcoABCDRSE* (Brown *et al.*, 1995). Though not fully characterized, homologues to identified copper resistance proteins are encoded including the CueO homologue PcoA, and CusR/S homologues PcoR and PcoS (Rensing *et al.*, 2003).

Several additional genes have been identified in *E. coli* that are thought to be involved in copper homeostasis and are designated as *Cu-t*olerance (*cutA, -B, -C, -D, -E, -F*). *CutA* has been cloned and contains three genes (*cutA1, -2, -3*) in an operon that when knocked out causes increased copper accumulation and sensitivity (Fong *et al.*, 1995). *CutA2* encodes a protein disulfide isomerase (Fong *et al.*, 1995). The function of *CutA3* is undetermined. *CutA1* is conserved and the *Arabidopsis*

homologue is a primary topic of this thesis. *CutC, CutE,* and *CutF* were also isolated in copper-sensitive mutants (Gupta *et al.*, 1995; Gupta *et al.*, 1997). The functions of *CutABCDEF* are not yet entirely clear and it is thought that they may play a minor role in *E. coli* copper homeostasis (Rensing *et al.*, 2003). Although several mechanisms of copper efflux have been characterized in *E. coli*, copper uptake in the bacterium is not yet understood.

Copper in cyanobacteria

It is thought that cyanobacteria were responsible for creating the oxidizing atmosphere in which most of life must operate. The genomes of several species of cyanobacteria have been fully sequenced with *Synechocystis* PCC 6803 serving as a common model system (sequence information at http://www.kazusa.or.jp/cyanobase/Synechocystis/index.html). Modern cyanobacteria are thought to share a common ancestor with plant chloroplasts and would have developed early mechanisms for copper homeostasis. Photosynthesis in cyanobacteria functions much the same as it does in higher plants and includes homologous proteins and photosynthetic machinery. Therefore, information about cyanobacterial copper homeostasis can be used to gain insight into chloroplast copper homeostasis in higher plants. Like chloroplasts, cyanobacteria contain membrane-bounded thylakoids where photosynthetic electron transport takes place and must acquire copper for the thylakoid membrane cytochrome oxidase and the thylakoid lumen photosynthetic electron carrier plastocyanin, although some cyanobacteria can replace plastocyanin with cytochrome c_6 under copper deficiency (Zhang *et al.*, 1992). Copper is delivered to the photosynthetic machinery in cyanobacteria by two P-type ATPases: CtaAp in

the plasma membrane and PacSp in the thylakoid membrane (Tottey *et al.*, 2001). A gene encoding an Atx1-like copper chaperone (see below) has been identified in *Synechocystis* PCC 6803 that specifically interacts with CtaAp and PacSp and functions supplying copper for both plastocyanin and cytochrome oxidase (Tottey *et al.*, 2002).

Copper in a model eukaryote

Saccharomyces cerevisiae has been used as a model for the study of copper homeostasis in eukaryotic organisms. Copper is acquired in yeast by the high affinity copper transporters CTR1p and CTR3p (Dancis *et al.*, 1994; Knight *et al.*, 1996). In the cytosol, Lys7p functions as a copper chaperone for a cytosolic Cu-Zn superoxide dismutase SOD1p (Culotta *et al.*, 1999). Copper is delivered to the Golgi-localized copper transporting P-type ATPase Ccc2p by the Atx1p copper chaperone (Lin *et al.*, 1997). Ccc2p supplies copper for Fet3p, a plasma membrane multi-copper oxidase that is in turn required for high-affinity iron assimilation (De Silva *et al.*, 1995; Yuan *et al.*, 1995). The third copper chaperone, Cox17p, delivers copper ions from the cytosol to mitochondrial cytochrome C oxidase (Beers *et al.*, 1997). Intracellular levels of copper in yeast are controlled by the transcriptional activators *MAC1* and *ACE1*. Under low copper conditions, *MAC1* stimulates transcription of copper transporters and reductases for high affinity copper uptake (Labbe *et al.*, 1997), while *Ace1* stimulates transcription of metallothionein genes in yeast at high intracellular copper levels (Culotta *et al.*, 1994).

As copper homeostasis is becoming increasingly characterized in eukaryotes, it is apparent that much of the machinery is conserved in bacteria, yeast, mammals, and higher plants. In humans, functional homologues of yeast Ctr1p (Zhou and

Gitschier, 1997), Atx1p (Klomp *et al.*, 1997), Cox17p (Punter *et al.*, 2000), and CCSp (Culotta *et al.*, 1997) have been found. In addition, two homologues of Ccc2p, Menkes Disease Protein (ATP7A) and Wilson Disease Protein (ATP7B) have been identified (for a recent review, see Cox and Moore, 2002). Despite a high degree of similarity (67% identity) and localization in the trans-Golgi network, mutations in ATP7A and ATP7B are markedly different, largely due to differences in expression patterns. Menkes Disease caused by mutations in ATP7A manifest as copper deficiency in the brain and liver by lacking function in essential copper enzymes (Shim and Harris, 2003). ATP7B mutations causing Wilson Disease are clinically apparent as copper excess, particularly in the liver, whereby copper is not effectively eliminated from the cell (Shim and Harris, 2003).

Copper homeostasis in plants

Plants have unique requirements for copper in that they must take up copper from the soil, transport it to sites distant from the soil, transport the ion across the cell membrane and accurately route the ion within the cell. The primary copper enzymes in the cell are the following: (Marschner, 1995) 1) plastocyanin in chloroplasts, 2) copper-zinc superoxide dismutases in cytosol, chloroplasts and peroxisomes, 3) cytochrome C oxidase in mitochondria, 4) cell wall oxidases such as laccase and polyphenol oxidase, and 5) ethylene receptors in the plasma membrane. Insight into copper trafficking in plants is emerging as plant homologues of copper machineries in bacteria and in other eukaryotes are discovered. For reviews of copper traffic in plants, see Fox and Guerinot (1998) and Noh *et al.* (2000). Homologues of Atx1p and Ccc2p have been identified in the green alga *Chlamydomonas rheinhardtii* (La

Fontaine *et al.*, 2002), functioning to provide copper in ferric reductase necessary for iron acquisition.

In *Arabidopsis*, *COPT1* and four homologues were identified as copper transporters complementing a yeast CTR mutant strain deficient in high affinity copper uptake (Kampfenkel *et al.*, 1995; Sancenon *et al.*, 2003). *COPT* proteins are expected to transport copper from the apoplast into cells, and its members are differentially expressed in root, stem, flower and leaf tissues, but not in roots. A different transporter likely moves copper into root cells. *Arabidopsis RAN1* (Responsive-to-antagonist1) encodes a P-type ATPase involved in ethylene signaling (Hirayama *et al.*, 1999) that is a homologue of the endomembrane-localized Ccc2p and human Menkes/Wilson proteins. *AtCOX17* has been shown to complement *COX17*, which supplies copper to yeast mitochondria (Balandin and Castresana, 2002). Himelblau *et al.*, (1998) identified *CCH* as an *Arabidopsis* homologue of *ATX1*. Expression of *CCH* and the metallothionein *MT1* is upregulated in senescing *Arabidopsis*, while copper stress reduces expression of *CCH* and increases expression of *MT1* (Mira, 2002). Therefore, roles for copper chaperones in both copper acquisition and possibly mobilization during senescence are possible. It is suggested that CCHp is stable even when the mRNA level is downregulated and the protein is utilized in intercellular transport to move copper from senescing to reproductive tissue (Mira *et al.*, 2001). It is also expected that one or more copper chaperones for copper-zinc superoxide dismutases exist in plants to supply copper to the enzymes (Kliebenstein *et al.*, 1998). A functional homologue of *Lys7* has been characterized in

Lycopersicon esculentum (tomato) and a sequence homologue in *Arabidopsis* has been identified (Zhu *et al.*, 2000).

Scope of this thesis

This thesis work was undertaken to provide new insight into the homeostatic mechanisms in plant chloroplasts with respect to copper. Many components of copper homeostasis have been identified in bacteria, yeast, humans and plants where much of the basic machinery is conserved. However, plants are unique in that they require copper in chloroplasts, particularly in photosynthetic electron transport. Several components of the machinery for chloroplast copper homeostasis have recently been identified, as shown in Figure 2.

Copper traffic in chloroplasts

Besides *RAN1*, three of eight heavy metal transporting ATPases identified in the *Arabidopsis* genome are putative copper-transporting proteins (Axelsen and Palmgren, 2001). The P-type ATPase PAA1p has been characterized with a functional chloroplast targeting peptide and data consistent with function in copper transport into chloroplasts through the envelope (Shikanai *et al.*, 2003). The encoded protein shows similarity to other copper binding P-type ATPases such as Ran1p and contains a consensus MXCXXC metal binding domain. In addition, *PAA2* encodes a P-type ATPase with chloroplast localization and supporting data indicating it is involved in transporting copper across the thylakoid membrane to supply copper to lumenal proteins such as plastocyanin (Abdel-Ghany *et al.*, unpublished). In addition to *PAA1* and *PAA2*, the *E. coli CUTA* homologue *AtCUTA* has been identified as encoding a chloroplast protein with copper binding properties and data consistent

with envelope localization (Burkhead *et al.*, 2003). However, like the *E. coli* gene, the exact biological function of *AtCUTA* remains unclear.

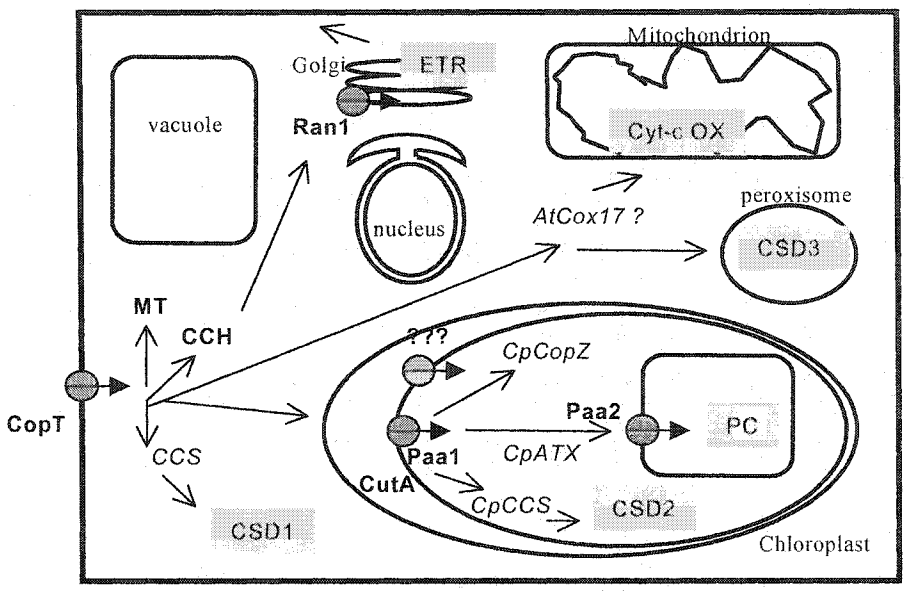

Figure 2. Copper traffic in a green *Arabidopsis* cell. Primary copper proteins are indicated in gray boxes. Membrane transporters are filled circles with arrows. Characterized components of copper traffic are indicated in boldface. Putative copper traffic proteins are in italics.

AtCUTA was identified as a homologue of the *E. coli CUTA1* gene, which is in an operon involved in copper homeostasis (Fong *et al.*, 1995). Cloning, expression, chloroplast localization, and copper binding in the purified protein are described for *AtCUTA* in Chapter 2.

Experiments documented in Chapter 3 were undertaken in order to gain additional information about the biological function of *AtCUTA*. We analyzed expression of the mRNA and protein in both wild type plants and those deficient in chloroplast copper transporters. In addition, we analyzed plants with altered *AtCUTA* expression levels.

Chapter 4 presents a biochemical characterization of the copper-binding AtCutAp, including construction and analysis of point mutants.

Since P-type ATPases have been identified to transport copper into chloroplasts and thylakoids and copper-containing proteins are known, it is expected that copper chaperones reside in chloroplasts to ensure safe and precise copper sorting. We suspect that there are at least two copper chaperones in chloroplasts, one with Atx1p-like function to transport copper from Paa1p to Paa2p, and one to supply copper to CSD2p. There may be other chaperones as well, either as redundant systems or for trafficking to other chloroplast structures. We searched the sequenced *Arabidopsis* genome for homologues of copper chaperones that also contained putative chloroplast targeting information and identified three candidates. Cloning and preliminary characterization for chloroplast localization for these candidate chaperones is described in Chapter 5.

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Chapter 2

The *Arabidopsis thaliana CUTA* Gene Encodes an Evolutionarily Conserved Copper Binding Chloroplast Protein

Summary

The *Arabidopsis thaliana AtCUTA* gene encodes a 182 amino acids long putative precursor of a chloroplast protein with high sequence similarity to evolutionarily conserved prokaryotic proteins implicated in copper tolerance. Northern analysis indicates that *AtCUTA* mRNA is expressed in all major tissue types. Analysis of cDNA clones and RT-PCR with total mRNA revealed alternative splicing of *AtCUTA* by retention of an intron. The intron-containing mRNA encodes a truncated 156-amino acid protein due to stop codons in the included intron. The sequence of AtCutAp encoded by the fully spliced transcript suggests that the precursor consists of three domains: An N-terminal chloroplast transit sequence of 70 residues, followed by a domain with prokaryotic signal sequence-like characteristics and finally a most conserved C-terminal domain. The N-terminal chloroplast transit

sequence was functional to route a passenger protein into isolated pea chloroplasts with possible sorting to the envelope. Chloroplast localization was confirmed by Western blot analysis of isolated and fractionated chloroplasts. Recombinant AtCutA protein was expressed in *Escherichia coli* without the N-terminal 70 amino acid chloroplast transit sequence. This recombinant AtCutAp was routed to the bacterial periplasm of *E. coli*. Purified recombinant AtCutAp is tetrameric and selectively binds Cu(II) ions with an affinity comparable to that reported for mammalian prion proteins.

Introduction

The transition metal copper is an essential element for all organisms. Despite being necessary for key functions, intracellular levels of transition metals such as copper must be precisely regulated to prevent damage due to reactivity with sulfhydryl groups and formation of free radical species (Nelson, 1999). Studies in the model organism yeast indicate that cytosolic free copper levels are indeed kept below one atom per cell (Rae *et al.*, 1999).

Copper functions within plant cells in a variety of processes including electron transfer in photosynthesis and respiration, and lignification of cell walls (Fox and Guerinot 1998). Copper homeostasis is also important to antioxidative responses and senescence (Mira, *et al.*, 2002). Key components of the photosynthetic machinery require specific metal cofactors including copper, as do other enzymes within chloroplasts. Plastocyanin is thought to contain more than 50% of the copper in chloroplasts (Marschner 1995).

The plastid genome does not contain all genes necessary for chloroplast function. A large number of proteins must be synthesized in the cytosol from nuclear transcripts, imported through the chloroplast envelope and targeted within the organelle based on information in an N-terminal transit peptide and secondary sorting information (Schnell, 1998; Keegstra and Cline, 1999; Bruce, 2000). Much of the posttranslational assembly of photosynthetic metalloproteins occurs within chloroplasts after import of the precursors (reviewed by Merchant and Dreyfuss 1998). We are interested in the molecular mechanisms that underlie the regulation of metal ion uptake, storage, and mobilization by plant chloroplasts. As a first step, we

need to identify specific gene products involved in chloroplast metal homeostasis or metal binding.

Copper homeostasis has been well-characterized in yeast and mammals and a number of genes involved in copper trafficking have been identified in plants including *RAN1*, a homologue of P-type ATPase Ccc2 (Hirayama, *et al.*, 1999), and *CCH*, a homologue of the copper chaperone *Atx1* (Himelblau, *et al.*, 1998; Mira, *et al.*, 2001). Much of what is known about copper trafficking in *Arabidopsis thaliana* has been characterized by complementation studies in yeast mutants (for an extensive review of copper traffic in plants, see Guerinot 2000).

One component that may be involved in divalent metal ion homeostasis in prokaryotes is *CUTA*. The *CUTA* region in *Escherichia coli* was characterized by evidence of increased copper uptake and sensitivity in mutant cells (Fong *et al.*, 1995; Crooke and Cole, 1995). The *E. coli CUTA* gene (also called *CUTA1* or Orf 112) encodes a soluble protein of 112 amino acids that may have a role in divalent metal tolerance (Fong *et al.* 1995). However, thus far, the exact biochemical role of the *CUTA* protein in *E. coli* is not clear. The Arabidopsis Genome Initiative (AGI, http://www.arabidopsis.org/home.html) revealed the presence of a *CUTA* homologue, *AtCUTA*, in *Arabidopsis thaliana*. Here, we present evidence that *AtCUTA* is expressed in root and shoot tissues, the encoded protein has specific copper-binding properties, and that the protein is localized to chloroplasts. In addition, we find that *AtCUTA* mRNA is alternatively spliced. Results are discussed in the light of a possible function of *AtCUTA* in plastid metal ion homeostasis.

Results

To identify chloroplast proteins involved in divalent metal homeostasis, we screened databases for genes encoding possible homologues of prokaryotic proteins involved in divalent metal homeostasis that are predicted to have plastid targeting sequences. *AtCUTA* was one of the genes identified as a homologue of *E. coli CUTA* (Fong *et al.*, 1995) using this approach. *AtCUTA* (Figure 1) is predicted to encode a precursor protein of 182 amino acids with three predicted domains. Amino acids 1-70 make up a chloroplast targeting sequence as predicted by the neural network-based programs ChloroP and TargetP (http://www.cbs.dtu.dk/services/; for literature reference see Emanuelsson *et al.*, 1999). Amino acids 71-106 include a number of hydrophobic residues and comprise a domain with signal sequence-like characteristics that when combined with the 70 N-terminal amino acids potentially functions as a bipartite targeting peptide (Smeekens *et al.*, 1986, Schnell *et al.*, 1998). The C-terminal domain of AtCUTAp (amino acids 107-182) is the most conserved region (Figure 1). Cleavage of the AtCutA precursor at amino acid 70 results in a 112 amino acid protein with 58% similarity to the 112-amino acid *E. coli* CutAp. Homologues of *CUTA* can be identified in numerous species by BLAST (Basic Local Alignment Search Tool; http://www.ncbi.nlm.nih.gov/BLAST/). An alignment of AtCutAp with several homologues is shown in Figure 1. Notable is a high degree of sequence conservation among phylogenetically divergent organisms including Bacteria, Archaea, and Eukarya. No other homologues of CutAp were detected by BLAST search in the completely sequenced genome of *A. thaliana*.

To begin a functional analysis of *AtCUTA*, two clones of ESTs for *AtCUTA* were obtained. The insert in one of the clones (92B37T) was very short (<200 base pairs as determined by PCR and restriction analysis) and thus determined to be incomplete. Restriction digest and PCR analysis indicated that the second clone (213A16T7) was complete. Analysis of the genomic sequence predicts six exons and five introns to give about a 700-base transcript. Surprisingly, sequence analysis revealed that this clone (213A16T7) retained the fifth intron and would predict a transcript of about 850 bases. Though the predicted protein translated from a fully spliced mRNA is 182 amino acids in length, translation of the EST clone would result in a protein lacking 26 C-terminal amino acids due to stop codons in all reading frames of the retained intron. The results suggest that *AtCUTA* is alternatively spliced. Indeed, RT-PCR revealed the presence of mRNA species corresponding to both fully spliced and intron-containing transcripts (Figure 2). The 700 base pair fragment shown in lane 4 of Figure 2 was cloned and its sequence was compared to that of the intron-containing clone. Sequence data confirmed that the shorter RT-PCR product was identical to the intron-containing clone except for the included intron and they are from transcripts of the same gene.

Northern blot analysis of poly $(A)^+$ RNA from different tissues was carried out to determine the expression levels of *AtCUTA* mRNA. All major tissue types contained a single transcript of about 1.35 kb that hybridized with the *AtCUTA* probe (Fig 3). The presence of this single transcript indicates that the intron-containing mRNA (which is less abundant by RT-PCR) is not expressed at high levels. These results, together with the RNA blot analysis of ubiquitin (Fig 3) suggest that *AtCUTA* is

expressed in all major plant organs and the mRNA level is comparable in all tested tissues.

We identified two independent T-DNA insertional knockout lines for *AtCUTA*. The first contained four insertions while the second contained three. RT-PCR confirmed that *AtCUTA* mRNA was absent in the homozygous plants of both lines. Neither homozygous knockout line showed an obvious phenotype when grown on soil, tissue culture, and tissue culture with 30 μM copper when compared to wild-type plants. The second line containing only three T-DNA insertions was used for preliminary tissue culture experiments. We found no difference between 14-day old knockout and wild-type plants grown in tissue culture with either no additional copper or 30 μM $CuSO_4$. Mean fresh weights are as follows: wild type control, 31.4 mg, knockout control, 27.56 mg; wild type copper-treated, 20.8 mg, knockout copper-treated, 19.0 mg. A second trial was performed with similar results. All analysis of variance p-values were greater than 0.10. We tentatively conclude that *AtCUTA* is not essential under normal conditions nor under high copper conditions.

In order to investigate biochemical and metal binding properties, recombinant full length AtCutAp (fully spliced, lacking the putative N-terminal chloroplast targeting sequence) was expressed in BL21-DE3 *E. coli.* A protein of 12.4 kDa was produced (Figure 4, lane 2). This size suggests that the putative signal sequence-like domain is not cleaved in *E. coli*. Recombinant full length AtCutAp was recovered from BL21-DE3 culture by osmotic shock and separated from the bulk of the cellular proteins, suggesting a periplasmic location. Recombinant full length AtCutAp was purified

from the periplasmic fraction by ion-exchange chromatography on a DEAE Sepharose column (Figure 4, Lane 3). The sequence of the N-terminal six amino acids of the purified protein as determined by Edman degradation was MEESSK. This result identified the purified protein as AtCutAp and confirmed that the putative 36-amino acid signal sequence-like domain was not processed by *E. coli*. Purified recombinant AtCutAp was analyzed by gel filtration. The protein eluted as a single peak with apparent molecular weight of 43 kDa, suggesting it is purified from *E. coli* as a tetramer. The intron containing clone was expressed in BL21-DE3, and it was present in the periplasmic fraction following osmotic shock. The truncated protein was purified with the same techniques as the fully spliced protein (Figure 4, Lane 6). SDS-PAGE indicated a molecular weight corresponding to the predicted 9.8 kD for the truncated protein. Gelfiltration analysis indicated that truncated AtCutAp is predominantly tetrameric (results not shown).

To test the hypothesis that *AtCUTA* may be involved in metal homeostasis, we analyzed the recombinant protein for interactions with metal ions by immobilized metal ion affinity chromatography (IMAC). Purified protein was incubated with metal-saturated resin. The unbound fraction was removed and the resin was washed with the binding buffer. The resin was washed with a pH 5.5 buffer, then finally eluted with EDTA to remove all metals and associated protein. Results are shown in Figure 5. No recombinant full length AtCutAp was recovered in the unbound fraction for copper-saturated resin, nor was AtCutAp released in the pH 5.5 wash. Full length AtCutAp was quantitatively recovered when the copper-saturated resin was washed with EDTA (Figure 5, panel a). In contrast, recombinant AtCutAp was

quantitatively recovered in the unbound fraction of all other metal-saturated resins and was not present in the EDTA washes. For the truncated AtCUTAp, some, but not all, protein was recovered in the unbound fraction of copper and nickel-saturated resin (Figure 5, panel b). Truncated AtCUTAp was recovered in the pH 5.5 wash of nickel and zinc saturated resin. Most of the loaded truncated AtCUTAp was recovered in the EDTA wash of copper saturated resin. Some truncated AtCUTAp was also recovered in the EDTA wash for nickel as well as zinc. These results indicate that fully spliced AtCUTAp interacts specifically with copper, while truncated AtCutAp has less specificity in metal binding.

Copper affinity and Cu(I) versus Cu(II) specificity were determined for purified fully spliced AtCutAp by incubation with copper ions in solution followed by unbound copper ion removal by ultrafiltration. The results indicate that purified AtCutAp preferentially binds Cu(II) ions in solution at an approximately 1:1 ratio. AtCutAp has low affinity for Cu(I) in solution by this test while Cu(I) binding was below the detection limits of the assay when EDTA was added to the buffer solution to remove divalent copper ions (Figure 7) (Rae *et al.* 1999). Therefore, the observed binding to Cu(I) may be due to partial oxidation to Cu(II). Half maximal binding under experimental conditions was at about 50 μM Cu(II) (Figure 6, inset). Aggregation of protein was visible as a precipitate at copper concentrations above 100 μM. The positive control BSA (Linder 1991) bound Cu(II) at approximately 3.5:1 ratio while lysozyme, the negative control, did not show Cu(II) binding within the detection limits of the test (not shown). Truncated AtCUTAp had a similar half-maximal binding specificity for Cu(II) as fully spliced AtCUTAp under the same experimental

conditions (data not shown). Unfortunately, the truncated protein showed an increased tendency to aggregate at high Cu(II) concentrations, which precluded the determination of a Kd.

Precursor-AtCutAp contains characteristics of a precursor protein with a targeting peptide for plastid localization. *In vitro* import studies were performed using isolated pea chloroplasts to analyze the presence of chloroplast targeting and possible suborganellar routing information in the AtCutA precursor. A fusion clone with plastocyanin mature sequence as a passenger was constructed to provide for efficient labeling with ^{35}S-methionine. The results, shown in Panel a of Figure 7, indicate that the plastocyanin control protein was imported to the thylakoid fraction and processed to its mature size (Figure 7, Panel a, top). Upon incubation of the AtCutA-plastocyanin fusion precursor with isolated chloroplasts, labeled proteins with smaller size than the precursor but larger than mature plastocyanin were detected in the chloroplasts (Figure 7, Panel a, bottom). This intermediate-sized protein was fully protected against thermolysin treatment of intact chloroplasts and thus fully translocated across the chloroplast outer envelope membrane. Quantification indicated that the import efficiency of the fusion was comparable to that of the authentic plastocyanin precursor. After hypotonic lysis of chloroplasts and centrifugation, the fusion protein was recovered in the soluble fraction and not in the membrane pellet. The lysis procedure separates thylakoids from soluble stromal and envelope fractions, indicating that the fusion protein was either in the stroma or in the envelope intermembrane space.

Protease treatments were used to distinguish stromal and envelope localized proteins in additional import experiments. Trypsin can pass through the chloroplast outer membrane, but not the inner membrane and will digest proteins exposed to the intermembrane space of the chloroplast envelope; thermolysin cannot pass through the outer envelope (Lübeck *et al.*, 1997). Chloroplasts were isolated and incubated with AtCutA-plastocyanin fusion as above, followed by fractionation and protease treatments designed to differentiate between stromal or chloroplast envelope localization. The results are shown in Panel b of Figure 7. The AtCutA-plastocyanin fusion protein was imported and protected from digestion by thermolysin. However, the protein was sensitive to trypsin even after import, indicating a possible chloroplast envelope location. The soluble nature of AtCutAp suggests that it is located in the intermembrane space rather than as a membrane protein in the inner or outer envelope. Degradation of the AtCutAp-plastocyanin protein in the stromal fractions by both thermolysin and trypsin confirmed susceptibility to both proteases. To directly analyze chloroplast localization of AtCutAp in *Arabidopsis*, we raised antibodies against the purified fully spliced protein. The antiserum was specific to AtCutAp and detected as little as 20 ng of purified AtCutAp. In wild-type plants grown under standard conditions, detection of AtCutAp required overloading of gels for Western blots. Therefore, we used AtCutAp-overexpressing line SPL-6B to detect the protein in subcellular fractions. Panel c of Figure 7 shows AtCutAp in total plant homogenate, intact chloroplasts, and a stromal/soluble envelope fraction, thus confirming chloroplast localization of AtCutAp. Positive control blots on the same fractions were performed with proteins for chloroplast thylakoids

(plastocyanin, Smeekens, *et al.*, 1986) and chloroplast stroma (AtNifS, Pilon-Smits *et al.*, 2002). Negative controls for chloroplast isolation and immunodetection from the overexpressing plants were for mitochondria (PMO35, Tom Elthon, personal communication), endoplasmic reticulum (AtSEC12, Bar-Peled and Raikhel, 1997), and cytosol (p28, Sebastian Bednarek, personal communication). The negative controls were present in the total homogenate but not in the chloroplast fractions as indicated in Panel d of Figure 7.

Discussion

The *Arabidopsis thaliana* gene *AtCUTA* was characterized in this study. Comparison of *AtCUTA* with sequences from other organisms showed that CutA protein sequences are evolutionarily conserved, which may reflect a critical function of the protein. *AtCUTA* mRNA is expressed in all major tissue types and the mRNA is alternatively spliced. Alternative splicing may have a role in controlling the functional characteristics of *AtCUTA*—altering the physiochemical properties of AtCutAp by inclusion or exclusion of an intron and translation of a truncated or full-length protein. Although numerous animal genes have been identified that have tissue- or developmental stage-specific alternative splicing (Green, 1991), the role of alternative splicing in gene expression in plants is a relatively new area of research, and the functional significance is not yet clear in the case of *AtCUTA*. Among the characterized examples of alternative splicing in plants, functions range from control of flowering time (MacKnight *et al.*, 1997) to controlling subcellular localization as influenced by light regulation (Mano *et al.*, 1999) and in one case a single mitochondrial targeting sequence carrying either of two mature proteins (Kubo *et al.*, 1999).

An important clue to the function of AtCutAp is obtained from its subcellular localization. Targeting sequences that route cytosolically synthesized proteins to plastids, and signal sequences for sub-organellar sorting have been documented in the literature (Smeekens *et al.*, 1986; Schnell, 1998; Keegstra and Cline, 1999). The domain structure of the AtCutA precursor is reminiscent of precursors with bipartite targeting peptides, such as pre-plastocyanin, that serve to localize the mature protein

to the thylakoid lumen (Smeekens, *et al.*, 1986). However, AtCutAp does not contain the AXA↑X motif described by Schnell (1998) as a consensus thylakoid cleavage site. *In vitro* uptake experiments with isolated chloroplasts indicated that AtCutAp is routed to chloroplasts and sorted to either the stroma or envelope. It is possible for an envelope protein to be sorted as a soluble stromal intermediate and exported to the envelope, as is the case of IEP110 (Lübeck, *et al.*, 1997), though we suspect AtCutAp will be in the intermembrane space rather than the inner envelope membrane. The observation that recombinant AtCutAp expressed in *E. coli* accumulated in the periplasm, a location that can only be reached by undergoing export from the cytosol, supports the assertion that the putative secondary signal sequence functions in export. Assuming that the signal sequence-like domain of AtCutAp was responsible for the periplasmic location in bacteria, a similar mechanism may be used to export AtCutAp to the chloroplast envelope after import into the stroma and cleavage of the N-terminal 70 amino acid transit sequence. Mammalian CutAp (mCutAp) is contains an N-terminal membrane anchor (Navaratnam *et al.*, 2000). This could also be the case with the similar putative secondary signal sequence in AtCutAp. Thus, it is feasible that the mature AtCutAp is localized to the intermembrane space and retained by the hydrophobic N-terminal region inserted in the inner membrane.

Purified recombinant AtCutAp showed a specific interaction with Cu(II) ions, both with copper ions bound to IDA-agarose resin and in solution. The IDA-agarose assay has been used to characterize other proteins that bind transition metals but the target proteins usually interact with several metals in this assay (Dykema *et al.*, 1999).

Therefore, the absolute specificity for Cu that is observed with AtCutAp stands out. Truncated AtCutAp showed more promiscuity in binding to metal-saturated IDA-agarose than did fully spliced AtCutAp, suggesting that the C-terminal region of the protein might help determine metal specificity.

Analysis of *AtCUTA* knockout lines indicated that *AtCUTA* is not essential for copper tolerance or accumulation. Nevertheless, *AtCUTA* may be involved in chloroplast copper homeostasis. The specific interaction with Cu(II) ions suggests that AtCutAp occupies a role other than a typical copper chaperone, since copper chaperones have been characterized as Cu(I) binding proteins (Harrison *et al.*, 2000). Thus, if AtCutAp does serve as a copper chaperone, it would be a novel Cu(II)-binding chaperone. AtCutAp does not contain a consensus metal binding motif (such as CXXC) commonly found in metal binding proteins such as copper chaperones or transporters (Fox and Guerinot, 1998). Furthermore, the single cysteine residue in the mature protein is insufficient to have sequence characteristics of a metallothionein (Zhou and Goldsbrough, 1995, Goldsbrough, 1999). Rather than having a role in metal sequestration, the observed interaction of AtCutAp and Cu(II) may suggest a regulatory or signaling role for AtCutAp, as has been suggested for the mammalian cellular prion proteins which bind Cu(II) with a comparable affinity (Stöckel *et al.*, 1998). CutA proteins lack the octarepeat peptides found in prion proteins, which have been reported to be involved in Cu(II) binding (Stöckel *et al.*, 1998). Therefore, AtCutAp may bind to copper by a novel copper-binding motif. A regulatory or signaling role may also be possible for bacterial CutAp since *E. coli* mutants in the

operon containing *CUTA* are more sensitive to increased copper levels than wild type and accumulate increased amounts of divalent cations (Fong *et al.*, 1995).

In conclusion, *AtCUTA* is expressed in all major tissues, it is alternatively spliced and codes for a chloroplast-localized copper binding protein. AtCutAp is a member of a class of copper binding proteins whose function has not yet been determined. AtCutAp is found in the chloroplast, an organelle containing peptides and enzymes that require copper for proper function. Interestingly, there is a human *CUTA* homologue localized to neural synapses in the brain (Navaratnam *et al.*, 2000). Thus, the human *CUTA*, like AtCutAp, has a posttranslational routing outside the cytosol. Further characterization of *AtCUTA* will include detailed analysis of phenotypes in plants with altered expression levels and may provide insight into chloroplast copper homeostasis in plants as well as other types of organisms.

Materials and Methods

Vectors and clones

Genbank accession numbers for fully spliced and intron-containing *AtCUTA* are AF327524 and AF327525, respectively. *AtCUTA* EST clones were obtained from the Ohio State University *Arabidopsis* Biological Resource Center as SalI/NotI inserts in pSPORT vectors (Life Technologies, Rockville, MD). pBluescriptKS is a Stratagene (San Diego, CA) product, pET11-d was obtained from Novagen (Madison, WI). Vector pSP64-PC was described by Smeekens *et al.* (1986). pMOG 18 and pMOG23 were described by Sijmons *et al.*, (1990).

Materials/Enzymes

MS salts were obtained from Sigma (St. Louis, MO). The RNeasy RNA isolation kit and Qiaquick Gel Extraction Kit were obtained from Qiagen (Valencia, CA). The High Fidelity PCR kit (HF-PCR) and Titan RT-PCR kits were obtained from Roche (Palo Alto, CA). The Ampliscribe *in vitro* transcription kit was purchased from Epicentre (Madison, WI). M-mLV reverse transcriptase and wheat germ extract *in vitro* translation reagents were obtained from Promega (Madison, WI). DEAE Sepharose and ^{35}S-labeled methionine (1000 Ci/mmol) was obtained from Amersham-Pharmacia (Uppsala, Sweden). Imino-diacetic acid agarose was obtained from Novagen (Madison, WI). All other reagents were of the highest quality commercially available. *Arabidopsis thaliana* ecotype Columbia seed was a gift from Dr. June Medford (Colorado State University). Antibodies to AtSEC12 protein and p28 protein were gifts from Dr. Sebastian Bednarek (University of Wisconsin). Antibodies to PMO35 were a gift from Dr. Tom Elthon (University of Nebraska).

The Salk Institute Genomic Analysis Laboratory provided the sequence-indexed Arabidopsis T-DNA insertion mutant (http://signal.salk.edu/index.html).

Cloning

Two cDNA clones were obtained from the Ohio State University *Arabidopsis* Biological Resource Center. The clones were analyzed by restriction digest and PCR in which general sequencing primers M13(-20) and M13rev amplified the inserts. The clone 92B3T7 insert was very short (<200 base pairs) and determined to be incomplete. It was not used for further analysis. DNA was sequenced on both strands (M13(-20) and M13rev primers and two internal primers) by the dideoxy method. To obtain a fully spliced cDNA clone, cDNA from 14-day old seedlings grown on 1/2-strength Murashige and Skoog (MS) medium, was generated from isolated RNA using RT-PCR. The following optimized PCR conditions were used: hot start at 94°C for 2 minutes; melting 94°C, 30 seconds; annealing 55°C, 45 seconds; extension 68°C with cycles 1-10, 2 minutes, 30 seconds; cycles 11-20, 4 minutes; cycles 21-30, 5 minutes; and cycles 31-35, 6 minutes. Primers A (5' CACAGTGATGGCTTCGTCTCT), B (5' TCATTTGACGAACTGGTCCAGT), and C (5' CGCATTACTCATGTTTTTATATG) were designed based on sequence data. The PCR product for a fully spliced *AtCUTA* transcript (700 base pairs) was extracted from a 1% agarose gel and modified to introduce NcoI and BamHI sites (HF-PCR). Primer Cl1 (5'GGGGTACCATGGCTTCGTCTCTCACCACTAG) was used to introduce KpnI and NcoI sites upstream and primer Cl2 (5' GGGGTACCGGATCCTGGTCCAGTATTACACTTCACA) introduced KpnI and BamHI sites downstream of the coding sequence. The PCR products were cloned

into the KpnI site of pBluescriptKS. This clone is identified as pBS-spl (*AtCutA* fully spliced) and was sequenced to confirm fidelity. The insert was then subcloned from pBS-spl into the pET11d expression vector and pMOG18 as a BamHI/NcoI restriction fragment to generate pET-spl and pMOG18-spl, respectively. The Cl1 and Cl2 primers were also used to introduce restriction sites into the EST clone 213A16T7. This cDNA was then subcloned to pBSKS to generate pBS-int and then to pET11d to generate pET-int.

HF-PCR was employed to remove the putative chloroplast targeting sequence from both the pBS-spl and pBS-int. The Mod1 (5' GGGGTACCATGGAGGAGAGCAGCAAAACTG) upstream primer was used to introduce KpnI and NcoI restriction sites while the downstream primer was the Cl2 primer used in cloning. These products were cloned as a KpnI fragments to pBluescriptKS vector to generate pBS-M5 (fully spliced) and pBS-M10 (intron containing). pBS-M5 and pBS-M10 were subcloned to pET11-d as a BamHI/NcoI restriction fragments to generate pET-M5 and pET-M10. The targeting sequence of *AtCUTA* was amplified from the pBS-spl clone using a primer for the upstream SP6 promoter and the Fus1 (5' GCGCAAGCT TGCAACAGCGATGGCTTCGTCTCTCAC) downstream primer to introduce an NcoI restriction site. This restriction fragment was inserted as a PstI/NcoI fragment in the PstI/NcoI digested pSP64 vector, which contained the mature plastocyanin sequence, to generate pCAPC.

Overexpression of AtCutAp in *Arabidopsis*

The fully spliced *AtCUTA* cDNA was subcloned from pMOG18 along with the Cauliflower Mosaic Virus 35S promoter and enhancer sequence to pMOG23 to generate pMOG23-spl. The pMOG23-spl binary vector was transformed to *Agrobacterium tumafaciens* strain C58C1 (An, *et al.*, 1988). *Arabidopsis thaliana* (Ecotype Columbia) plants were transformed with this strain by the floral dip method (Clough and Bent, 1998). Transgenic lines were selected for kanamycin resistance. Twelve independent lines were confirmed by PCR to include the *AtCUTA* construct. None of these lines showed an obvious phenotype. Western blotting was used to identify lines with increased expression of AtCutAp. Of four homozygous lines with significant increased expression, one line was chosen for the experiment shown in Figure 8c.

Copper tolerance in *AtCUTA*-knockout

To examine the tolerance of *AtCUTA*-knockout seedlings to excess copper, WT and knockout seeds were surface-sterilized essentially as described by Pilon-Smits *et al.* (1999). For each treatment, approximately 25 seeds of each line were sown in a grid pattern in a single Magenta box (Sigma, St. Louis, USA) on medium containing half-strength Murashige and Skoog (MS) salts and vitamins (Murashige and Skoog, 1962), 10 g L^{-1} sucrose, and 4 g L^{-1} agargel (Sigma), with or without 30 μM $CuSO_4$. After 14 days at 25°C and 16/8 L/D photoperiod, individual seedlings were harvested and weighed.

Expression in *E. coli* and protein isolation

The pET-M5 and pET-M10 plasmids were transformed to *E. coli* BL21-DE3 (Studier *et al.*, 1990). Cultures were grown in 500 mL batches of LB medium at 37°

C with vigorous shaking to an OD_{600} of 0.3 and induced by adding IPTG to 0.3 mM. Incubation continued to an OD_{600} of 1.7 when cells were collected by centrifugation at 4000x*g* in a fixed angle rotor at 4°C. Cells were subjected to osmotic shock to isolate periplasmic proteins, essentially as described by Neu and Heppel (1965). Proteins were further purified at 4°C on a 1.5 cm x 14 cm DEAE Sepharose column equilibrated with 25 mM Tris/HCl pH 7.5 + 10% (w/v) glycerol. After allowing the protein to bind, the column was washed with 10 mL of the same buffer. Elution began with 10 mL of 200 mM NaCl in 25 mM Tris/HCl pH 7.5 + 10% (w/v) glycerol and continued in a continuous gradient from 250 mM NaCl to 500 mM NaCl in the same buffer for 500 mL. The recombinant *AtCUTA* protein expressed from pET-M5 eluted at 425 to 475 mM NaCl and was free from contaminants as observed by SDS-PAGE with Coomassie Brilliant Blue staining. Peak fractions were pooled for a yield of approximately 2.5 mg protein/500 mL of original culture. The recombinant *AtCUTA* protein expressed from pET-M10 eluted from the column at 275 to 300 mM NaCl. Peak fractions were pooled for a yield of approximately 2.0 mg protein/500 mL of original culture. Gelfiltration used a Sephacryl S-200 column, 60x1.6 cm, (Amersham-Pharmacia) in 10 mM $NaPO_4$ pH 7.5, 150 mM NaCl.

Immobilized metal binding chromatography

Purified full length AtCutAp and truncated AtCutAp were analyzed using a metal-saturated IDA-agarose (Novagen, Madison, WI). 100 μL aliquots (settled volume) of resin were stripped by incubation in 1 mL of 100 mM EDTA, 500 mM NaCl, 20 mM Tris pH 7.5, washed 3 times with sterile distilled water and saturated with one of the following 400 mM solutions in water: NaCl, $CuSO_4$, $NiSO_4$, $ZnSO_4$, $MnSO_4$, or

$CdSO_4$. Resins were washed first 3 times with 1 mL distilled water and then 3 times with 1 mL binding buffer (50 mM potassium phosphate, pH 7.5, 250 mM NaCl). 50 μL of each resin was nitric acid digested and analyzed by ICP-AES (Zarcinas *et al.*, 1987) to confirm metal saturation. The remaining 50 μL resin portions were then incubated with 20 μg of the purified AtCutAp (lysozyme (Sigma-Aldrich, St. Louis, MO) and Bovine Serum Albumin (Sigma-Aldrich) were used as a controls) in a total of 300 μL of buffer for 10 minutes on ice. The resin was collected by centrifugation (5000 rpm/45 seconds in a bench top microfuge) and the supernatant collected as the unbound fraction. Three additional washes with the binding buffer were followed by a low pH wash (50 mM KPi, pH 5.5, 250 mM NaCl) whereby the supernatant was collected as the pH 5.5 wash. The final wash was with 100 mM EDTA (50 mM KPi, pH 6.0, 250 mM NaCl) to release metal ions and associated protein from the resin. Protein in each fraction was concentrated with a final concentration of 0.0015% w/v deoxycholic acid and 15% w/v trichloroacetic acid. Precipitations were incubated on ice for ten minutes and pelleted by centrifugation at 13,000*g*, 4°C for 10 minutes. Pellets were washed with ice-cold 100% acetone and dried in a vacuum desiccator before dissolution in SDS-PAGE sample buffer (125 mM Tris/HCl pH 6.8, 10% w/v glycerol, 2% SDS, 10 mM dithiothreitol). Each fraction was analyzed by SDS-PAGE followed by staining with Coomassie Brilliant Blue.

Copper affinity assay

To determine Cu(II) binding in solution, 800 μg of full length or truncated AtCutAp was incubated for 5 minutes at room temperature in 10 mM Tris pH 7.5, 100 mM NaCl, 0-1.5 mM $CuSO_4$ in a volume of 500 μL. The solution was loaded into a

Nanosep Centrifugal device (Pall Gelman Laboratory, Ann Arbor, MI) with a molecular weight cutoff of 10 kD. The device was centrifuged in a bench top microcentrifuge for 10 minutes at 7000 rpm after which less than 50 μL remained. The retentate was then diluted back to 500 μL in 10 mM Tris pH 7.5, 100 mM NaCl and centrifuged as before. This was repeated 2 additional times and the final retentate brought back to 500 μL in the same buffer. 10 μL was used for protein determination by Bradford assay. 100% w/v TCA was added to the remaining retentate to a final concentration of 9.2% and the reaction was placed on ice for ten minutes. The tube was then centrifuged at 14,000*g* for 10 minutes at 4°C to collect the protein. The supernatant containing released Cu ions was then neutralized with 80 μL 6 M NaOH and 100 μL of 1 M untitrated Tris buffer. To this, 10% (w/v) ascorbic acid was added to 0.6% final concentration to reduce Cu(II) ions to Cu(I) ions. Bathocuproindisulfonic acid (BCS) (Sigma) was added to a final concentration of 0.65 mM to determine Cu(I) concentration by OD_{485} (Zak, 1958) with a standard curve from 0-200μM Cu(I). Cu(II) binding by lysozyme as a negative control and BSA as a positive copper binding control were performed simultaneously under the same conditions. Cu(I) binding was obtained by reducing 1 mM $CuSO_4$ in 1% (w/v) ascorbic acid and using this as a stock to repeat the copper binding test as described. A second Cu(I) binding test was performed with the addition of 1 mM EDTA to remove any Cu(II) ions that might be present (Rae *et al.*, 1999). Various buffer conditions were tested to determine suitable conditions for Cu(II) affinity tests. Cu(II) affinity was determined for AtCutAp by using the described procedure with $CuCl_2$ concentrations of 0, 10, 20, 30, 40, 60, and 80 μM in 50 mM MES pH 6.0,

150 mM NaCl (Stöckel et al, 1998). Standard curves for spectrophotometric determination of Cu content with BCS were made by adding TCA, NaOH, Tris, ascorbic acid and BCS as described for experimental tests.

Tissue expression

To analyze the expression of AtCUTA, total RNA was isolated from roots, stems, leaves and flowers by the TRIzol reagent method (Life Technologies). Poly $(A)^+$ RNA was isolated using the oligotex mRNA kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Twelve μg poly $(A)^+$ RNA was electrophoresed on a 1% agarose gel containing 4% formaldehyde, transferred to a nylon membrane, and probed with a ^{32}P-labeled 700 bp *AtCUTA* cDNA. Prehybridization and hybridization were performed at 65°C in a solution containing 0.5M sodium phosphate and 0.7% SDS (w/v). Following hybridization, the membrane was washed with 0.1X SSC and 0.1% SDS at 65°C, wrapped in cling film, and placed in a phosphoimaging cassette (Molecular Dynamics). The screen was scanned in a Storm 840 phosphoimager (Molecular Dynamics, Sunnyvale, CA).

The hybridized probe was removed by washing the membrane three times at 65°C, 5 min each, with stripping buffer (0.1X SSC, 1% SDS, 40 mM Tris, pH 7.5) containing 50% (v/v) formamide and once with stripping buffer without formamide according to Ausubel et al., 1994. The membrane was re-probed with a ^{32}P-labelled 2 kb cDNA fragment that encodes for *Arabidopsis* ubiquitin. Prehybridization, hybridization and washing were performed as before.

In vitro transcription and translation

Plasmids pSP64-PC (pre-plastocyanin, Smeekens *et al.*, 1986), and pCAPC (this study) were linearized by EcoRI and PvuII, respectively, and transcribed *in vitro* using the Ampliscribe SP6 transcription kit with Cap analog (Epicentre, Madison, WI) according to the manufacturer's instructions. 0.2 μg of *in vitro* transcribed messenger RNA from each transcription was used in a wheat germ extract kit (Promega) with ^{35}S methionine (25 μCi/50 μL reaction). Translations were analyzed with SDS-PAGE and visualized using a STORM phosphor imaging system (Amersham Pharmacia, Uppsala, Sweden).

Chloroplast import and fractionation

Pea chloroplasts (from cultivar Little Marvel) were isolated using procedures detailed in Pilon *et al.* (1992). 500 μL import reactions contained 30 μL of translation mixture (see above) incubated with the isolated chloroplasts as described in Pilon *et al.* (1992). Procedures to obtain crude stromal and thylakoid fractions are detailed in Smeekens *et al.* (1986).

For selective protease treatment experiments, incubated chloroplasts (150 μg chlorophyll) were reisolated by centrifugation through a 40% (w/v) Percoll cushion, resuspended in 600 μL import buffer. Half of these were kept on ice and subjected to hypotonic lysis to obtain a stromal fraction. The remaining chloroplast portion was subdivided to three 100 μL fractions, which were treated with either thermolysin or trypsin (Lübeck *et al.*, 1997) or left untreated. Thermolysin treatment (125 μg/mL) for chloroplasts was at 4°C for 15 minutes. The thermolysin-treated chloroplast reaction was stopped by addition of EDTA in import buffer to a concentration of 100 mM. Trypsin treatment (235 μg/mL) for chloroplasts was 30 minutes at 20°C and

was stopped by addition of trypsin inhibitor to 920 μg/mL. Intact and protease-treated chloroplasts were collected by centrifugation (3 minutes, 2500 rpm) and dissolved in SDS-PAGE sample buffer. Similarly, the stromal fraction was divided to three fractions for thermolysin, trypsin, or control treatment at the same protease concentrations and conditions. Stromal protease treatment was stopped by TCA precipitation (0.0015% w/v deoxycholic acid and 15% w/v trichloroacetic acid) on ice. Fractions in TCA were centrifuged at 13,000*g* for 30 minutes, supernatants removed, and washed with 100% acetone. Pellets were air dried and resuspended in 40 μL of sample buffer. Precursor and import fractions were separated by SDS-PAGE and the dried gel was analyzed with a STORM 840 phosphor imaging system.

***Arabidopsis* chloroplast isolation**

To determine localization of AtCutAp in *Arabidopsis*, chloroplasts were isolated essentially as described by Rensink et al. (1998). Chloroplasts were isolated from rosette leaves (3-4 weeks old) of either wild type or AtCutAp-overexpressing line SPL-6B plants. Chloroplasts were precipitated by centrifugation and resuspended in 330 mM sorbitol, 50 mM Hepes-KOH (pH 8). One half of each fraction was again precipitated and lysed by resuspension in 300 μL of 10 mM Tris-HCl (pH 8). After 2 minutes on ice, an equal volume of 660 mM sorbitol, 100 mM Hepes-KOH (pH 8) was added. Thylakoid membranes were precipitated by centrifugation and resuspended in 330 mM sorbitol, 50 mM Hepes-KOH (pH 8). Total plant homogenate, chloroplasts, stroma (including envelopes), and thylakoid fractions were precipitated in 80% acetone, incubated on ice for 30 minutes and collected by centrifugation at 12,000*g* for 20 minutes. Pellets were air-dried and suspended in

SDS-PAGE sample buffer. Proteins were separated by 15% SDS-PAGE with each sample containing protein equivalent to 3 μg chlorophyll as determined by OD_{652} (Bruinsma 1961). Proteins were transferred to a nitrocellulose membrane by Western blot and visualized by immunodetection using AtCutAp-specific antiserum.

General methods

Protein concentrations were determined by a protein assay (Bradford, 1976) that was corrected to the absorption at 280 nm due to the presence of aromatic amino acids (Perkins, 1986).

Statistical Analyses

Statistical analyses for tolerance and accumulation were performed using the statistical software program JMP-IN from the SAS Institute (Cary, NC, U.S.A.).

Anitbody production

For AtCutAp antibody production the purified protein was dialyzed to 25 mM Na-phosphate pH 7.0, 150 mM NaCl. Polyclonal antibodies were raised in chickens at a commercial facility (Aves Labs, Tigard, OR).

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Figure legends

Figure 1. Sequence alignment of CutA isologs. *Arabidopsis thaliana, Aquifex aeolicus Methanobacterium thermoautotrophicum, Homo sapiens,* and *Escherichia coli.* Identical residues are denoted with an asterisk (*). Similar residues are denoted with dots. Predicted chloroplast targeting sequence for *Arabidopsis* is underlined. The secondary signal sequence-like domain is boxed. Sequence alignment performed by DN*Assist* software.

Figure 2. Alternative splicing shown by RT-PCR. PCR experiments using AtCutA specific (A, B, and C) primers that hybridize to the regions are indicated with arrows in the diagram. Lane 1: 1 kb marker. Lane 2: A and B primers with EST clone template. Lane 3: A and C primers with EST clone template. Lane 4: A and B primers with cDNA template. Lane 5: A and C primers with cDNA template. cDNA was synthesized from mRNA isolated from 14 day old *Arabidopsis thaliana* seedlings grown on ½ MS agar medium. The size for the cloned fully spliced *AtCUTA* cDNA is denoted with an asterisk (*).

Fig 3. Northern blot analysis. Poly (A)+ RNA was isolated from different tissues, electrophoresed and blotted to a nylon membrane. The blot was successively probed with ^{32}P-labelled *AtCUTA* specific and ubiquitin (UBQ) specific probes, respectively. Molecular weight markers are shown at the left in kb. Arrows indicate *AtCUTA* and ubiquitin transcripts.

Figure 4. Purification of Recombinant AtCutAp. Lane 1: Molecular weight standard. Lane 2: Total protein fraction from induced *E. coli* culture containing pET-M5 plasmid. Lane 3: Purified recombinant full length AtCutAp protein from *E. coli* culture. Lane 4: Molecular weight standard. Lane 5: Total protein fraction from induced *E. coli* culture containing pET-M10 plasmid. Lane 6: Purified recombinant truncated AtCutAp protein from *E. coli* culture.

Figure 5: Binding to metal IDA-Agarose. Panel a: recombinant AtCutAp-M5 (full length protein). **Panel b:** recombinant AtCutAp-M10 (truncated protein). 50 μL of resin was equilibrated with the indicated metals and washed four times with binding buffer (50 mM KPi, pH 7.5, 250 mM NaCl) before the addition of 20μg protein. Gel I: unbound fraction. Gel II: Proteins released in a low pH (5.5) wash. Gel III: EDTA wash to remove bound metal ions and proteins from IDA-agarose resin. Analysis was by SDS-PAGE, visualization by Coomassie Brilliant Blue staining.

Figure 6: Copper ion specificity and affinity of full length AtCutAp. Bar graph: moles Cu/mole AtCutAp by incubation with 1 mM Cu(I) or Cu(II) and ultrafiltration to remove unbound Cu ions. Inset: Cu(II) affinity plot.

Figure 7: Localization of AtCutAp in chloroplasts. Panel a: Import of AtCutAp-plastocyanin fusion protein into isolated pea chloroplasts. Precursor translations in wheat germ system with ^{35}S-methionine. Proteins were separated by

SDS-PAGE, gels dried and visualized by a phosphor imaging system. Fractions loaded on gel: PR: precursor; C: reisolated chloroplasts; CP: thermolysin-treated reisolated chloroplasts; S: stroma from thermolysin-treated chloroplasts; T: thylakoids; TP: thermolysin-treated thylakoids. **Panel b: Import of AtCutAp-plastocyanin fusion protein into pea chloroplasts with selective protease treatments.** Proteins visualized as in Panel A. Fractions loaded on gel: precursor; intact chloroplasts; thermolysin-treated chloroplasts; trypsin-treated chloroplasts; untreated stroma; SP: thermolysin-treated stroma; STR: trypsin-treated stroma. **Panel c: Western blot analysis of *Arabidopsis* homogenate and chloroplast fractions.** Proteins separated by SDS-PAGE. Each lane corresponds to 3 μg of chlorophyll. SPL: AtCutAp-overexpressing plants; WT: wild-type plants. Fractions loaded on gel: H: plant homogenate; CI: intact chloroplasts; S: stromal proteins; T: thylakoids; +: *E. coli*-expressed positive control. Bands corresponding to precursor-sized proteins are denoted by asterisks (*). **Panel d: Immunological controls for chloroplast localization.** Proteins separated and blotted. Fractions labeled as in Panel c. Controls: PC, plastocyanin (thylakoid), AtNifS (stroma), PMO35 (mitochondria), p28 (soluble cytosol), SEC12 (endoplasmic reticulum).

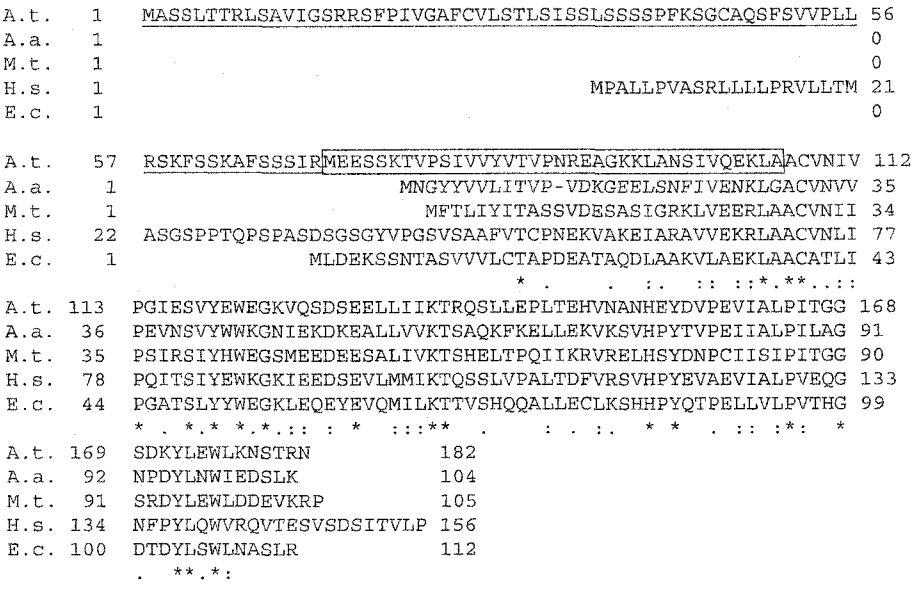

Figure 1

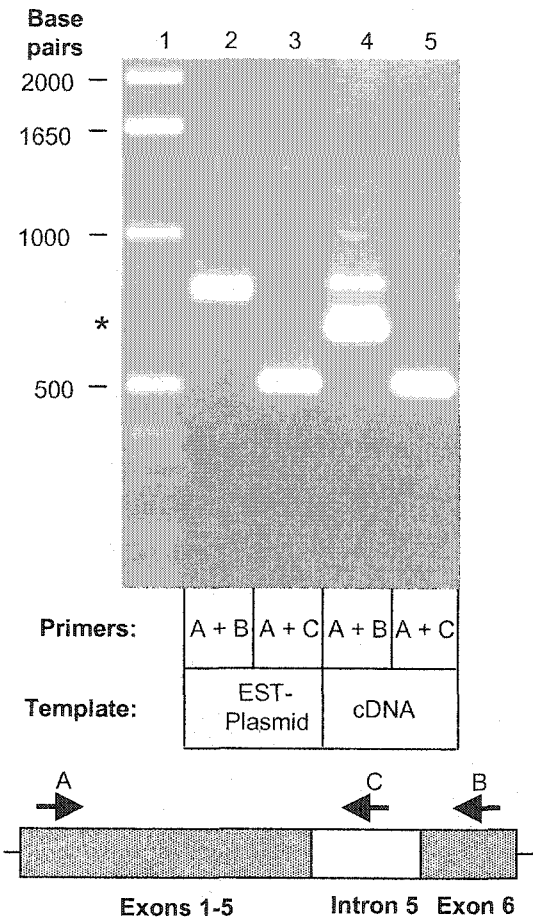

Figure 2

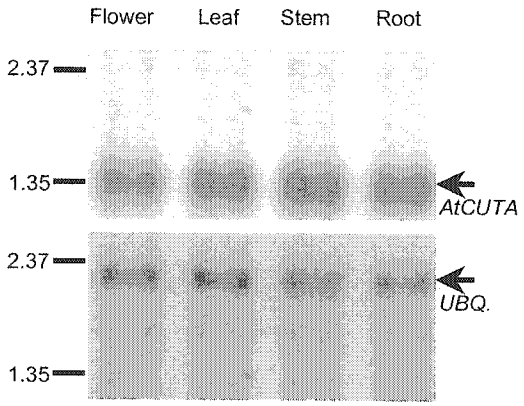

Figure 3

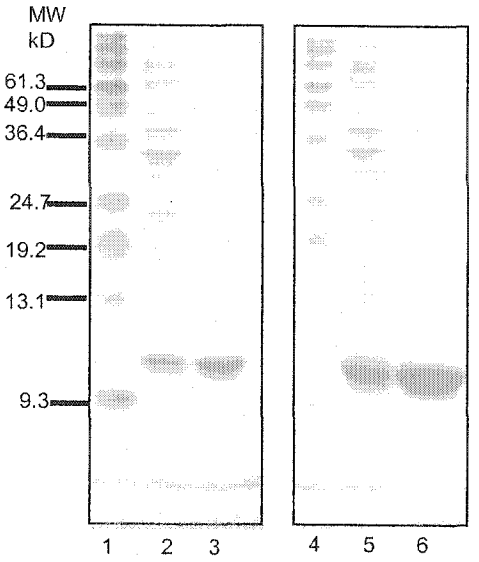

Figure 4

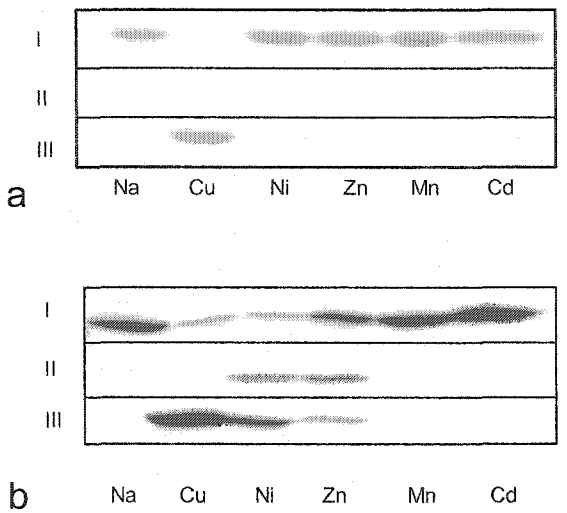

Figure 5

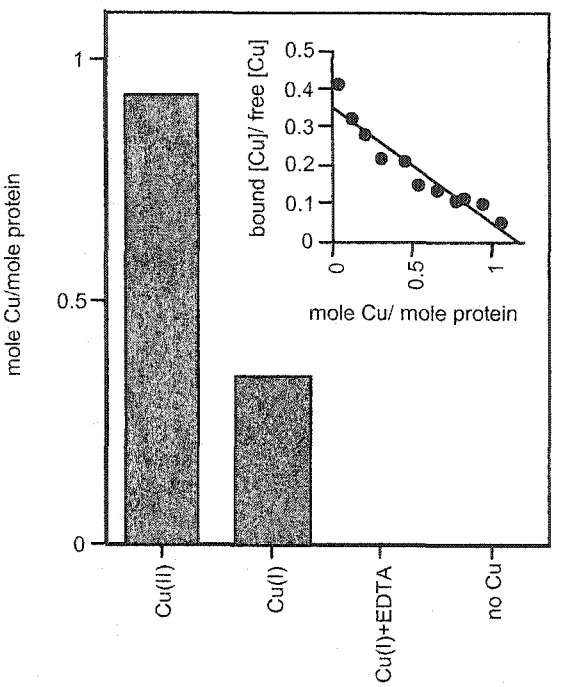

Figure 6

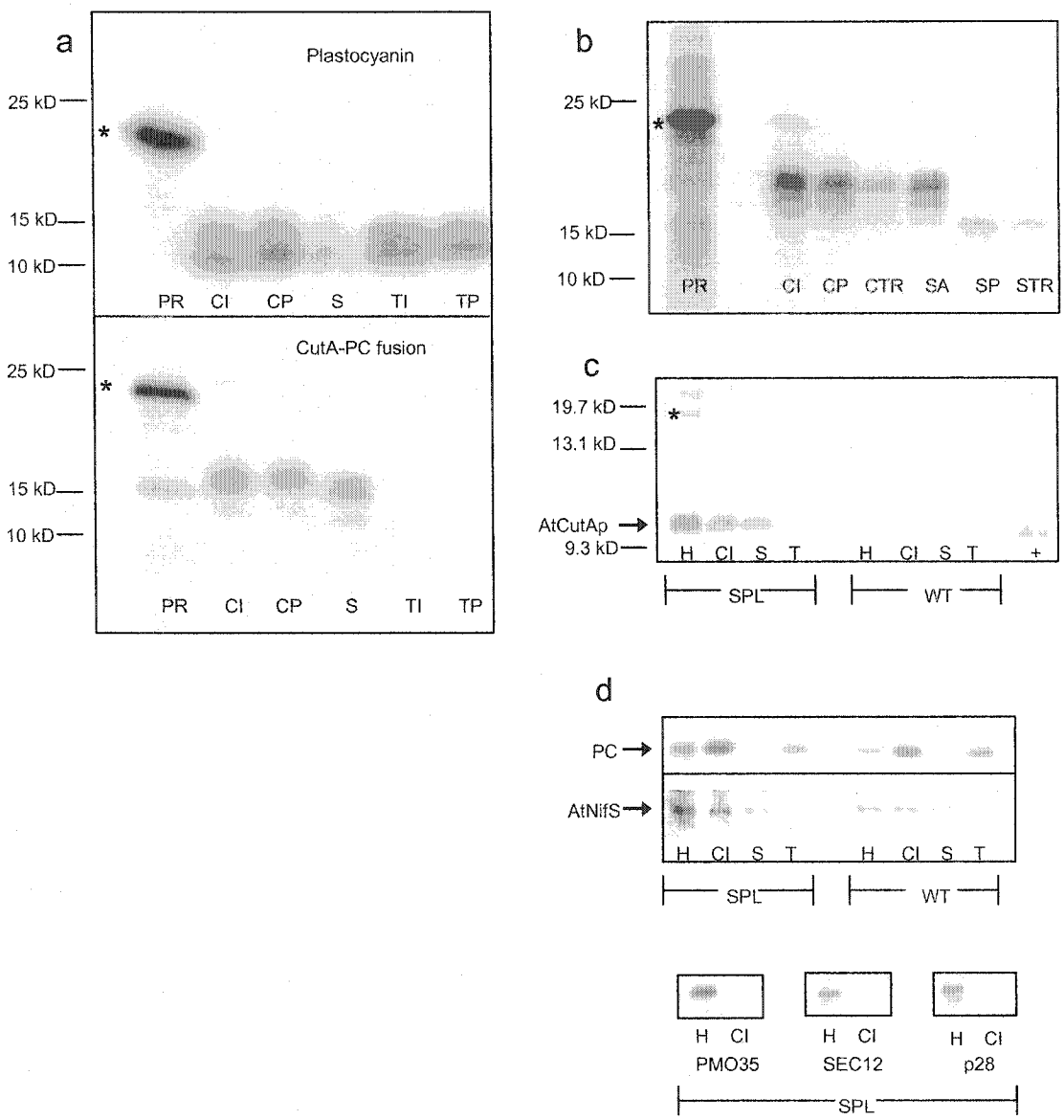

Figure 7

Chapter 3

Characteristics of *AtCUTA*: expression and analysis of plants with artificially altered expression levels

Summary

AtCUTA encodes a protein with copper binding properties that is located in chloroplasts. For functional analysis of *AtCUTA*, we obtained insertional knockout mutants in *AtCUTA* and transformed *Arabidopsis* plants with either *AtCUTA*-antisense or *AtCUTA*-overexpressing constructs. Plants with altered levels of *AtCUTA* expression had little or no difference in terms of growth and development on soil, tissue culture, or tissue culture with excess copper when compared to wild type plants. Thus, under the conditions tested, the physiological role remains unclear.

Antibodies were raised against recombinant AtCutAp. The antibody preparation was tested and optimal conditions for detection of the protein in plant samples were determined. We found that both senescence and deficiency in chloroplast copper transporters increases levels of AtCutAp in shoot tissue, even though no differences in mRNA levels were observed. These results indicate that

AtCUTA expression may be regulated at the translation level. We mapped the 5'UTR of *AtCUTA* within 10 bases by RT-PCR and found a 60-70-base 5' region with a predicted stable secondary structure that may influence translation of the protein *in situ*.

Introduction

Copper levels must be tightly regulated in cells and copper is required for photosynthesis in *Arabidopsis* as a component of plastocyanin (Weigel *et al.*, 2003). Therefore, there must be specific proteins for routing copper to chloroplasts, transport of the metal across the envelope, sorting within the organelle, and regulation of copper levels within chloroplasts.

Expression and function of genes implicated in chloroplast copper homeostasis is a relatively new area of study. Chloroplasts must acquire copper from the cytosol and must have machinery for import across the chloroplast envelope to supply plastid copper proteins.

Several key components of the copper trafficking machinery in chloroplasts have recently been identified. *PAA1* has been identified as a P-type ATPase that functions in copper transport across the envelope into chloroplasts (Shikanai *et al.*, 2003). Another chloroplast localized P-type ATPase in *Arabidopsis* is *PAA2* (Abdel-Ghany *et al.*, unpublished data). The copper binding protein AtCutAp has been localized to chloroplasts with data that implies retention in the envelope (Burkhead *et al.*, 2003). *AtCUTA* may have a role in chloroplast-specific copper homeostasis. Data presented here further support this assertion. Even though AtCutAp binds copper and is localized to plastids, its exact biological role is unclear. This study was designed to

gain further insight into the biological function of *AtCUTA*. To this end, we have analyzed expression characteristics of *AtCUTA* in wild type plants along with *paa1* and *paa2* mutants as well as general growth and copper tolerance phenotypes for plants with altered expression levels of *AtCUTA*. These studies suggest that *AtCUTA* may be involved in senescence and copper homeostasis in *Arabidopsis*.

Materials and methods

Plant growth conditions: *Arabidopsis* plants were grown on Metromix 350 potting soil (Scotts, Hope, AR). Plants grown in tissue culture for experiments shown in Figures 2 and 4 were surface-sterilized essentially as described by Pilon-Smits *et al.* (1999) and sown on 0.4% agar-solidified media with 1% sucrose and 1/2 MS salts (Murashige, 1962). Copper tolerance in AtCUTA-overexpressing lines was analyzed as described for the *AtCUTA*-insertional knockout line in Burkhead *et al.* (2003).

Plant material: *Paa1-1, Paa2-1, and Paa2-2* seeds were provided by Dr. T. Shikanai (Nara Institute of Technology, Nara, Japan). *Paa1-3* seeds were provided by Dr. C. Niyogi (University of California, Berkeley). The CUTA-Wisc T-DNA knockout line was obtained from the University of Wisconsin *Arabidopsis* Knockout Facility (http://www.biotech.wisc.edu/arabidopsis/default.htm; (Krysan *et al.*, 1999)). This line contained three insertions in addition to one in the *AtCUTA* gene (abbreviation: CutA-Wisc) and was used only to analyze preliminary growth phenotypes. The CUTA-SALK knockout line was obtained from The Salk Institute Genomic Analysis Laboratory (http://signal.salk.edu/index.html) as line # SALK 029578. All plant lines were propagated in the lab on potting soil.

RT-PCR expression analysis and 5'UTR analysis

To analyze tissue specific expression of *AtCUTA* transcripts in *AtCUTA*-knockout compared to wild type seedlings in Figure 2, Panel a seedlings were grown for 14 days on ½ MS agar and total RNA was isolated using the RNeasy kit according to the manufacturer's instructions. DNase treatment resulted in recovery of about 10 μg at 0.2 μg/μL RNA (in 50 μL) as measured by OD_{260}. 2μg of total RNA was used in cDNA synthesis with the poly-T primer and M-mLV reverse transcriptase (Promega) according to the manufacturer's instructions. The original reaction was diluted 1:1 with water. From that dilution, serial dilutions were made resulting in 1:4, 1:16, 1:64, and 1:256 dilutions of cDNA. The dilutions served as templates for PCR. PCR was performed with *AtCUTA*-specific primers hybridizing in Exon 1 (5' CACAGTGATGGCTTCGTCTCT) and Exon 6 (5' TCATTTGACGAACTGGTCCAGT) as well as *Actin-2*-specific primers (An *et al.*, 1996) as an internal control in the same tube. PCR conditions are as follows: hot start at 96° C for 3 minutes; 30 cycles of 96°C for 15 seconds, 55°C for 30 seconds, 72°C for 2 minutes. The reactions were diluted in a loading buffer, electrophoresed on a 1% agarose gel containing ethidium bromide and DNA visualized with a UV trans-illuminator.

To analyze expression of *AtCUTA* transcription by RT-PCR in *Paa1-3*, *Paa2-1*, and wild type plants grown with excess copper (Figure 4), seeds were surface-sterilized and plants were grown in tissue culture as described above as either control (1/2 MS, 1% sucrose) or on the same media with 25 μM $CuSO_4$. RNA was isolated and reverse transcribed to cDNA as described above with 1:10 and 1:20 serial

dilutions of cDNA used as template for PCR. *AtCUTA*-specific and *Actin-2*-specific primers were used as described for Figure 2.

5'UTR analysis

PCR primers for mapping are shown in Table 1. 5' untranslated region secondary structure predictions were made using RNAfold in a web-interface format at http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi.

Table 1. Primers for 5' UTR mapping

Exon 2	5' CATAGACAACAATGCTGG CAC
UTR 1	5' GACGGCTGAGATACACAGTGAT
UTR 2	5' GACATTTCGCAGCTTCCAGAAA
UTR 3	5' TTATGCAATTTCGTTTTAGACCC
UTR 4	5' TTTTCCTCCTCTCGTCACTGTG
UTR 5	5' CACATTCTCCAGAGCAAACAAAC
UTR 6	5' CCTCGAAATCGAAAAAATCGAAG
UTR A	5' ACAGTTAAAGGACATTTCGCA
UTR B	5' TTACATTATTACAGTTAAAGG
UTR C	5' CCCTAAACTATTACATTATTAC
UTR D	5' CGTTTTAGACCCCTAAACTATT

Protein expression analysis

Soil or tissue culture-grown plants were harvested and immediately ground in liquid nitrogen. Soluble protein was extracted from ground plant tissue in 50 mM Tris-HCl pH 7.5, 50 mM NaCl, 0.1 mM EDTA, 20 mM $MgCl_2$, 10 mM PMSF and 10% glycerol. Protein concentrations were determined by the method of Bradford (1976). Chloroform extractions were performed by adding an equal volume of water-saturated chloroform to each sample, vortexing for five minutes, centrifuging in a benchtop microcentrifuge for five minutes at 10,000*g*, and retaining the aqueous layer for analysis. Chloroform-extracted samples were normalized for protein content using the protein concentrations acquired prior to chloroform extraction. Following SDS-

PAGE, proteins were transferred to a nitrocellulose membrane by Western blot for immunodetection with AtCutAp-specific antiserum.

Results

Characteristics of plants with altered expression of *AtCUTA*

In order to gain information of the biological function of *AtCUTA*, an insertional knockout mutant was obtained and *AtCUTA*-antisense lines were constructed along with *AtCUTA*-overexpressing lines first described in Burkhead *et al.*, (2003). Figure 1 shows a schematic diagram of the T-DNA insertion, overexpressor, and antisense constructs for *AtCUTA*. The *Arabidopsis* knockout line used was from the Salk collection (line # 029578) and is abbreviated CutA-Salk. This T-DNA insertion is in the second intron, resulting in no *AtCUTA* message detectable by RT-PCR in a homozygous line (Figure 2, Panel a). Serial dilutions of cDNA from the CutA-Salk line and wild type *Arabidopsis* were analyzed with *AtCUTA*-specific primers and primers for *Actin-2* in the same reaction. A strong signal is detectable in the wild type cDNA, while no signal is detectable in CutA-Salk cDNA.

Seven independent homozygous *AtCUTA*-antisense lines were identified from *Arabidopsis* plants transformed by *Agrobacterium*-mediated plant transformation. Exons 1-5 of the *AtCUTA* gene were inserted into the genome under control of the Cauliflower Mosaic Virus 35S promoter as described for the *AtCUTA-overexpressor* by Burkhead *et al.* (2003). RT-PCR analysis of these lines indicated no appreciable reduction in *AtCUTA* transcripts (data not shown); therefore, experiments to investigate effects of reduction of *AtCUTA* transcript levels were focused on using

insertional knockout lines. Representative *AtCUTA*-antisense lines used for initial analysis were abbreviated as follows: as1.2, as2.1.3, and as5.2.

Four independent homozygous *AtCUTA*-overexpressor lines were identified. Relative expression levels of AtCUTAp were analyzed by Western blot of total protein from 21-day old whole seedlings (Figure 2, Panel b). Expression levels of AtCUTAp in spl6B and spl11.4 have the highest expression, while spl5.8 had intermediate expression compared to wild type. Spl13.1 showed similar expression of AtCUTAp compared to the low-expressing wild type. Higher molecular weight proteins visible on this blot appear to be a result of cross-reactivity in the antibody.

In order to compare growth characteristics and analyze possible phenotypes plants were grown on soil and monitored over the entire life cycle. None of the *Arabidopsis* lines with altered *AtCUTA* expression showed a visible phenotype under standard soil growth conditions. A photograph of representative overexpressor, antisense, and knockout plants grown along with wild type *Arabidopsis* is shown in Figure 3.

AtCutA-overexpressing lines were tested for copper tolerance as described for the AtCutA-knockout line in Burkhead *et al.* (2003). No significant difference in fresh weight was found for *AtCUTA*-overexpressors spl5.8, spl6B, and spl11.4 grown on 1/2 MS media or 1/2 MS supplemented with 30μM $CuSO_4$. Only spl6B, the most extreme overexpressor, showed reduced copper tolerance in one of two independent tests when grown on MS media with 30 μM $CuSO_4$. Mean fresh weights for spl6B and wild type seedlings are listed in Table 2. For Trial 1, the One-way ANOVA P-value for copper-treated wild type vs. spl6B seedlings is 0.0249. For Trial 2, the One-

way ANOVA P-value for copper-treated wild type vs. spl6B the copper-treated seedlings is 0.45. There was no significant difference in the control treatment for either trial. Therefore, though there is a trend toward smaller seedlings for spl6B

		Trial 1	Trial 2
Line	Cu (uM)	Fresh weight	Fresh weight
wild type	0	50.47	40.41
spl6B	0	38.28	35.7
wild type	30	23.14	21.16
spl6B	30	17.19	19.57

Table 2. Mean fresh weight of 14-day old *Arabidopsis* seedlings grown in tissue culture.

compared to wild type under copper stress, the difference was not reproduced.

AtCUTA transcript expression

Messenger RNA expression patterns may provide clues about the function of *AtCUTA*. *AtCUTA* mRNA was detected in all major tissues (Burkhead *et al.*, 2003). Since *AtCUTA* has been implicated in copper homeostasis, RT-PCR analysis of mRNA from tissue culture-grown plants was used to determine if *AtCUTA* transcript levels were influenced by excess copper. The mutant lines *PAA1-3* and *PAA2-1* deficient in envelope and thylakoid copper transport (Shikanai *et al.*, 2003); (unpublished data), respectively, were also analyzed. *AtCUTA*-specific PCR product was detected in wild type, *PAA1-3*, and *PAA2-1* lines for both the control treatment and the 25 μM $CuSO_4$ treatment (Figure 4). It may be noted that there is no *AtCUTA*-specific PCR product for the *PAA2-1* template cDNA of copper-treated plants, while there is for the direct serial dilution of the same template. This appears to be a PCR artifact. Specific PCR product in a lower dilution of cDNA indicates presence of the

transcript in the original cDNA pool. The presence of the *Actin-2* control in the same reaction indicates that template cDNA was indeed present. Both *AtCUTA*-specific and *Actin-2* PCR products were also present in wild-type, *PAA1-3* and *PAA2-1* lines grown in 1/2 MS media with the copper chelator cuprizone (data not shown). These results indicate that *AtCUTA* mRNA is present under normal, excess and restricted copper conditions. Thus, there does not appear to be any difference in the expression response to copper, at least as can be judged by this semi-quantitative method.

Expression of AtCutAp

We next aimed to investigate the expression of *AtCUTA* at the protein level. Due to low expression of AtCutA protein in wild type plants as described in Burkhead *et al.* (2003), it was necessary to optimize protocols for antibody detection of the protein in total plant extracts. In addition to low expression of the protein in wild type plants, the significant problems in antibody detection of AtCutAp were cross reactivity with other proteins and the interference of lipids and pigments, which may influence resolution in gel electrophoresis. We used a chloroform extraction procedure to remove remaining hydrophobic proteins and pigments from protein extracts. The aqueous fraction was then dissolved in SDS-PAGE sample buffer and used for Western blot analysis. The ability to quantitatively recover AtCutAp in the procedure was tested using *AtCUTA*-overexpressing line spl11.4. Figure 5, Panel a shows that the chloroform extraction does not affect levels of AtCutAp detected in spl11.4 plants. The protein could be detected with anti-AtCutAp antisera in both control and chloroform-extracted samples with 5.0 and 0.5 μg or equivalent protein loaded. Five nanograms of purified AtCutAp could be easily detected on the same

blot. In protein samples from overexpressing plants, the antibody detected two AtCutAp-specific bands, with one corresponding to the purified protein molecular weight and one at a higher molecular weight. We suspect this doublet is the result of a posttranslational modification in the plant cell. As a control for chloroform extraction, the same protein samples used for Western analysis were analyzed by SDS-PAGE. Fifteen, 1.5, and 0.15 μg total protein or extracted equivalent were separated on a polyacrylamide gel and stained with Coomassie Brilliant Blue. Enhanced resolution of protein bands and reduction in overall protein in chloroform-extracted samples is visible in Figure 5, Panel b, showing that indeed the total protein, lipid and pigment loaded is reduced by chloroform extraction. Distinct protein bands can be seen in the highest-concentration of chloroform-extracted sample, as opposed to the unextracted sample.

To further characterize detection of AtCutAp by Western blot and immunodetection with a polyclonal antibody, a blot was performed with 30 μg equivalent chloroform-extracted protein from wild type, AtCutA-knockout, and two AtCutA-overexpressing lines. Figure 5, Panel c shows AtCutAp detectable in the 5 ng positive control, wild type, spl6B and spl11.4. There was no detectable AtCutAp in the AtCutA-knockout line. Therefore, detection of AtCutAp was specific.

Additional tests were performed to determine the influence of copper and oxidative stress on the expression of AtCutAp. Wild type plants were grown in tissue culture on agar-solidified media for 14 days and subsequently treated in aqueous solutions of 10 μM $CuSO_4$, 10 μM paraquat (methyl viologen), 300 μM ferrozine (to complex iron), and water. No differences were seen in AtCutAp expression by

Western blot (not shown). Two-week old seedlings were also tested in 50, 100, and 200 μM Cu and 100, 200 and 400 nm paraquat and for 24 hours to induce acute copper and/or oxidative stress. Again, no differences in AtCutAp expression were detected as compared to untreated or water-treated seedlings (data not shown).

In order to determine AtCutAp expression in different plant tissues, Western blot analysis was used to analyze shoot, silique, and root tissue from mature plants in addition to senescing shoot tissue. The results are shown in Figure 6 along with positive controls. Significant AtCutAp could be detected in senescing tissue, migrating at the same molecular weight as the upper band in the AtCutAp-overexpressing positive control. This higher molecular weight species is indicated in Figure 6 as AtCutAp*. Mature shoot green tissue had little to no detectable expression, while silique and root tissue contained no detectable AtCutAp. Therefore, senescence appears to promote expression of *AtCUTA* at the protein level.

To determine AtCutAp expression in mutant *Arabidopsis* plants deficient in chloroplast copper transport, Figure 7 shows Western analysis of shoot tissue from five-week old plants grown on soil. Arrows indicate the two migration positions of AtCutAp-specific bands on the blot. For plants deficient in a chloroplast envelope transporter (*PAA1-1*), shoots and the corresponding wild-type ecotype (Landsberg *erecta*) had no detectable expression. *PAA1-3*, also deficient in the *PAA1* copper transporter, showed expression of the lower molecular weight AtCutAp, along with its corresponding wild type ecotype (Columbia). Both *PAA2-1* and *PAA2-2*, deficient in a putative thylakoid copper transporter, had detectable quantities of both the lower and higher molecular weights of AtCutAp. Therefore, it appears that mutation of the

thylakoid membrane copper transporter Paa2p corresponds with upregulation of AtCutAp translation.

Wild type Columbia root tissue had a detectable quantity of the lower molecular weight AtCutAp, but expression in root tissue was only seen in this experiment and may have been an unrelated band. Senescing tissue consistently contained the higher molecular weight form of the protein. Expression of AtCutAp in *PAA1-3, PAA2-1, PAA2-2*, wild type Columbia, and senescing shoot tissue was confirmed by multiple experiments.

5'UTR analysis

If the abundance of *AtCUTA* transcripts in all major tissue types and under both high and low copper stress is considered with the relatively low amount of detectable protein in plant tissue, it is possible that regulation of AtCutAp expression is posttranscriptional. Transcript levels as indicated by RT-PCR (Figure 4) are comparable in wild type, *PAA1-3*, and *PAA2-1* plants. These results suggest constitutive expression of *AtCUTA* mRNA and potential posttranscriptional regulation. The 5' untranslated region of specific mRNA can influence translation initiation, possibly by forming a stem-loop secondary structure. Of five EST clones with sequence available from the TAIR database (The Arabidopsis Information Resource, http://www.arabidopsis.org/), only a single clone contained 5'UTR sequence. This clone (213A16T7) contained 9 bases of 5'UTR. Therefore, RT-PCR was used to determine the 5'UTR of *AtCUTA* represented in cDNA from *Arabidopsis* seedlings. A downstream primer hybridizing in exon 2 of *AtCUTA* was used with a series of upstream primers. Figure 8, Panel a, left shows the results of coarse mapping

with PCR primers in 50-base increments as indicated Figure 8, Panel c. PCR products for the exon 2 primer and both UTR 1 and UTR 1 indicate a 5' endpoint between UTR 2 and UTR 3. PCR products in Panel a, left for the UTR 5 primer are non-specific PCR artifacts, as they do not correspond to the size expected for an mRNA transcript (450 bp). (Figure 8, Panel a, right shows positive control PCR products with the indicated primers and a genomic DNA template. Fine mapping in 10-base increments was performed with another set of upstream primers used in conjunction with the Exon 2 primer. The results shown on the left of Figure 8, Panel b, indicate a PCR product of expected mRNA size for UTR A, with the UTR 2 primer as a positive control. Bands at approximately 320 and 330 base pairs correspond to the expected mRNA size. Stronger signals are noted at approximately 380 and 390 bp, indicating contamination of the cDNA with genomic DNA. Positive control for the primers is shown in Figure 8, Panel b, right, indicating all primers used will amplify the expected product from genomic DNA. These results indicate a 5'UTR of between 60 and 70 bases in length.

The secondary structure of mRNA can be predicted with the web-based program RNAfold (Mathews, 1999). RNAfold predicts a stable secondary structure for the 5'UTR of *AtCUTA* for the AUG start codon and 65 bases upstream. The secondary structure is shown in Figure 9 as a highly base-paired (22 base pairs) structure with potential implications in translation initiation due to its assumed stability. As a control for a chloroplast copper protein, the 5'UTR of plastocyanin was also analyzed by RNAfold. The program does not predict a stable secondary structure for the 5'UTR of plastocyanin (Vorst *et al.*, 1988), as shown in Figure 9, panel b.

AtCAB1 has been characterized with posttranscriptional regulation (Saha *et al.*, 2003). The 64-base 5'UTR of *AtCAB1* was analyzed with RNAfold. Results for *AtCAB1* are shown in Figure 9, panel c indicate a potentially stable a stem-loop structure with 11 base pairs. Further indication of a role for the *AtCUTA* 5'UTR in translational regulation stems from the observation of no beta-glucoronidase (GUS) activity from an *AtCUTA::*GUS promoter fusion in transformed *Arabidopsis* plants, while the control CAMV35S::GUS promoter showed high activity (Amsbaugh and Pilon, unpublished). High levels of AtCutAp are seen in overexpressing lines (Figure 2), which lack the 5'UTR between the CAMV35S promoter and the start codon for *AtCUTA* translation.

Discussion

Despite high levels of mRNA expression, *AtCUTA* is not essential for normal growth. An insertional knockout line for *AtCUTA* has no detectable transcript by RT-PCR, yet exhibits normal growth and has copper tolerance—comparable to the wild type as described (Burkhead *et al.*, 2003). Likewise, *AtCUTA* lines overexpressing the gene product showed normal growth and comparable tolerance to copper stress when compared to wild type. These results indicate that the gene product is not essential under standard growth conditions as tested here. *AtCUTA* may be a redundant component of a copper homeostasis (or other, unidentified) system in *Arabidopsis*. In addition, *AtCUTA* may be required for survival only under specific conditions not tested herein. Despite a lack of significant growth phenotype in *AtCUTA*-knockout plants, we suspect the gene serves a precise function, given its retention in the *Arabidopsis* genome. As suspected for *CUTA* in E. coli, it is possible *AtCUTA* may

play only a minor role in copper homeostasis. The developed transgenic plant lines, however, will be useful in discerning chloroplast-specific phenotypes. Specifically, varied levels of AtCutAp in overexpressors (Figure 2) can contribute to elucidation of a specific biological function for *AtCUTA*.

An antibody was raised to purified AtCutAp to analyze expression of the protein in both wild type and *paa1* and *paa2* mutants. Despite high levels of transcription, protein levels remain very low in wild type plants under normal conditions. As shown in Figure 3, transcript levels remain high despite copper treatment or copper transport deficiency. Given the low levels of protein in wild type plants, it was necessary to optimize immunodetection of AtCutAp in Western blots. AtCutAp is a highly soluble protein as evidenced by the ease of extraction of recombinant AtCutAp from the soluble periplasmic fraction of *E. coli* (Burkhead *et al.*, 2003). Therefore, protocols for investigating AtCutAp expression in plants included using a native protein extraction buffer (detergent-free) to leave as much hydrophobic protein as possible with the tissue pellet and removal of remaining hydrophobic protein along with lipids and pigments by means of chloroform extraction. Figure 5, panel c indicates that these methods allow for detection of AtCutA-specific protein by Western blot and immunodetection. In this blot, soil-grown wild type plants show very low expression, at approximately 0.5 to 1.0 ng based on comparison of band intensity in the blot with 10 ng of the purified protein loaded. This would correspond to approximately 0.003% of the total protein extracted with detergent-free buffer. Samples of overexpressing lines shown on the same blot contain approximately 50 ng of AtCutAp, representing 0.025% of the total extracted

protein. These results indicate a highly sensitive antibody, capable of detecting low levels of AtCutAp in wild type plants.

The protein extraction techniques described above were used to investigate AtCutAp expression in shoot, silique, senescing, and root tissue from soil-grown wild type *Arabidopsis* ecotype Columbia. Expression was very low in both shoot tissue and root tissue of mature plants (rosette leaves, shoots and early inflorescences). Senescing plants had an AtCutAp-specific band at a slightly higher molecular weight than the purified recombinant protein and the larger portion of AtCutAp in overexpressing plant extract loaded as a positive control. This suggests both an upregulation of AtCutAp translation in senescing plants and a posttranslational modification of the protein. Other copper-related genes are regulated by senescence. The copper chaperone *CCH* is downregulated by copper stress (Mira, 2002) and upregulated during senescence (Himelblau *et al.*, 1998) and the metallothionein *MT1* is upregulated by both copper stress and senescence (Mira, 2002). In Western Blots, the observation that AtCutAp that migrates as a higher molecular weight (AtCutAp*, Figure 6) than the purified recombinant protein suggests a modification that may be concurrent with increased expression in senescing plants. One could speculate that this higher molecular weight version is an active form of the protein, while the lower molecular weight form is inactive. Proteins of both molecular weights can be seen for the overexpressing control shown in Figure 6, with the higher molecular weight as a minor proportion.

With evidence of posttranscriptional regulation of *AtCUTA* expression, suggesting a need for swift translation of the protein, one possible modification would

be protein phosphorylation. Indeed, AtCutAp contains serine, tyrosine, threonine and histidine residues as potential phosphorylation sites. We suspect that lipid modifications are less likely, due to the soluble nature of the protein and its recovery in the aqueous fraction during chloroform extraction of plant samples. AtCutAp contains several asparagine residues in addition to serine and threonine residues that may be glycosylation sites, though glycosylation of chloroplast proteins has yet to be identified.

High transcription levels of *AtCUTA* along with low protein levels and an apparent posttranslational modification implies that activity of AtCutAp may be tightly regulated. Its activity may be restricted to specific conditions in plant cells. Genes involved in copper homeostasis that are induced during both senescence and copper stress, are likely to reclaim copper from senescing tissue in the former and to protect from copper-induced oxidative stress in the latter (Mira, 2002). In addition to senescing plants, AtCutAp expression was enhanced in *paa1* and *paa2* mutants. The mutants in *Arabidopsis* Col background (*paa1-3, paa2-1* and *paa2-2*) showed expression of AtCutAp in its lower molecular weight form in Figure 7, with the higher molecular weight also present in *paa2* mutants. These plants were grown on soil and beginning to flower, but not near senescence. Older *PAA1-3* plants (near senescence) grown on soil showed increased expression of AtCutAp in its high molecular weight form at levels above those in senescing wild type plants, while concurrently grown *PAA2-2* plants expressed AtCutAp in high molecular weight at similar levels to senescing wild type plants. It is possible that translation and modification of AtCutAp is regulated by copper levels in chloroplasts and during

senescence. Specifically, induction of *AtCUTA* in *paa1* and *paa2* mutants, which have been shown to have reduced levels of copper and copper proteins (Abdel-Ghany *et al.*, unpublished; Shikanai *et al.*, 2003) in chloroplasts indicates translation when chloroplasts are copper deficient. Translation during senescence is likely due to involvement in remobilization of copper from senescing tissue, as is suggested for *CCH* and *MT1*. Taken together with localization to chloroplasts and copper binding, these results support a role for *AtCUTA* in chloroplast copper homeostasis.

Posttranscriptional regulation

Regulatory elements in gene transcripts, particularly the 5'-untranslated region, have been identified in eukaryotic cells. Iron Responsive Elements (IREs) of iron-responsive proteins are stem-loop structures that can be located in the 5'-or 3'-untranslated regions of mRNA and are a canonical example of regulatory elements at the translational level. In humans, ferritin translation is regulated by a stem-loop structure in the 5' UTR (Haile *et al.*, 1989). Specifically, translation is regulated by IRE-binding protein interacting with the 5'UTR stem-loop and inhibiting translation.

The stable stem-loop structure predicted *in silico* for the 5'UTR of *AtCUTA* might regulate translation either by protein binding as in IREs or by slowing ribosomal entry and thereby slowing translation of the protein. In contrast, no stable secondary structure is predicted *in silico* for the 5'UTR of plastocyanin, which is regulated mainly at the transcription level (Vorst *et al.*, 1993). *AtCAB1* has been reported as regulated at the translational level (Saha *et al.*, 2003), implicating properties in the 5'UTR that affect mRNA stability and ribosome entry. The apparent stability of the predicted secondary structure of the *AtCUTA*-5'UTR suggests that

ribosome entry could be retarded unless another (yet unknown) factor assists in ribosome entry. The structure may also contain a protein-associated region allowing regulation by (as yet unknown) cytosolic proteins, as is the case of IREs.

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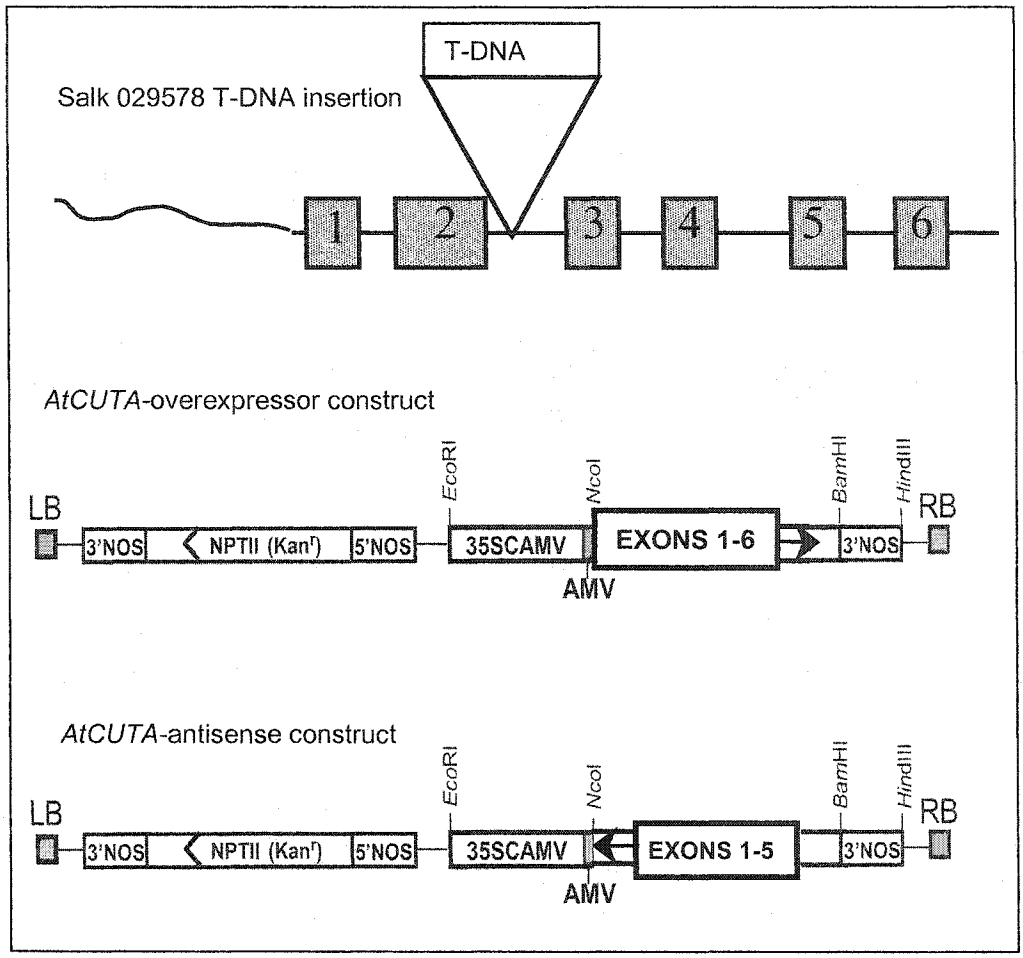

Figure 1. Schematic diagram of constructs to alter expression levels of *AtCUTA*.

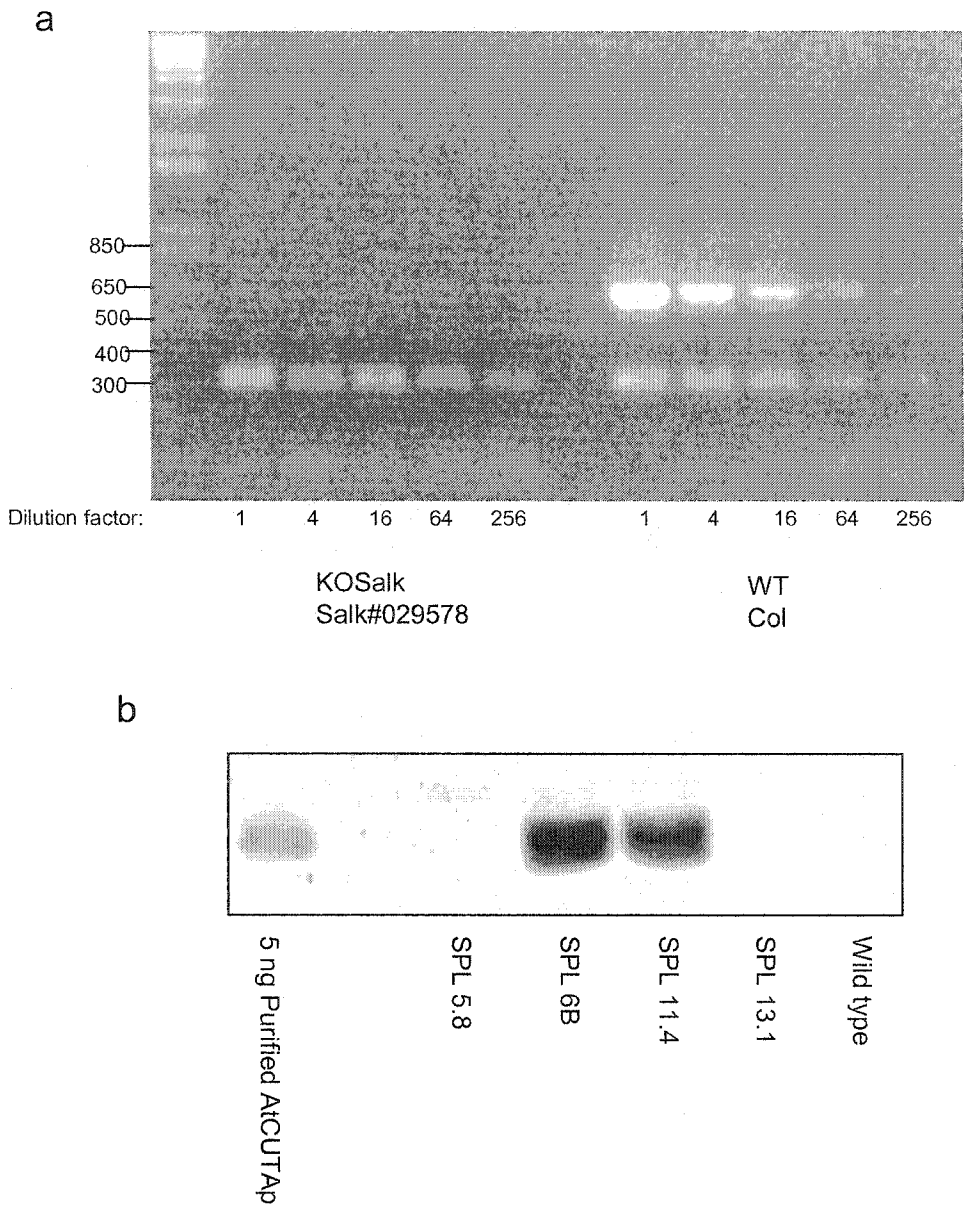

Figure 2. Panel a, RT-PCR analysis of *AtCUTA* and *Actin-2* transcripts in homozygous *AtCutA*-knockout and wild type *Arabidopsis* seedlings. Panel b. Western blot analysis of *AtCUTA*-overexpressing (Spl) lines. 10 micrograms equivalent of chloroform-extracted protein loaded per lane.

Figure 3. *Arabidopsis* lines with altered expression of *AtCUTA* are sown on soil as shown in the table.

as.12	spl5.8	wild type	spl5.8	as2.1.3	CutA-Wisc
as5.2	spl6B	CutA-Wisc	wild type	spl13.1	spl6B
CutA-Salk	as2.1.3	spl13.1	as1.2	CutA-Salk	as5.2

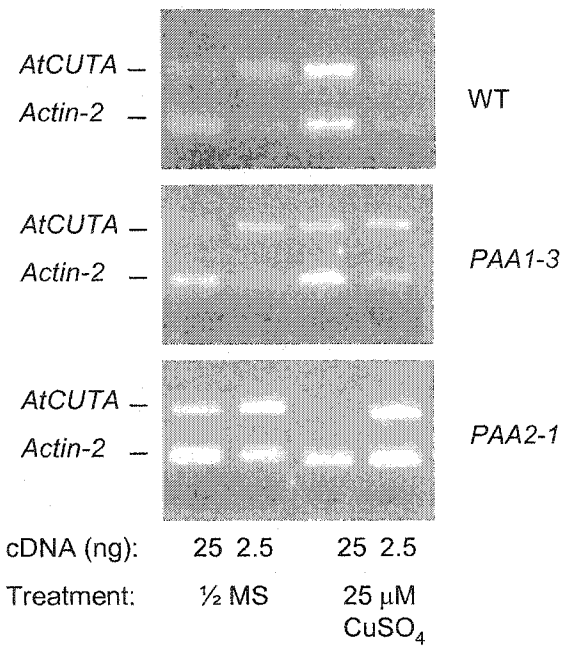

Figure 4: RT-PCR using primers for *AtCUTA* transcript and *Actin-2* transcript. RNA isolated from 21-day old seedlings grown on ½ MS media or ½ MS +25 μM $CuSO_4$.

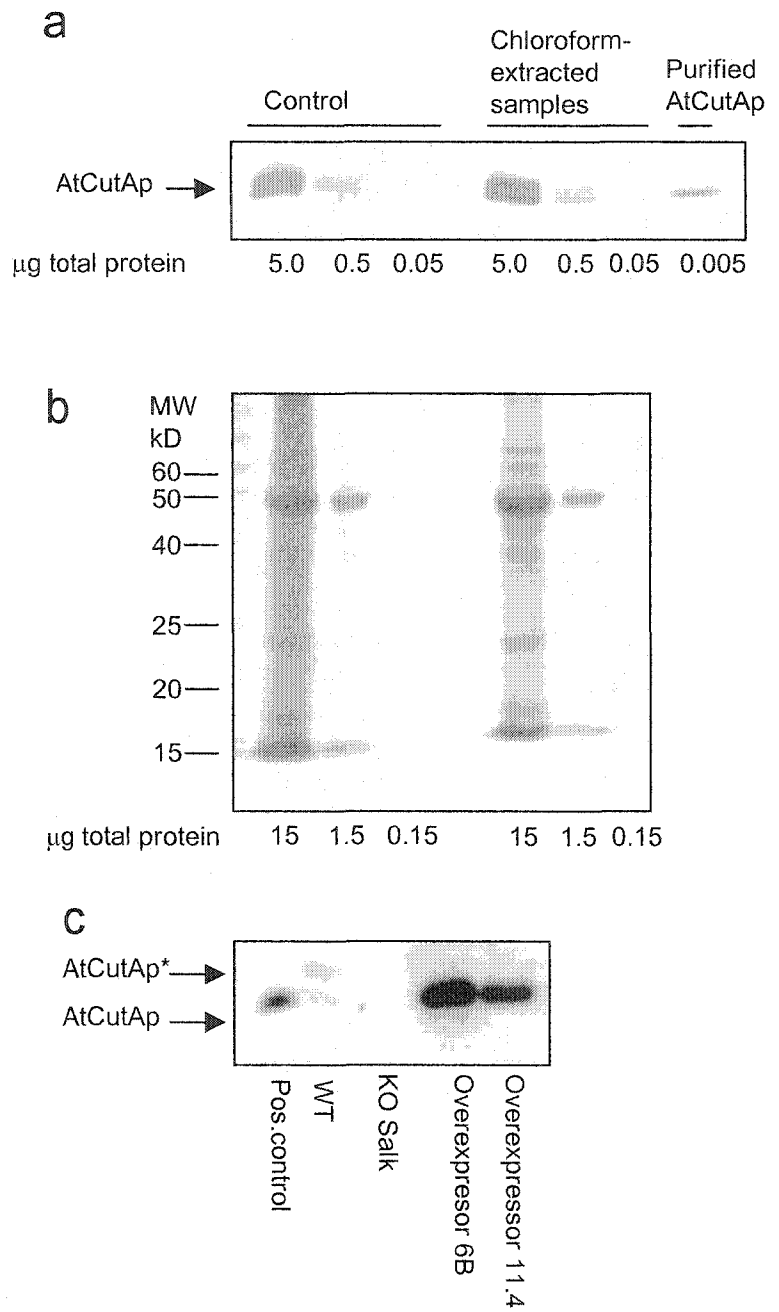

Figure 5. Panel a: Detection of AtCutAp with polyclonal antibody. Soluble protein was extracted from *AtCUTA*-overexpressing line SPL 11.4 was left untreated or extracted with chloroform prior to addition of SDS sample buffer. Serial dilutions of samples were made and the proteins were separated by 15% SDS-PAGE. Panel b: AtCUTA-Overexpressing line SPL 11.4 protein extractions from Panel a, proteins separated by SDS-PAGE and stained with Coomassie Brilliant Blue. Panel c: Western blot and immunodetection of AtCutAp in *Arabidopsis* plants with altered AtCutA expression.

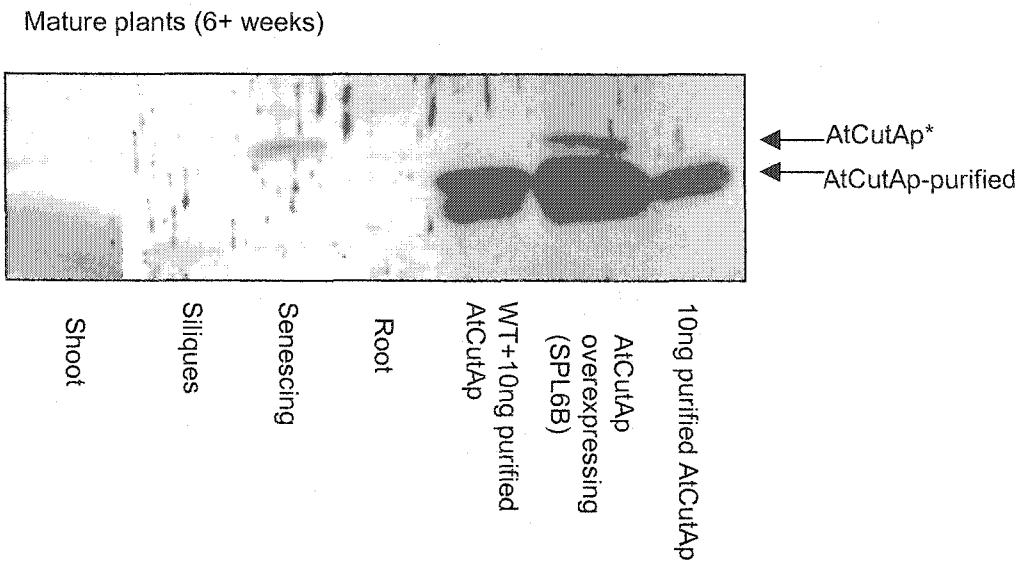

Figure 6. AtCutAp expression in mature plant tissues.

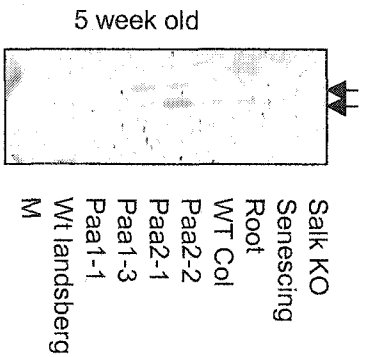

Figure 7. AtCutAp expression in *Arabidopsis* lines deficient in chloroplast copper transport. Five-week old plants with inflorescences grown on soil.

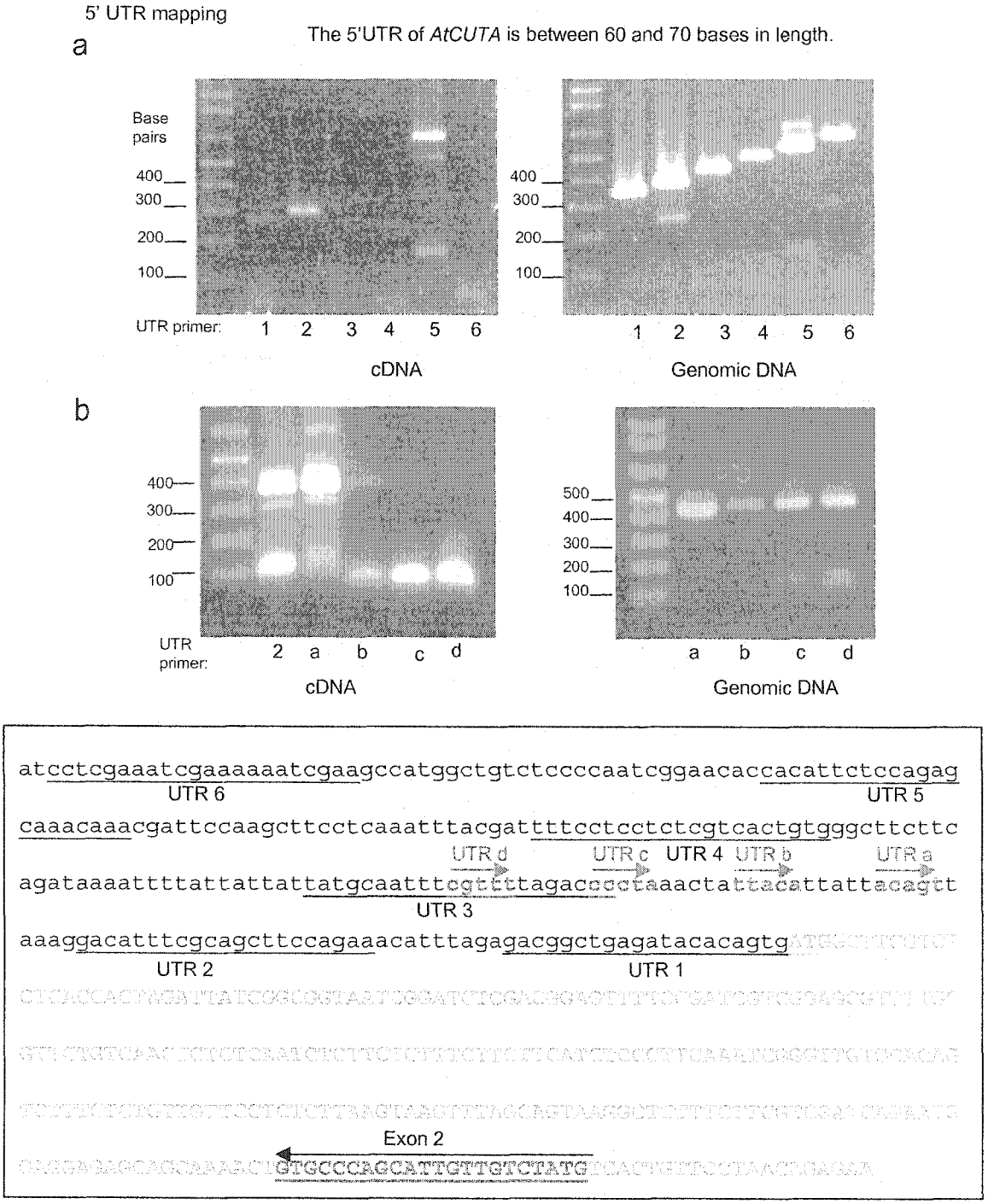

Figure 8. 5' UTR mapping of AtCUTA mRNA. Panel a: mapping in 50 –base increments, left: Exon 2 primer incremental upstream primers and cDNA template; right: same primers, genomic DNA template. Panel b, fine mapping to 10 –base increments, left Exon 2 primer coupled with indicated upstream primer and cDNA template. Right, same primers with genomic DNA template. Panel C: primer hybridization locations.

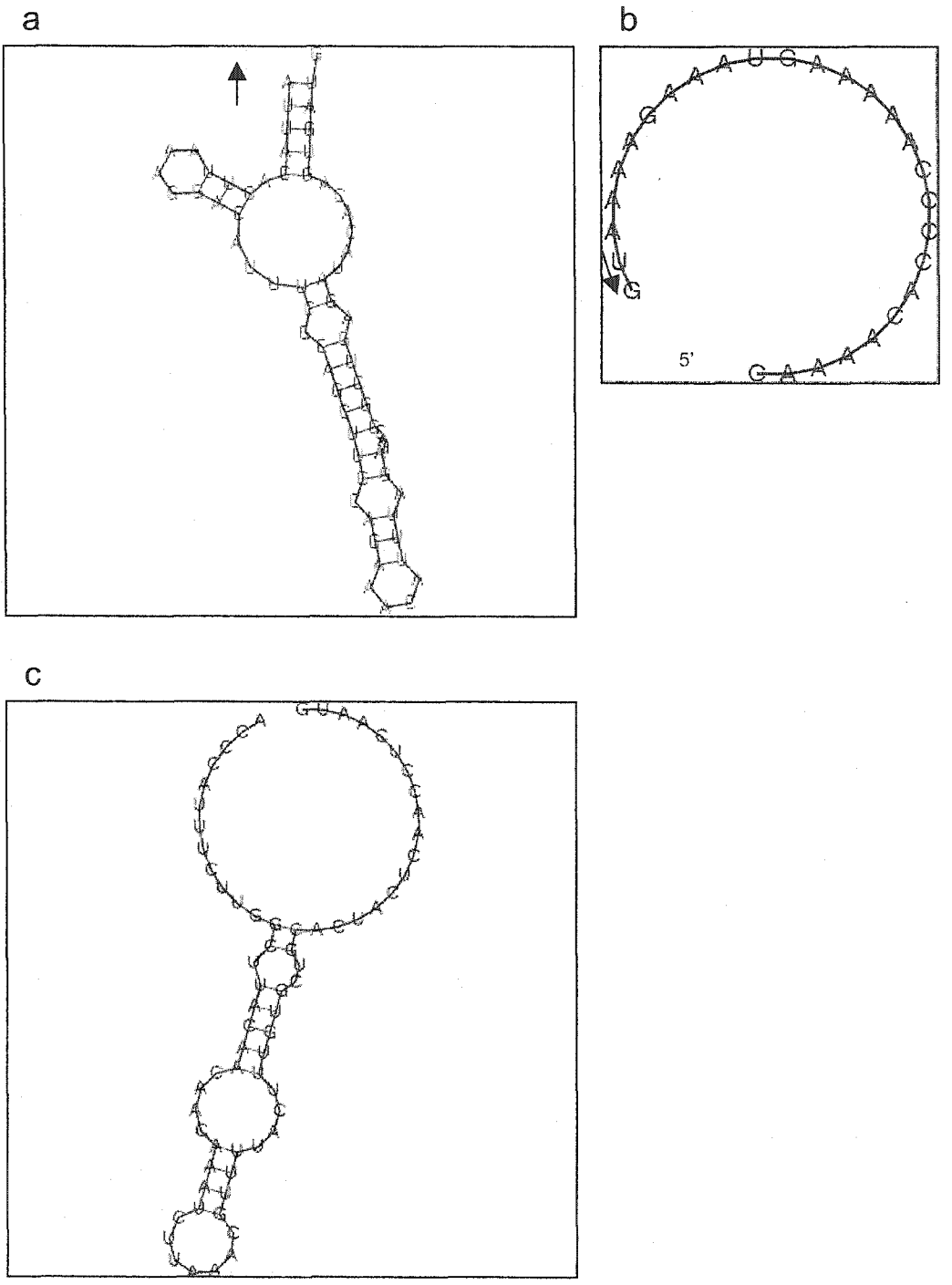

Figure 9. RNAfold predictions for stable secondary structure of the 5'UTR of *AtCUTA* (panel a) and plastocyanin (panel b). Start codons (AUG) are indicated with arrows. A stem-loop structure is predicted for the translationally-regulated *AtLhcb1*3* 5'UTR in Panel *c*.

Chapter 4

Biochemical properties of the AtCutA protein

Summary

AtCutAp is a conserved Cu(II) binding protein lacking any known copper binding motif. We used structural modeling based on solved crystal structures of prokaryotic homologues to predict a tertiary structure for AtCutAp. The predicted tertiary structure revealed protein folding with similar characteristics of Cu(I) metal binding domains, particularly the copper chaperone Atx1p. We analyzed biochemical properties of AtCutAp, AtCutAp-tr (lacking 26 C-terminal amino acids), and ten point mutants to obtain information about the protein. Based on protease sensitivity measurements, AtCutAp and the point mutants appear to be tightly folded and resistant to Proteinase K digestion, while AtCutAp-tr is susceptible to protease attack. Gelfiltration analysis indicated that AtCutAp is a trimer, as are the point mutants, while AtCutAp-tr appears to have multiple oligomerization numbers. AtCutAp and the ten point mutants specifically bind copper(II)-saturated IDA agarose in IMAC tests. Two mutants substituting alanine for glutamic acid exhibit some affinity for

Ni(II)-saturated IDA agarose in addition to Cu(II) affinity. Point mutations in AtCutAp resulted in either similar or increased copper binding when compared to wild type AtCutAp, suggesting that AtCutAp may have an optimal affinity for Cu(II) related to its biochemical function.

Introduction

Copper is essential to life as a cofactor in enzymes and electron carriers. Free copper, however, must be strictly avoided due to its toxicity. There must be precise mechanisms in place to transport copper and deliver it to target proteins along with regulatory mechanisms to control movement of copper within the cell and its organelles.

Many proteins involved in copper homeostasis contain methionine-rich regions involved in copper binding (Arnesano *et al.*, 2002; Huffman *et al.*, 2002) or the canonical MxCxxC metal binding motif of copper chaperones and copper transporting P-type ATPases (Harrison *et al.*, 1999; Jones *et al.*, 1999). These regions are involved in coordinating copper in its reduced state.

Retention of oxidized copper in a protein is less well characterized. Prion proteins have been shown to bind Cu(II) through octarepeats of PHGGGWGQ, with histidine and possibly one carbonyl group from glycine coordinating the Cu(II) ion (Stockel *et al.*, 1998). Histidine residues along with acidic residues have also been implicated in copper binding in CueO, PcoC, and CopC (Arnesano *et al.*, 2003; Huffman *et al.*, 2002; Roberts *et al.*, 2003), yet the residues may be in diverse portions of the primary structure, whereby no consensus sequence can be determined.

The blue-copper protein plastocyanin coordinates copper by methionine, cysteine and histidine on one loop and a second histidine on another loop (Guss *et al.*, 1992). Aromatic amino acids phenylalanine and tryptophan have been shown to influence both copper binding and metal specificity in plantacyanin (Babayan *et al.*, 1983) and in the human carbonic anhydrase II protein (Cox *et al.*, 2000), though methionine and histidine are thought to be the primary ligands in these proteins.

AtCutAp has been shown to bind Cu(II) and is likely localized to the chloroplast envelope (Burkhead *et al.*, 2003). It contains only a single methionine in the mature protein and no consensus metal binding domain. Therefore, it is likely that AtCutAp binds Cu(II) through a novel mechanism. Like CueO, and CopC, *E. coli* CutAp is thought to be in the periplasm (Fong *et al.*, 1995), while mammalian CutAp has been localized as an extracellular protein copurifying with acetylcholinesterase (Navaratnam *et al.*, 2000). This study determines biochemical properties of AtCutAp in order to gain insight in its mechanism of copper interaction and its functional characteristics.

Materials and methods

Materials: Proteinase K and PMSF were obtained from Sigma (St. Louis, MO, USA). DEAE Sepharose was obtained from Amersham-Pharmacia (Uppsala, Sweden). Imino-diacetic acid agarose (His-Bind) was obtained from Novagen (Madison, WI). Ultrafiltration was performed with Nanosep 10K ultracentrifuge filters (Pall Gelman Laboratory, Ann Arbor, MI, USA).

Protein modeling and alignment

Multiple protein alignments were performed with the ClustalW program (Thompson *et al.*, 1994) at Pôle Bio-Informatique Lyonnais (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_clustalw.html). Protein modeling was performed with the web-based Swiss-Model program (http://www.expasy.org/swissmod/SWISS-MODEL.html) based on the structures deposited in the Protein Data Bank (http://www.rcsb.org/pdb/) for *Thermotoga martima* (Protein Data Bank ID: 1KR4) (Savchenko *et al.*, 2002), *Thermus thermophilus* (1NZA) (Bagautdinov *et al.*, 2003), and *Archaeoglobus fulgidus* (1P1L) (Kniewel *et al.*, 2003).

Amino acid substitutions in recombinant AtCutAp

Amino acid substitutions in AtCutAp were constructed in a cDNA clone of mature *AtCUTA* in a pET11-d *E. coli* expression plasmid (Novagen, Madison, WI). Two rounds of HF-PCR (Roche, Palo Alto, CA, USA) were used to introduce base-pair substitutions appropriate to change single amino acids. The Mod1 and Cl2 primers described by Burkhead et al. (2003) were used with internal overlapping primers containing directed base changes either downstream of Mod1 or upstream of Cl1. A first round of PCR was performed with each downstream primer and Mod1 and each upstream primer with Cl2. PCR products were extracted from a 1% agarose gel with the Qiaquick Gel Extraction Kit, (Qiagen, Valencia, CA, USA). These combined first-round products were used as template for a second round using only the Mod1 and Cl2 primers. The PCR product with substituted base pairs was cloned to pET11-d in Nco1 and BamH1 restriction sites for expression of mutant proteins.

Primers used are listed in Table 2. HF-PCR was performed with parameters as described by Burkhead et al. (2003). Mutations were confirmed by DNA sequence analysis (Davis Sequencing, Davis, CA; www.davissequencing.com).

***E. coli* expression and protein purification**

Plasmids containing inserts of mutated recombinant *AtCUTA* were transformed to BL21-(DE3) CodonPlus®*E. coli* cells (Stratagene, La Jolla, CA) for expression under control of the T7 promoter (Studier *et al.*, 1990). Cultures were grown in 500 mL of LB medium with vigorous shaking to an OD_{600} of 0.3 and induced by adding IPTG to 0.4 mM. Incubation continued to an OD_{600} of 0.7 to 1.0 when cells were collected by centrifugation at 4000*g* in a fixed angle rotor cooled to 4°C. The periplasmic proteins were separated from the bulk of cellular proteins by osmotic shock (Neu and Heppel, 1965). Proteins were further purified by anion exchange chromatography at 4°C on a DEAE Sepharose column equilibrated with 25 mM Tris-HCl (pH 7.5) + 10% (w/v) glycerol. After allowing the protein to bind, the column was washed with 20 mL of the same buffer. Proteins were eluted by a continuous gradient in the same buffer with 200 to 500 mM NaCl for 150 mL. Fractions were tested for purity and protein content by SDS-PAGE and CBB staining. Peak pure fractions were pooled and stored in aliquots at -80°C for use in biochemical experiments. Protein concentrations were measured by the absorbance at 280 nm (Yunis and Perkins, 1986) and by the method of Bradford (1976). A conversion factor was obtained for each protein in order to correct Bradford Assay values in copper binding assays to corresponding OD_{280}-derived concentrations (Table 2).

Gelfiltration

Protein complex size was analyzed by gel-filtration on a Superdex-200 column (30 x 1 cm, Amersham-Pharmacia, Piscataway NJ). The column was connected to a Dionex Summit HPLC system controlled by a PC with Chromeleon software (Dionex, Sunnyvale CA). The injection loop size was 100 µl. The column was equilibrated and eluted in 50 mM Tris-HCl pH 7.5, 150 mM NaCl, at a flow rate of 0.75 ml/min at room temperature. Elution was monitored by absorbance at 280 nM and the elution volume (V_E) and peak area were determined by the Chromeleon software (Dionex). The following markers (Amersham-Pharmacia) were used to calibrate the column: Blue dextran (void volume, V_0, 8.15 ml), Chicken IgY (180 kDa), BSA (67 kDa), Ovalbumin (43 kDa), Chymotrypsinogen (25 kDa), RNAse (14 kDa). The column volume (V_h) was determined by monitoring the elution of an equimolar mix of DTT and Ellman's reagent at 420 nM and was found to be 20.85 ml. The calibration curve used to estimate complex sizes was produced by plotting the ^{10}Log of the Mw as a function of the K_{AV}. The K_{AV} is defined by this formula: $K_{AV} = (V_E-V_0)/V_h-V_0)$. The K_{AV} is directly related to the elution volume and retention time but is independent of column size and tubing volumes.

Protease treatment

Proteins were tested for Proteinase K sensitivity in 50 mM Tris-HCl pH 8.0, 5 mM $CaCl_2$, 1 mM DTT. Stock solutions of Proteinase K were made at 1 mg/mL and 0.1 mg/mL in water and frozen at -80°C. A stock solution of 500 mM Tris-HCl pH 8.0, with 10 mM DTT was used as a 10X reaction buffer. $CaCl_2$ was added to each reaction from a 50 mM stock solution. 40 µg of each protein was treated with

increasing concentrations of Proteinase K (0 to 100 μg/mL). Reactions were incubated at 37°C for 30 minutes. The protease was inactivated by addition of 100 mM PMSF in 96% ethanol to a concentration of 2 mM followed by addition of an equal volume of SDS-PAGE sample buffer and immediate heating to 95°C for five minutes. Protease treatment was analyzed by 16% SDS-PAGE.

Immobilized metal affinity chromatography.

AtCutAp and AtCutAp mutants were analyzed for affinity to Cu-, Ni-, and Zn-saturated imino-diacetic acid agarose as previously described for AtCutAp and AtCutAp-tr (Burkhead *et al.*, 2003).

Copper binding

Copper binding per mole of protein for AtCutAp, AtCutAp-tr and mutants was determined by incubation of 50 μM protein with 100 μM $CuCl_2$ essentially as described for affinity determination of AtCutAp (Burkhead *et al.*, 2003). Two assays of copper binding were performed for each protein, including the wild type. Changes to the assay from Burkhead *et al.* (2003) were as follows: Tris-HCl, NaCl, and glycerol in storage buffer were exchanged for 10 mM Na-Phosphate buffer (pH 7.2) by ultrafiltration in Nanosep 10K microcentrifuge filters (Pall Gelman Laboratory, Ann Arbor, MI) and two washes of buffer. All filtration was performed in Nanosep 10K filters at 7000 rpm in a benchtop microcentrifuge. 10 mM Na-Phosphate buffer was used as binding buffer and as a wash buffer. All other parameters for the assay remained unchanged.

Results

The crystal structures for three prokaryotic homologues of *AtCUTA* have been solved and are deposited in the Protein Data Bank (Berman *et al.*, 2000) (http://www.rcsb.org/pdb/). The *CUTA* protein structures from *Thermotoga maritima* (Savchenko, et al., 2002), *Thermus thermophilus* (Bagautdinov, et al. 2003), and *Archaeoglobus fulgidus* (Kniewel, et al. 2003) can be used as templates to generate a model of the mature AtCutAp structure. AtCutAp is aligned with its homologues in Figure 1, panel a with the following similarities: *Thermotoga maritima*: identity 29%, similarity 45%; *Thermus thermophilus*: identity 49%, similarity 70%; and *Archaeoglobus fulgidus*: identity 38%, similarity 65%. The web-based program Swiss Model (http://www.expasy.org/swissmod/SWISS-MODEL.html) was used to generate models of both the full length and truncated AtCUTAp. An alignment of AtCutAp with the proteins it was modeled on is shown in Panel a of Figure 1. Figure 1, Panel b shows the Swiss-Model structure of full length mature AtCutAp. Characteristic structures are three alpha-helical domains and four beta-sheet domains. Panel b shows the model of mature truncated AtCutAp (AtCutAp-tr), lacking the c-terminal beta-sheet and helix. In its predicted structure, though not in sequence, AtCutAp shares some similarity with the solved crystal structure of *ATX1*, a copper chaperone (Rosenzweig *et al.*, 1999) shown in panel d. Single crystal structure is available for the CutA from *Thermotoga martima* and is shown in Figure 1, Panel e, indicating a trimeric structure and predicted biological molecule. The expected molecular weight of a trimeric AtCutAp would be 37.44 kD, while tetrameric would be 49.92 kD.

AtCutAp and AtCutAp-tr were analyzed by gelfiltration to determine the molecular weight and thus oligomerization state of each native protein. The elution profiles for AtCutAp and AtCutAp-tr are shown in the top two panels of Figure 2. AtCutAp elutes as a single, sharp peak, indicating a single oligomerization state with a retention time consistent with a trimer of AtCutAp. AtCutAp-tr elutes with multiple peaks of higher molecular weight oligomers as shown in Figure 2. The lower panel of Figure 2 shows the gelfiltration calibration curve and comparison of the elution (K_{AV}) of AtCutAp compared to marker proteins. Equivalent amounts of AtCutAp and AtCutAp-tr as determined by absorbtion at 280 nm were used for gelfiltration. Despite a difference in absolute height for each peak, they both represent roughly equal area, given the broad retention time for AtCutAp-tr. Retention times for AtCutA and the two main peaks of AtCutAp-tr are shown in Table 1. The assignation of a molecular weight for AtCutAp by gelfiltration that is consistent with a trimeric form is based on the assumption that the protein and standards do not interact with the gelfiltration column and that the proteins are globular. The single crystal structure for *Thermotoga CUTA* is consistent with the assumption of a globular protein, though AtCutAp may vary slightly in its structure.

The predicted structure of AtCutAp indicates a relatively compact protein. Such a protein should be resistant to attack by protease. In order to gain experimental support for the predicted structure of AtCutAp, a series of protease treatments was performed. Aliquots of AtCutAp were treated with the increasing quantities of the protease at 37°C for 30 minutes and analyzed by SDS-PAGE. AtCutAp was tested for protease sensitivity to trypsin, thermolysin, and Proteinase K. AtCutAp was found to

be sensitive to Proteinase K, showing susceptibility to increasing concentrations for a 30-minute treatment as indicated in Figure 3, Panel a. The gel in Panel a indicates that AtCutAp contains small Proteinase K-sensitive region, apparent at 30 and 100 μg/mL protease. AtCutAp-tr was sensitive to Proteinase K even at the lowest concentration. As AtCutAp-tr lacks 26 C-terminal acids, it is possible the tertiary or quaternary structure is disrupted.

AtCutAp and AtCutAp-tr have been shown to bind Cu(II). In order to investigate possible conformational changes in AtCutA species due to copper binding, we again used a protease sensitivity assay. Addition of 40 μM $CuCl_2$ did not appreciably change Proteinase K sensitivity of either AtCutAp or AtCutAp-tr (Figure 3, Panel b).

In order to investigate which amino acid residues are involved in the copper binding characteristics of AtCutAp, a series of single amino acid substitutions were made in recombinant *AtCUTA*. The criteria used to select residues were the following: conservation in different CutA proteins, and potential to coordinate a metal cation. The amino acids substituted by alanine are underlined in the sequence in Figure 4, Panel a. With the first methionine of the mature AtCutAp as residue number 1, substituted residues are as follows: cysteine 38, serine 47, tyrosine 49, tryptophan 51, glutamic acid 60, glutamic acid 73, histidine 78, histidine 83, tyrosine 102, and tryptophan 105. The residues that were substituted are indicated as black side chains on the model of AtCutAp in panel b of Figure 4.

Point mutations resulting in amino acid substitutions in AtCutAp were introduced into an existing *AtCUTA* mature clone previously used for expression in

Escherichia coli BL21 cells (Studier *et al.*, 1990). Individual *E. coli* lines were identified harboring pETlld plasmid with appropriate mutations. Clones containing expected inserts were sequenced and upon confirmation of appropriate nucleotide substitution, were used for protein expression. Figure 5 panel a shows both a Western blot (top) and Coomassie-stained polyacrylamide gel indicating expression of appropriate AtCutAp mutant proteins. Expression of the mutant proteins in *E. coli* is similar to that of the wild type in the same *E. coli* strain. Periplasmic proteins were separated from the bulk of cellular protein by osmotic shock and AtCutA mutant proteins were purified from the periplasmic fraction by anion-exchange chromatography. Purified proteins were analyzed by SDS-PAGE and Native-PAGE with the results shown in Figure 5, Panel b. The mutant proteins migrated in SDS-PAGE at similar rates, while elimination of negatively charged glutamic acid residues 60 and 73 appears to alter migration of those proteins—probably due to a reduction in overall charge of the protein and a reduced migration in the gel towards the positive electrode.

In order to determine if the tertiary and quaternary structure of each mutant AtCutA protein was similar to the wild type, the gelfiltration and Proteinase K sensitivity tests were performed. Gelfiltration indicates no change in the structure as identical retention times were observed for each of the mutant proteins and the AtCutAp wild type (see Table 1). Each of the mutated proteins were sensitive to increasing Proteinase K concentrations, giving a similar profile to the wild type as shown in Figure 6. Thus, the mutations do not significantly affect the overall structure of AtCutAp.

To investigate the effects of single amino acid substitutions in AtCutAp on metal affinity, we used immobilized metal affinity chromatography (IMAC). IMAC can give insight about the potential interactions with a protein and immobilized metal. Lack of interaction with a specific metal immobilized on IDA-agarose suggests interaction in solution is unlikely. Such analysis can be applied to the mutant *AtCUTA* proteins by creating a binding profile for each protein with respect to IDA-M(II) binding. Mutant proteins were analyzed for retention on IDA-agarose saturated with Cu(II), Ni(II) and Zn(II). Sodium is included as an unsaturated resin negative control, confirming that the proteins do not interact with the resin itself. As indicated in Figure 7, the majority of mutant AtCutA proteins share a retention profile with the wild type. Significant differences can be seen in the Glu-60 substitution and the Glu-73 substitution. These proteins have some retention on IDA-Ni(II) in addition to IDA-Cu(II). The interaction with nickel is weaker as evidenced by recovery of the majority of protein in the unbound fraction; however, it is significant in that protein was recovered in both cases in the pH-5.5 wash (after three binding buffer washes) and in the EDTA wash (after pH-5.5 wash). Both AtCutAp and AtCutAp-tr have been shown to bind Cu(II) in solution by incubating the proteins with $CuCl_2$ and removing unbound ions by ultrafiltration. The ten mutant AtCutA proteins were tested by this assay alongside wild type and AtCutAp-tr. The results shown in Table 2 are the average Cu(II)/protein ratio from two separate binding experiments for each protein. All of the mutants retain copper binding capacity by this assay. A number of the proteins bind an increased amount of copper compared to the wild type, while one, Tyr-49 showed a decreased capacity for binding. In this test, the wild type protein

also had a slightly decreased molar ratio (Cu/protein) compared to the original reported ratio (Burkhead *et al.*, 2003).

Discussion

AtCUTA represents the higher plant form of a conserved copper(II) binding protein with a primary structure dissimilar to other Cu(II) binding proteins. *CUTA* proteins do not contain consensus metal binding motifs of copper chaperones (MXCXXC) nor do they contain regions rich in thiol ligands characteristic of metallothioneins. Therefore, AtCUTAp must coordinate copper by ligands yet to be identified.

Crystal structures of three prokaryotic *AtCUTA* homologues have been solved and the structures are available in the Protein Data Bank (http://www.rcsb.org/pdb/). These solution structures can be used to generate a model of the mature AtCutAp protein. High primary structure similarity allows for precise model fit with the Swiss Model program. Despite a lack of sequence homology, the models of AtCutAp and AtCutAp-tr in Figure 1 show similarity in secondary and tertiary structure with the copper chaperone *ATX1*. Notably, two alpha-helical domains are juxtaposed on four beta strands in an *ATX1*-like configuration of β-α-β-β-α-β. An additional alpha-helical domain is present in the C-terminal portion of the AtCutAp model, but is not predicted to be present in the AtCutAp-tr, suggesting it is not necessary for copper binding. This structure indicates a possible interaction of AtCutAp with other copper proteins. Indeed, the N-terminus of Ccc2p contains a domain with sequence similarity to Atx1p. An analogous example of copper protein interactions may be the copper chaperone for CuZnSOD (Lys7p), which contains a domain with structural similarity

with the SOD itself (Lamb *et al.*, 2001). Some specific characteristics of these interactions have recently been identified (Lamb *et al.*, 2001; Wernimont *et al.*, 2000), though the exact nature of most protein-protein interaction remains to be determined.

The original identification of a *CUTA* protein was in the gram-negative bacteria *E. coli* (Fong *et al.*, 1995). Recombinant AtCutAp expressed in *E. coli* is exported to the bacterial periplasm. The periplasm of cyanobacteria may be considered evolutionarily homologous to the chloroplast envelope, where it is suspected that AtCutAp is localized. Other Cu(II) binding proteins have been identified in bacterial periplasm, notably copC, which is shown to bind both Cu(II) and Cu(I) in physically distinct locations (Arnesano *et al.*, 2003). CopCp is proposed to interact with the inner membrane-localized CopAp through a structurally similar domain (Arnesano *et al.*, 2003), whereas *CopA* encodes the primary copper efflux transporter in *E. coli* (Outten *et al.*, 2001). It is thought that CopCp coordinates Cu(II) by means of two histidine residues and two other ligands that are most likely conserved aspartic acid and glutamic acid side chains in the same region. The nickel metallochaperone UreE also coordinates Ni(II) or Co(II) by a similar arrangement of ligands (Colpas *et al.*, 1999). Examination of the model for AtCutAp reveals both histidine and acidic residues in close proximity in a region with similar secondary and tertiary structure to the metal binding site of Atx1p.

Clues about structural features in a protein can be gained by assaying protease sensitivity. AtCutAp is largely resistant to Proteinase K, with cleavage of only a small portion of the protein. AtCutAp-tr was sensitive to even the lowest concentration of

Proteinase K used in the assay, indicating an increased number of available cleavage sites and perhaps improper protein folding when compared to the wild type. The resultant size of Proteinase K-digested AtCutAp is close to that of AtCutAp-tr, yet it is more probable that the portion of AtCutAp cleaved by Proteinase K is near the N-terminus. If a C-terminal fragment were cleaved to leave a protein similar to AtCutAp-tr, it would be expected to have the same destabilizing effect and leave the protein entirely susceptible to the protease.

Presence or absence of copper in the reaction does not appear to influence the sensitivity of either AtCutAp or AtCutAp-tr to Proteinase K. With the assumption that the protein can bind copper under these conditions, it is likely that copper binding does not largely affect the stability or folding of either form of wild type AtCutAp. For comparison, apoCopCp does not have a significant structural divergence from Cu(II)CopCp or Cu(I)CopCp (Arnesano *et al.*, 2003).

Single amino acid substitutions were made in recombinant AtCutAp to gain insight about potential copper binding elements in AtCutAp. Mutant proteins retained the localization with periplasmic *E. coli* proteins and were detectable with anti-AtCutAp antibodies. The single amino acid mutations used did not largely affect the folding of AtCutAp as demonstrated by gelfiltration, Proteinase K sensitivity and Native-PAGE.

Protein binding to metal-saturated IDA-agarose can also give clues about surface topography with respect to surface histidine residues. Mature AtCutAp contains two histidine residues (His-83 and His-87). According to Sharma and Agarwal (2001), to be retained on Ni-saturated IDA resin (IDA-Ni(II)), two surface

histidines are required, retention on IDA-Cu(II) requires only one histidine, and binding to IDA-Zn(II) requires two vicinal histidine residues (Sharma and Agarwal, 2001). However, it is unclear which residues in addition to surface histidines might be involved in retention of AtCutAp with Cu(II)-saturated resin.

In tests for affinity in mutant AtCutAp for metal ions immobilized on IDA-agarose, all proteins were retained on Cu(II)-saturated resin. Two proteins, the mutated in the conserved Glu-60 and non-conserved Glu-73, respectively, were found to bind Ni(II)-saturated agarose in addition to Cu(II)-agarose. These results indicate that topology near histidine residues is affected by substitution of Glu-60 and Glu-73 with alanine. Glu-60 and Glu-73 are in close proximity on the modeled AtCutAp to His-83 and His-87, respectively. It is possible that a substitution of the Glu residues results in newly accessible ligands that are not available for binding in wild type AtCutAp. Therefore, the acidic residues Glu-60 and Glu-73 may have a role in retaining copper specificity, as absence results in some Ni(II)-IDA agarose affinity. Presence of AtCutAp-Glu-73 in the unbound Cu fraction is likely due to saturation of the resin with protein.

The molar ratios (Cu/protein) of wild type AtCutAp, AtCutAp-tr and mutants were determined by incubation in solution with $CuCl_2$ and removal of unbound copper by ultrafiltration. Curiously, some substitutions (Tyr-49 and Trp-51) reduced the Cu/protein ratio in this test, one (Cys-38) had little effect, and all others significantly increased it. One would typically expect a reduction in function due to replacement of conserved charged/acidic/aromatic residues with alanine, but it is possible that AtCutAp functions in a regulatory or sensing role where copper binding

is maintained in a defined range. It has been reported that the copper-resistance (Cu(II)-binding) PcoC protein typically binds a single Cu(II) ion per monomer and copper binding can cause formation of higher-order oligomers (Huffman *et al.*, 2002). Although gelfiltration analysis of copper-free mutant AtCutA proteins indicates wild type-like trimeric states, it may be possible that substitution of conserved residues in AtCutAp allows for higher order oligomerization upon copper interaction and increased copper binding capacity within oligomers.

Copper binding could not be eliminated in AtCutAp by single amino acid substitution. It is likely that Cu(II) is coordinated in the protein by multiple ligands, as is the case for other copper proteins including Cu(II)-binding CopC (Arnesano *et al.*, 2003). Multiple mutations may be required to construct a true loss-of-function mutant. Ultimately, a solved crystal structure with bound copper will provide the best information about a binding site. It is also possible that amino acids other than those mutated in these experiments are involved in AtCutAp copper binding. Since the purified protein is a trimer and it is likely that it exists in plants as a trimer, multiple monomers may be involved in copper binding using ligands from separate monomers to coordinate a single copper ion.

Copper(II) binding has not been identified as a characteristic of copper chaperones, copper transporters or metallothioneins. Copper remains bound to plastocyanin and CuZnSOD in both oxidized and reduced states where it functions as an electron carrier or acceptor. As copper is transported into and within cells in a reduced state, which is more toxic than the oxidized state, assigning a role for an intracellular Cu(II) binding protein can prove challenging. CopC has been implicated

for its role in copper resistance in *E. coli* (Arnesano *et al.*, 2003), yet it functions in the bacterial periplasm. CueO is a multicopper oxidase that binds Cu(II) in the periplasm and functions in copper resistance (Roberts *et al.*, 2003). The Cu(II) bound in CueO performs a regulatory role in the oxidase. By analogy to periplasmic proteins, it is possible that AtCutAp has a regulatory or transfer function in the chloroplast envelope.

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Table 1. Gelfiltration retention times for wild type and mutant AtCutA proteins.

Protein	**Retention time**
WT	20.45
AtCutAp-tr	18.40
AtCutAp-tr	19.39
Cys 38	20.45
Ser 47	20.47
Tyr 49	20.45
Trp 51	20.38
Glu 60	20.59
Glu 73	20.60
His 78	20.43
His 83	20.38
Tyr 102	20.47
Trp 105	20.41

Table 2. Copper binding in mutant proteins by ultrafiltration assay. Binding is reported in the table as moles Cu(II)/mole AtCutAp. Protein concentrations were measured by Bradford Assay and corrected by an experimentally determined conversion factor. Theoretical molar extinction coefficients at 280 nm are included with the conversion factor for each protein that corrects Bradford Assay protein measurements for protein concentrations as measured at 280 nm.

Protein name	Cu/protein (moles)	Molar extinction coeff. 280 nm	Conversion factor
Cys 38	0.89	16000	2.09205
Ser 47	1.68	16000	2.10084
Tyr 49	0.60	14800	3.378378
Trp 51	0.50	10400	1.976285
Glu 60	1.45	16000	1.510574
Glu 73	1.21	16000	1.851852
His 78	1.58	16000	1.672241
His 83	1.69	16000	1.754386
Tyr 102	1.22	14800	1.779359
Trp 105	1.88	10400	1.886792
WT	0.83	16000	2.403846
AtCutAp-tr	1.37	9800	1.694915

Table 3. Oligonucleotide primer sequences.

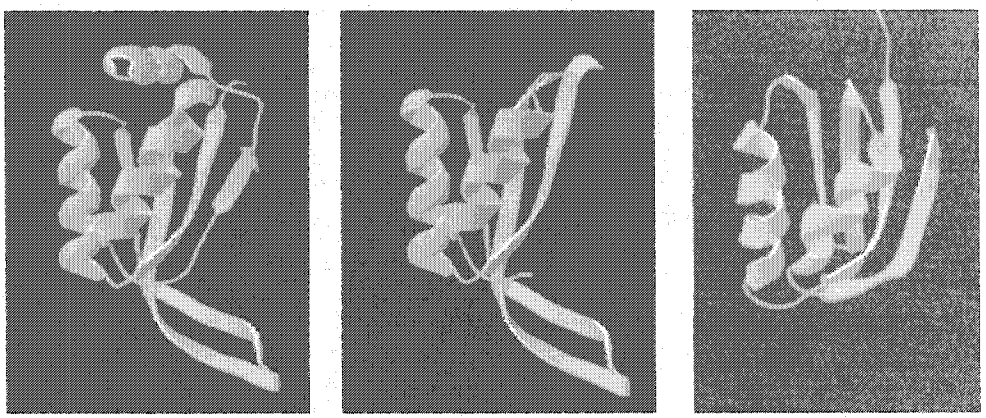
Name	Clone name	Sequence (5'-3')
Mod1		GGGGTACCATGGAGGAGAGCAGCAAAACTG
Cl2		GGGGTACCGGATCCTGGTCCAGTATTACACTTCACA
Cys 38	Mut1	CAATGTTCACCGCCGCTGCAAG; CTTGCAGCGGCGGTGAACATTG
Ser 47	Mut2	CGTACACCGCTTCAATGCCAGGC; GCCTGGCATTGAAGCGGTGTACG
Tyr 49	Mut3	CCCCTCCCACTCAAACACCG; CGGTGTTTGAGTGGGAGGGG
Trp 51	Mut4	CTTCCCCTCAAACTCGTACAC; ATCGGTGTACGAGTTTGAGGG
Glu 60	Mut5	CAGGAGCTCCGCTGAATCAC; GTGATTCAGCGGAGCTCCTG
Glu 73	Mut7	GGTTAGAGGCGCTACAAGGG; CCCTTCTAGCGCCTCTAACC
His 78	Mut8	GCATTGACCGCCTCGGTTAG; CTAACCGAGGCGGTCAATGC
His 83	Mut9	GTATTCCGCATTTGCATTG; CAATGCAAATGCGGAATAC
Tyr 102	Mut10	CCATTCTAAAAACTTGTCGCTC; GAGCGACAAGTTTTTAGAATGG
Trp 105	Mut11	CTGTTTTTCAAAAATTCTAAATACTTG; CAAGTATTTAGAATTTTTGAAAAACAG

a

```
                    10        20        30        40        50        60
                    |         |         |         |         |         |
AtCutA              MEESSKTVPS----------IVVYVTVPNREAGKKLANSIVQEKLAACVNIVPGIES
1NZA-A              MEE----------------VVLITVPSEEVARTIAKALVEERLAACVNIVPGLTS
1P1L-A              MHN----------------FIYIIAPSLEEAERIAKRLLEKKLAACVNIFP-IKS
1KR4-A              MGSSHHHHHHSSGREALYFXGHXILVYSTFPNEEKALEIGRKLLEKRLIACFNAFE-IRS
                    * .                .: * *. * .  :.. :::::* **.* .  : *
Prim.cons.          MEES222222SSGREALYFXGHXIVVYITVP2EE4A44IAK4L2E22LAACVNI2PGI4S

                            70        80        90        100       110       120
                            |         |         |         |         |         |
AtCutA              VYEWEGKVQSDSEELLIINTRQSLLEPLTEHVNANHEYDVPEVIALPITGGSDKYLEWLK
1NZA-A              IYRWQGEVVEDQELLLLVKTTHAFPKLKERVKALHPYTVPEIVALPIAEGNREYLDWLR
1P1L-A              FFWWEGKIEAATEFAMIVKTRSEKFAEVRDEVKAMHSYTTPCICAIPIERGLKEFLDWID
1KR4-A              GYWWKGEIVQDKEWAAIFKTTEEKEKELYEELRKLHPYETPAIFTLKVENILTEYXNWLR
                      : *:*:;    *   :.**        : :.:  * * .* : :: :      :: .*:
Prim.cons.          4YWWEG22V4D4E42LIVKT24EKF4EL4EEVKALHPYT2PEI4ALPIE4GL4EYLDWLR

AtCutA              NSTRN-
1NZA-A              ENTG--
1P1L-A              ETVE--
1KR4-A              ESVLGS
                    ...
Prim.cons.          ES242S
```



b AtCutA c AtCutA-tr d Atx1

e *Thermotoga* CutA single crystal

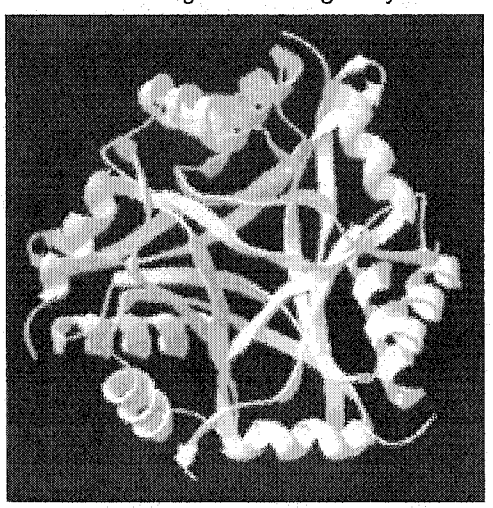


Figure 1. Modeling AtCutAp with Swiss-Model. Panel a: alignment of mature AtCutAp with bacterial homologues *Thermotoga martima* (Protein Data Bank ID: 1KR4) *Thermus thermophilus*, (1NZA), and *Archaeoglobus fulgidus* (1P1L). Panel b: Mature AtCutA model. Panel c: Mature truncated AtCutA model. Panel d: Atx1 sructure Panel e: single crystal of *Thermotoga* CutA indicating a Homotrimer.

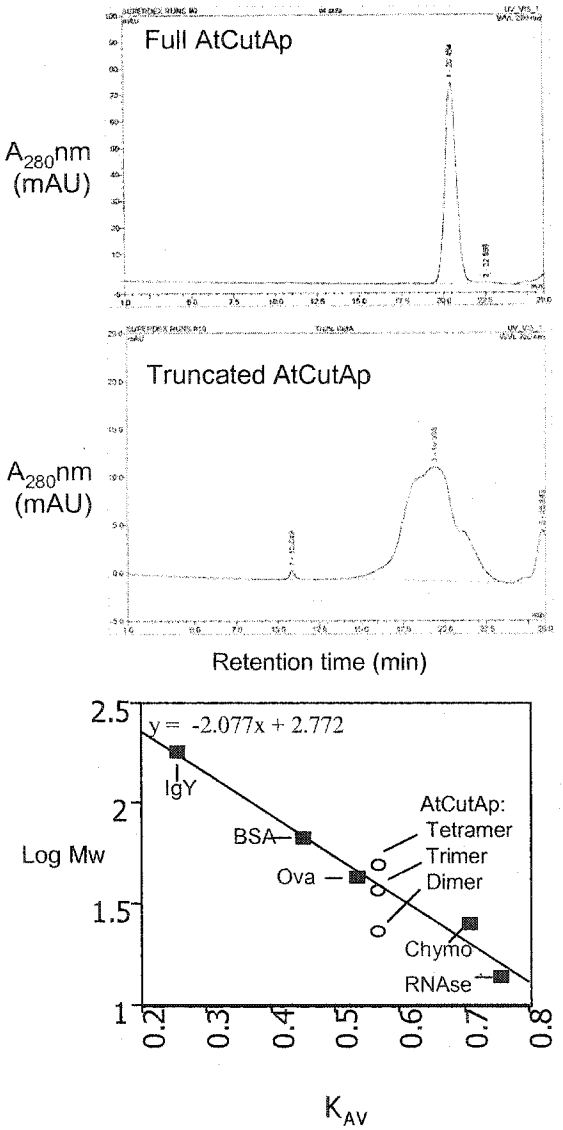
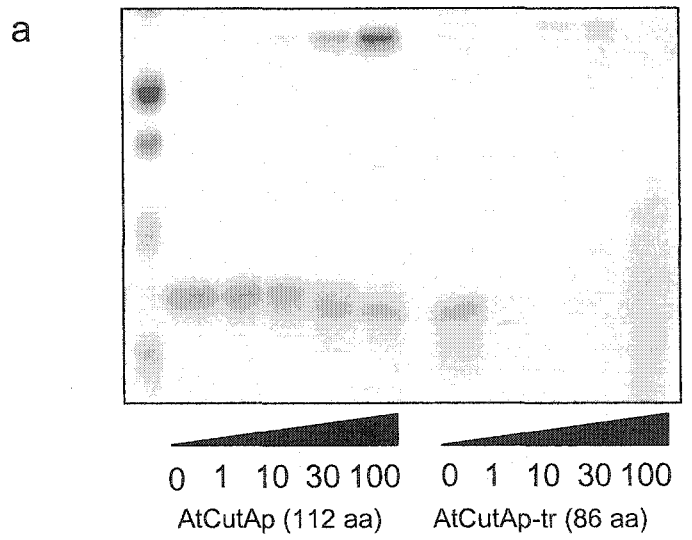


Figure 2. Gelfiltration analysis of AtCutA proteins on a Superdex-200 column. Top two panels: Elution profiles of full AtCutAp and truncated AtCutAp. Lower panel: Calibration curve and comparison of the elution (K_{AV}) of full AtCutAp compared to marker proteins.



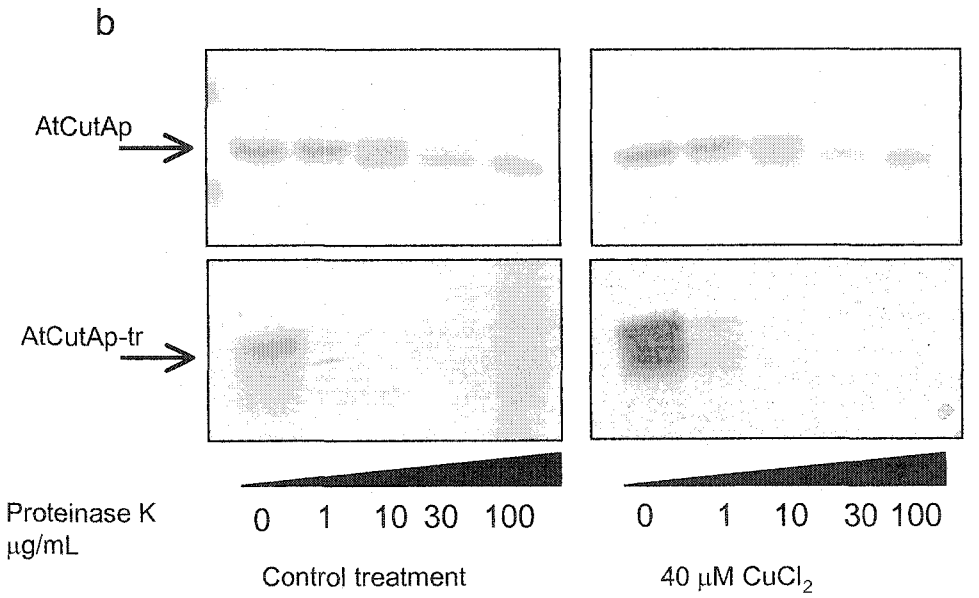


Figure 3. Panel a:,Protease sensitivity in AtCutAp. Purified recombinant AtCutAp incubated with increasing concentrations of Proteinase K for 30 min. 4 ug of protein used for SDS-PAGE and CBB staining. Left: recombinant AtCutAp; right, recombinant AtCutAp-tr. Panel b: protease treatment with added $CuCl_2$ Left, control treatment; right, proteinase K treatment with 40 µM $CuCl_2$.

a

1 MEESSKTVPSIVVYVTVPNREAGKKLANSIVQEKLAA**C**VN

41 IVPGIE**S**V**Y**E**W**EGKVQSDS**E**ELLIIKTRQSLL**E**PLTE**H**VN

81 AN**H**EYDVPEVIALPITGGSDK**Y**LE**W**LKNSTRN

b

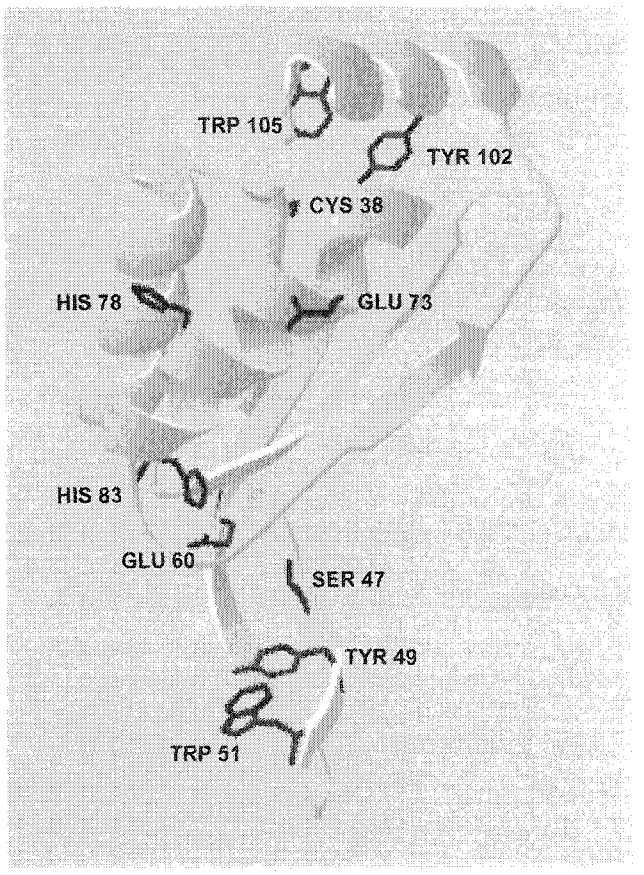


Figure 4. Panel a: Sequence of mature AtCutAp with mutated amino acids underlined and boldface. Panel b: AtCutA model with mutated amino acid side chains indicated.

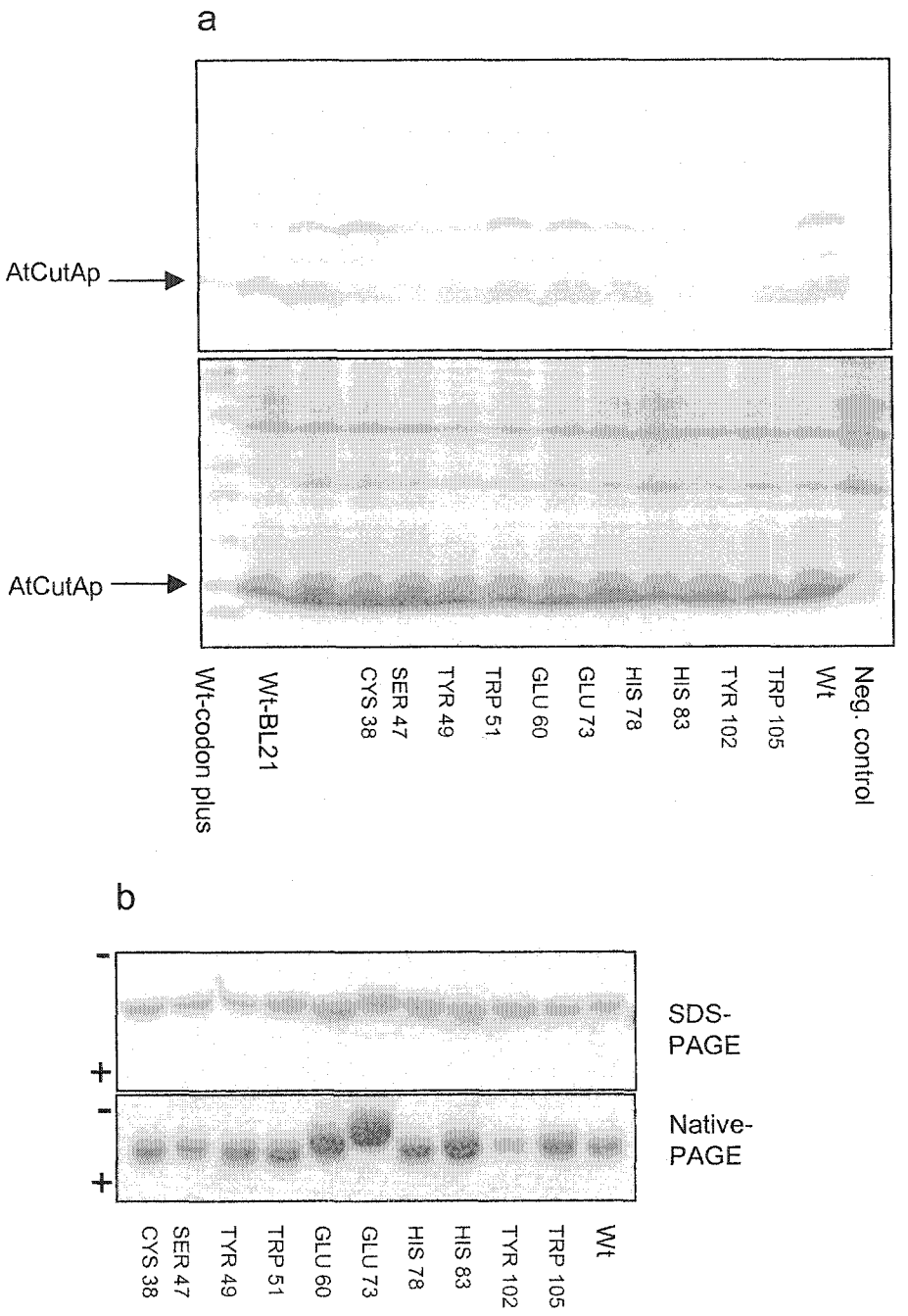


Figure 5. Expression and purification of point mutant AtCutA proteins. Panel a, top, Western blot and immunodetection of AtCutAp in expressed *E. coli* total cell lysate; bottom, SDS-PAGE of total cell lysate. Panel b, top, SDS-PAGE of purified AtCutA mutant proteins stained with CBB; bottom, Native-PAGE of purified proteins stained with CBB.

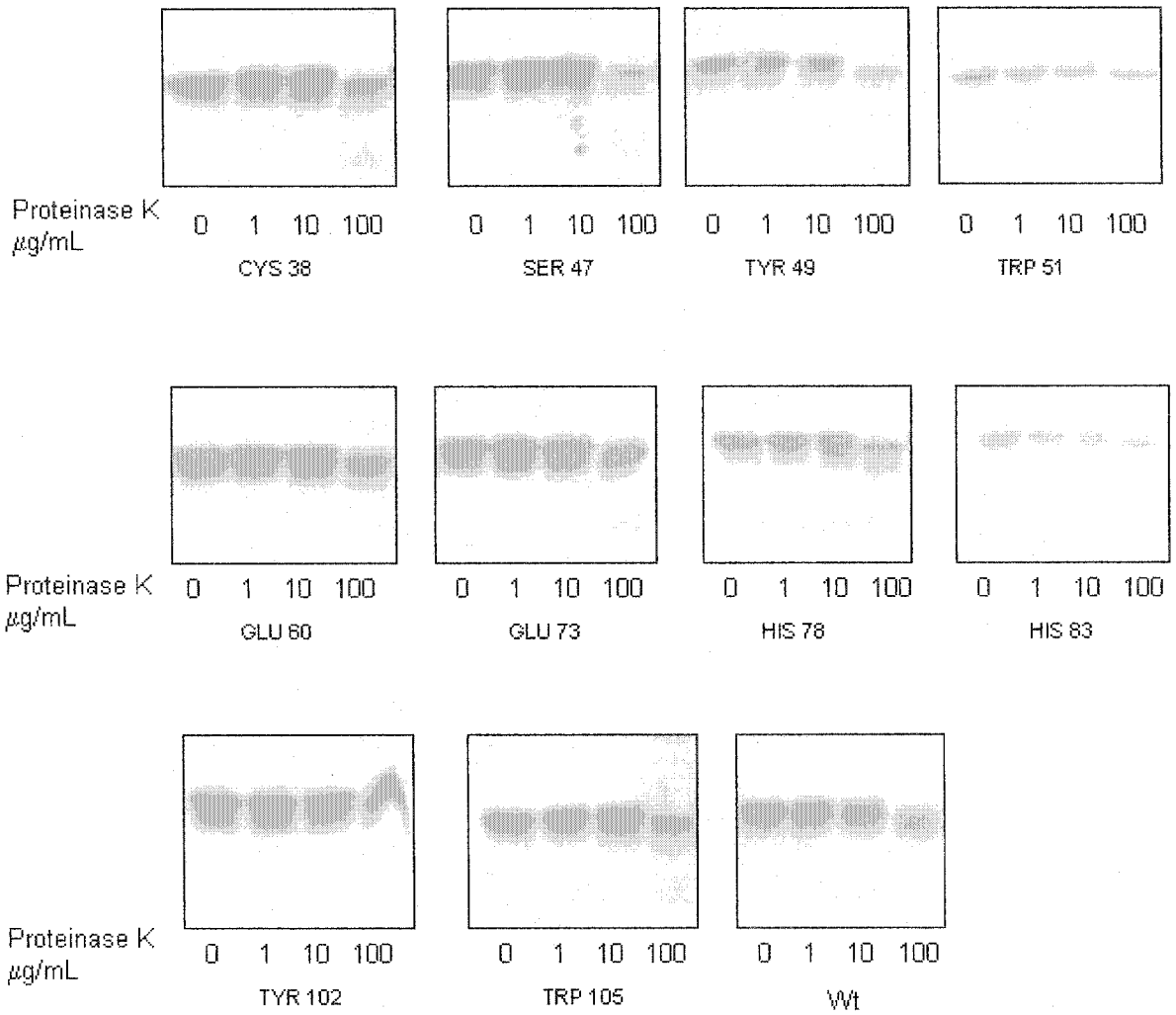


Figure 6. Protease sensitivity of AtCutA mutant proteins.

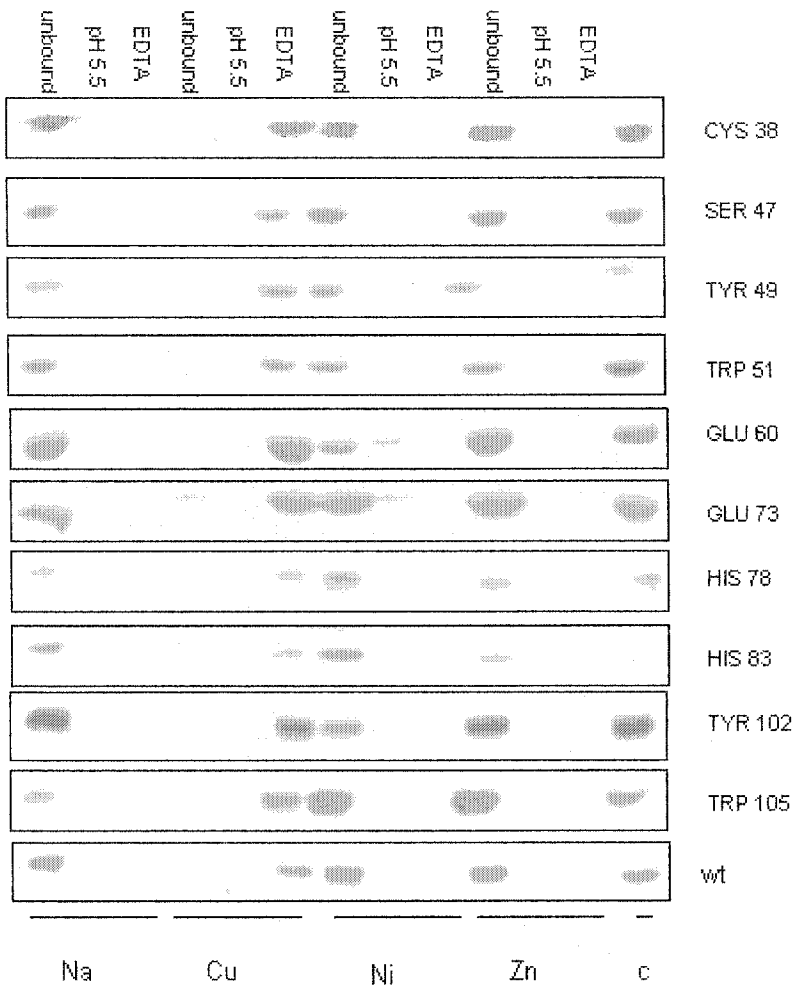


Figure 7. IMAC assay for CutA proteins. Metal binding assay with Na, Cu, Ni, Zn for mutant and WT proteins. Fractions loaded on each gel for each metal: unbound, pH 5.5 wash, and EDTA elution.

Chapter 5

Potential chloroplast copper chaperones

Summary

Copper is an essential cofactor in cells, but it is also highly toxic. Studies in the model organism yeast indicate that there is essentially no free copper in cells (Rae *et al.*, 1999), thus, copper must always be complexed to other molecules. Copper chaperones are soluble proteins that deliver copper to specific targets while keeping the reactive ion from interacting with other cellular components. Copper chaperones have been identified in prokaryotic and eukaryotic organisms, including cyanobacteria and higher plants. As copper transporters have been identified to supply copper to chloroplast proteins, it is expected that at least two copper chaperones should function in the organelles. Using BLAST searches, we have identified three candidate genes encoding proteins with homology to known copper chaperones that also have predicted chloroplast localization and cloned each by RT-PCR. *CpCOPZ* was identified with predicted chloroplast targeting information followed by a hydrophilic domain and a C-terminal ATX1-like metal binding domain. The predicted chloroplast targeting peptide of cpCopZp was able

to localize a GFP passenger protein to chloroplasts. CpCopZp is expected to function in chloroplasts to transfer copper from an acquisition point, such as Paa1p, to a target, such as Paa2p or the copper chaperone for superoxide dismutase. *CpCCS* was identified as a homologue to yeast CCS, a copper chaperone for superoxide dismutase. Full-length cpCCSp fused to a GFP passenger protein has been localized to chloroplasts. It is expected that cpCCSp will supply copper to CSD2p, the stromal CuZnSOD. The yeast *ATX1* homologue *ATX2* has been identified and the protein localized to cytosol rather than chloroplasts. We suggest that ATX2p may be functional in the cytosol or may require additional protein sequence to be imported into chloroplasts.

Introduction

Plant cells require precise delivery of copper to specific intracellular locations including soluble proteins and organelles. Plant chloroplasts must import copper from the cytosol and distribute it to precise locations within the organellar structure. Free copper ions are redox reactive and potentially toxic; therefore, copper ions must be transferred while bound to copper chaperones. Two principal types of copper chaperones have been characterized in yeast and humans, with highly conserved homologues in other organisms including plants (Field *et al.*, 2002). In yeast, the copper chaperone Atx1p can acquire copper directly from the C-terminal domain of the plasma membrane copper transporter Ctr1p (Xiao and Wedd, 2002) and deliver it to Ccc2p (Lin *et al.*, 1997), a copper-specific pump of the P-type ATPase family in the membrane of the Golgi. The human homologue of Atx1p directly interacts with the N-terminal Atx1-like domains of its target P-type

ATPases, suggesting copper transfer from chaperone to target by interaction of similar protein structures (Wernimont *et al.*, 2000). Lys7p/CCSp, which was originally implicated in lysine biosynthesis in yeast, delivers copper to the copper-zinc superoxide dismutase (CSD) by a direct protein-protein interaction (Casareno *et al.*, 1998; Rae *et al.*, 2001).

Plant chloroplasts must acquire copper for stromal copper-zinc superoxide dismutase (CSD2) (Kliebenstein *et al.*, 1998) and as a required component of photosynthetic electron transport in plastocyanin (Weigel *et al.*, 2003). Copper is imported into the stroma by the P-type ATPase Paa1p (Shikanai *et al.*, 2003), and moved from stroma to the thylakoid lumen by the P-type ATPase Paa2p (Abdel-Ghany, *et al.* unpublished). Therefore, we propose the existence of chloroplast chaperones to deliver copper to the CSD2p in the stroma and to Paa2p for transport into the thylakoid lumen and formation of holoplastocyanin. It is expected that an Atx1-like protein will acquire copper from Paa1p and transfer it to Paa2p for import into the thylakoid lumen. Similarly, it is expected that a CCS-like protein will acquire copper from either Paa1p or an Atx1-like protein in the chloroplast and incorporate it into CSD2p. We have selected three genes identified in the *Arabidopsis* genome with predicted chloroplast targeting information in addition to homology to characterized copper chaperones for analysis as chloroplast copper chaperones.

Materials and Methods

Vectors used in cloning: pET38-b was obtained from Novagen (Madison, WI, USA); pBluescript-SK was obtained from Stratagene, (San Diego, CA, USA). The GFP

reporter plasmid 35Ω-SGFP(S65T) was a generous gift from Dr. Rolland Norbert (Université Joseph Fourier, France) and is described in Miras *et al.* (2002).

ATX2 (AGI: At1g66240), *cpCOPZ* (AGI: At2g28660) and *cpCCSA* (AGI: At1g12520) were cloned by RT-PCR using mRNA isolated from 14-day old *Arabidopsis thaliana* ecotype Columbia. RNA isolation and cDNA synthesis are described for RT-PCR expression analysis in Chapter 3 of this thesis. Primers to amplify cDNA by PCR are as follows (upstream/downstream):

ATX2, 5'TATGCGCCATGGCGATGCTTAAAGACTTGTTCCAA /

5'TGCCAATGCGCGGCCGCAGCCTTAGCAGTTTCACCTTC;

cpCOPZ, 5'GCAGTCGTCGACATGAGATTTAGTGATATTTTCTG /

5'CAGTGCAAGCTTAAGATTTGAGGAGTGAATAAT;

cpCCS, 5'TATGCGCCATGGCATCAATTCTCAGGTCA /

5'TGCCAATGCGCGGCCGCAACCTTACTGGCCACGAAATC. Both *ATX2* and cpCCS were cloned to pET38-b as Nco1/Not1 restriction fragments. *CpCOPZ* was cloned to pBluescript-SK (Stratagene, San Diego, CA, USA) as a Sal1/BamH1 fragment. Original cDNA clones were sequenced in two directions at Davis Sequencing (Davis, CA, USA). Primers to introduce restriction sites for subcloning predicted chloroplast targeting information (30 N-terminal amino acids, including 26-a.a. predicted transit peptide) of *ATX2* GFP reporter plasmid as a Sal1/Nco1 fragment are the following:

ATX2: 5'TATGCGGTCGACCGATGCTTAAAGACTTGTTCCAA /

5'CATGCCATGGTAGCTTTCGATTCAACCACCGACAA. For cloning the *cpCOPZ* targeting peptide (90 N-terminal amino acids including 83-a.a. predicted transit peptide) to the GFP reporter plasmid, the upstream cloning primer to introduce a Sal1 restriction

site was used in conjunction with

5'CATGCCATGGCATCGCTACCGCGAGCCGAAGC introducing Nco1. The full coding sequence of *cpCCS* was amplified from cDNA to introduce Sal1/Nco1 restriction sites for use in the GFP reporter plasmid, as sequence analysis revealed an error in the original cDNA clone. Primers to amplify *cpCCS* with Sal1/Nco1 restriction sites are as follows (upstream/downstream):

5'CATCGTCGACATGGCATCAATTCTCAGGTCAGT /

5'CATGCCATGGAAACCTTACTGGCCACGAAATCG.

Sequencing: pET38b clones of *ATX2* and *cpCCS* were sequenced in two directions with CBD-LEAD (5'TAGGTGCAACTGTTGTTC) and ASCBD (5'GGTGTTCCACTGGTTGAC) primers. The pBSSK clone of CpCOPZ was sequenced in two directions with T7 and T3 primers.

Sequence alignments: Multiple protein alignments were performed with the ClustalW program (Thompson *et al.*, 1994) with default parameters at Pôle Bio-Informatique Lyonnais (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_clustalw.html).

Expression of Green Fluorescent Reporter Protein in Isolated *Arabidopsis* protoplasts.

Enzymes: cellulase Onozuka R-10 and Macerozyme R-10 were obtained from Karlan Research Products (Santa Rosa, CA, USA). Protoplasts were isolated and transformed essentially as described (Sheen, 2001). A confocal laser-scanning microscope (FVX-IHRT Fluoview Confocal LSM, Olympus, Melville, NY, USA) with Kr/Ar laser excitation (488 nm) was used to monitor green fluorescence (530 nm) and red

chlorophyll auto fluorescence (660 nm). Image captures and processing were by Fluoview software (Olympus). Images were captured at 90X magnification at a scan speed of 0.45 seconds for 256X256 pixel area. Scan slices were 1.0 μm thick.

Results

The entire *Arabidopsis* genome has been sequenced by the *Arabidopsis* Genome Initiative and can be accessed through The Arabidopsis Information Resource (tair, http://www.arabidopsis.org) to search for homologues of copper chaperones with potential chloroplast targeting. Three potential chaperones were identified based on sequence similarity and putative chloroplast targeting information.

One homologue of *ATX1*-like *Arabidopsis CCH* (Himelblau *et al.*, 1998) was identified (AGI: At1g66240) and contains a putative 26-amino acid chloroplast targeting sequence as predicted by ChloroP (http://www.cbs.dtu.dk/services/ChloroP). *CCH* is the *Arabidopsis* functional homologue of the highly conserved yeast cytosolic copper chaperone *ATX1*. ATX1p routes copper to P-type ATPases (Pufahl *et al.*, 1997), specifically to the endomembrane system. Not including the putative chloroplast targeting domain, the predicted protein for At1g66240 (Atx2p) shares 39% identity and 68 % similarity with yeast Atx1p.

A second putative chaperone with chloroplast targeting information is encoded in At2g28660. ChloroP predicts an 83-amino acid chloroplast targeting sequence for this protein. The mature protein contains a C-terminal region with a conserved heavy-metal associated domain including a CxxC metal binding motif. The N-terminal domain of the mature protein does not have similarity to other proteins with characterized function. This

putative chaperone is identified as cpCOPZ. An alignment of protein sequence for the predicted metal-binding domain of cpCopZp along with the sequences of Atx2p and the proteins encoded by *ATX1* homologues from yeast (*Saccharomyces cerevisiae*), higher plants (CCH, *Arabidopsis thaliana*), algae (*Chlamydomonas reinhardtii*), and cyanobacteria (*Synechocystis* PCC 6803) is shown in Figure 1a. The conserved heavy-metal associated domain of cpCopZp shown in the alignment is most closely related to the ATX1p homologue of *Synechocystis* with 23% identity and 62% similarity over the domain. The ATX2 protein shows highest similarity with the *Arabidopsis* CCH protein with 50. % identity, 63% similarity (not including chloroplast targeting information).

At third potential chloroplast copper chaperone is At1g12520. It was identified as a potential homologue of *CCS/Lys7* (Zhu *et al.*, 2000). However, the clone lacked a significant portion of the N-terminal sequence. The complete N-terminal region of the protein includes a predicted 67-amino acid chloroplast targeting sequence. The predicted protein from At1g12520 (cpCCS) contains high sequence similarity with *LYS7* (Zhu *et al.*, 2000), a yeast copper chaperone for CuZn superoxide dismutase (CSD). Not including the chloroplast targeting sequence, cpCCSp and Lys7p contain 24% sequence identity and 62% similarity. Copper-zinc superoxide dismutases (CSD) have been identified in *Arabidopsis* in cytosolic (Csd1p), chloroplastic (Csd2p) and peroxisomal (Csd3p) isoforms (Kliebenstein *et al.*, 1998). Therefore, there may be one or more chaperones to deliver copper to these enzymes in *Arabidopsis*. Figure 1b shows an alignment of cpCCSp with Lys7p, human CCSp, and a putative CCSp from tomato (*Lycopersicon esculentum*). Both the *Arabidopsis* and tomato proteins contain predicted N-terminal chloroplast targeting sequences. These Lys7p-like proteins retain high

sequence similarity including a conserved Atx1-like heavy-metal binding domain with canonical MxCxxC binding motifs and a C-terminal domain with similarity to a portion of CuZnSOD.

Cloning

We used RT-PCR to obtain the protein-coding sequence for *ATX2, cpCOPZ,* and *cpCCS*, and confirm that the mRNA for each is expressed. Amplified fragments are shown in Figure 2; sizes corresponding to *ATX2*, *cpCOPZ*, and *cpCCS* are at approximately 330, 800, and 1000 base pairs, respectively.

Subcellular localization

In order to test localization of Atx2p, cpCopZp, and cpCCSp to chloroplasts, the coding sequences for chloroplast transit peptides (tp) or full-length proteins were fused to coding sequence for green fluorescent protein in a transient expression vector under control of the Cauliflower Mosaic Virus 35S constitutive promoter. Isolated *Arabidopsis* protoplasts were transformed with these plasmids and analyzed for GFP expression. Green, red, green-red overlay and brightfield images are shown for a representative transformed cell with each construct in Figure 3 along with a control protoplast transformed with CAMV35S:GFP.

Protoplasts transformed with CAMV35S:GFP exhibited GFP fluorescence in the cytosol as shown in Figure 3. The Atx2-tp:GFP protein is clearly localized in the cytosol as shown in row 1. The localization of this protein is distinct from the chloroplasts in the same cell that are visualized by chlorophyll auto fluorescence (red, row 2). The overlay of red and green fluorescent images confirms a cytosolic location for Atx2-tp:GFP.

Protoplasts transformed with cpCOPZ-tp:GFP showed green fluorescence localized with the red fluorescence of chlorophyll, indicating that the cpCopZ-transit peptide is effective to import a GFP passenger into chloroplasts shown in Figure 3. The overlay of Green and red fluorescence in row 3 for cpCopZ-tr:GFP confirms chloroplast location.

A full-length cpCCS was fused to GFP for transformation of isolated protoplasts. Like the cpCopZ-tp:GFP, cpCCS:GFP is localized in structures corresponding to chloroplasts when compared to red chlorophyll fluorescence, indicating a that the cpCCSp is imported along with a GFP passenger into chloroplasts.

Discussion

The genes *ATX2*, *cpCOPZ*, and *cpCCS* were identified as encoding potential copper chaperones in chloroplasts by two main characteristics: an Atx1-like heavy-metal binding domain and putative chloroplast targeting information. Atx1-like domains are highly conserved across numerous species in both chaperones and as domains of transporters; Atx2p shares close homology with CCH, a cytosolic Atx1-like protein in *Arabidopsis*.

In yeast, Atx1p delivers Cu(I) to the Ccc2, a copper transporting P-type ATPase that transfers copper into the endomembrane system for incorporation into proteins (Pufahl *et al.*, 1997). *ATX2* was cloned as a candidate copper chaperone in chloroplasts, transferring copper from the envelope membrane transporter Paa1p to the thylakoid transporter Paa2p. The cytosolic location of the Atx2-tp:GFP fusion protein suggests an

alternative function for *ATX2*. One possibility is a transfer of copper from the plasma membrane to Paa1p, yet there could be alternate locations as well.

Another candidate protein for copper trafficking in chloroplasts is cpCopZp. The predicted mature protein contains a C-terminal Atx1-like domain and a hydrophilic N-terminal domain with unknown function. It is not clear if this hydrophilic domain is present in cpCopZp once it is imported into the chloroplast. It is possible it is a secondary signal peptide and is cleaved to result in a mature protein. A BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) search with the N-terminal domain of cpCopZp returns only cpCOPZ as hits. The conserved metal binding motif in cpCOPZ (IxCxxC) includes an isoleucine residue in place of the canonical methionine. The isoleucine residue in a metal binding motif can be found in other Atx1-like proteins (Arnesano *et al.*, 2002). The heavy metal binding domain of cpCopZp has close similarity with the protein encoded by the *ATX1* of of *Synechocystis* PCC 6803 (*ssr2857*) (Tottey *et al.*, 2002). Cyanobacterial Atx1p has metallochaperone function and can acquire copper from CtaAp or other locations and deliver it to PacSp, a copper transporting P-type ATPase in the thylakoid membrane (Tottey *et al.*, 2002). Since chloroplasts are thought to share a common evolutionary ancestor with cyanobacteria, the stroma can be considered homologous to cyanobacterial cytoplasm and similar functional relationship may exist for the copper targeting machinery. Indeed, the copper transporters for chloroplasts, Paa1p and Paa2p, share close homology with CtaAp and PacSp, respectively (Shikanai *et al.*, 2003; Abdel-Ghany, *et al.* unpublished). Chloroplast localization of the cpCOPZ-tp:GFP protein and the soluble nature suggests a function for cpCOPZ in the stroma, perhaps transferring copper from Paa1p to Paa2p.

The protein encoded by At1g12520 (cpCCSp) is closely related to the yeast copper chaperone for superoxide dismutase Lys7p/CCSp. *Arabidopsis* has three isoforms of the CuZnSOD: cytosolic (CSD1), chloroplastic (CSD2), and peroxisomal (CSD3). We have localized cpCCSp to chloroplasts, though it is the only potential candidate in the *Arabidopsis* genome with high conservation as a copper chaperone for superoxide dismutase. It is possible that cpCCSp delivers copper to both CSD1 and CSD2, perhaps utilizing an alternative translation start site that skips the chloroplast targeting peptide. Our data indicate expression of mRNA for *cpCCS* follows the pattern of CSD2 in wild type plants and *paa1* and *paa2* mutants, indicating coregulation (data not shown). We predict that *cpCCS* encodes a chloroplast-localized copper chaperone for superoxide dismutase.

Identification of *cpCOPZ*, *cpCCS*, and *ATX2* has provided further insight into plant copper homeostasis, particularly with respect to chloroplasts. Further experimentation will be required to extensively characterize these genes with respect to expression and protein function. If Atx2p is indeed a cytosolic protein, it may be a functional duplicate of CCH. Alternatively, it could function in another compartment of the cell. Function as an *ATX1*-like protein could be confirmed by complementation in yeast mutants. Expression analysis at the mRNA and protein level will also give clues to the function of *ATX2*.

It is apparent that *cpCOPZ* encodes a chloroplast-targeted protein with a conserved metal-binding domain. To function as a copper chaperone in chloroplasts, cpCopZp must acquire copper from one protein and deliver it to another. Defining a functional interaction with other proteins by either co-localization or possibly two-hybrid

studies will be critical in defining a role. Candidate target proteins for cpCopZp should include Paa1p and Paa2p. Analysis of phenotypes in *Arabidopsis* mutants in *cpCOPZ*, particularly with respect to chloroplast metal status, will also provide clues about its specific function. Complementation of a yeast mutant may be difficult due to the *Arabidopsis*-specific N-terminal domain, which has an unknown function. Removal of the N-terminal domain would leave a protein much like Atx1p in sequence, but it might also lose some of its biological relevance. It may be useful to determine an approximate length for the mature protein by *in vitro* chloroplast import and thus determine if indeed the N-terminal domain is retained.

It has been reported that cpCCS encodes a potential copper chaperone for copper-zinc superoxide dismutase (Zhu *et al.*, 2000). The homologue of cpCCS in tomato can functionally complement Lys7p in yeast (Zhu *et al.*, 2000). It will be informative to confirm this complementation with cpCCS and determine its ability to deliver copper to CuZnSOD. In addition, detailed expression analysis of cpCCS, particularly with respect to senescence and copper status will provide information about copper traffic and homeostasis in chloroplasts. The cytosolic copper chaperone CCH is upregulated during senescence and downregulated under copper stress (Mira *et al.*, 2002), so it will be informative to know if cpCCS expression follows a similar pattern. It will also be useful to determine exactly how cpCCS acquires copper. Possible sources for cpCCS copper acquisition should are Paa1p and any chloroplastic Atx1-like proteins—including cpCopZp.

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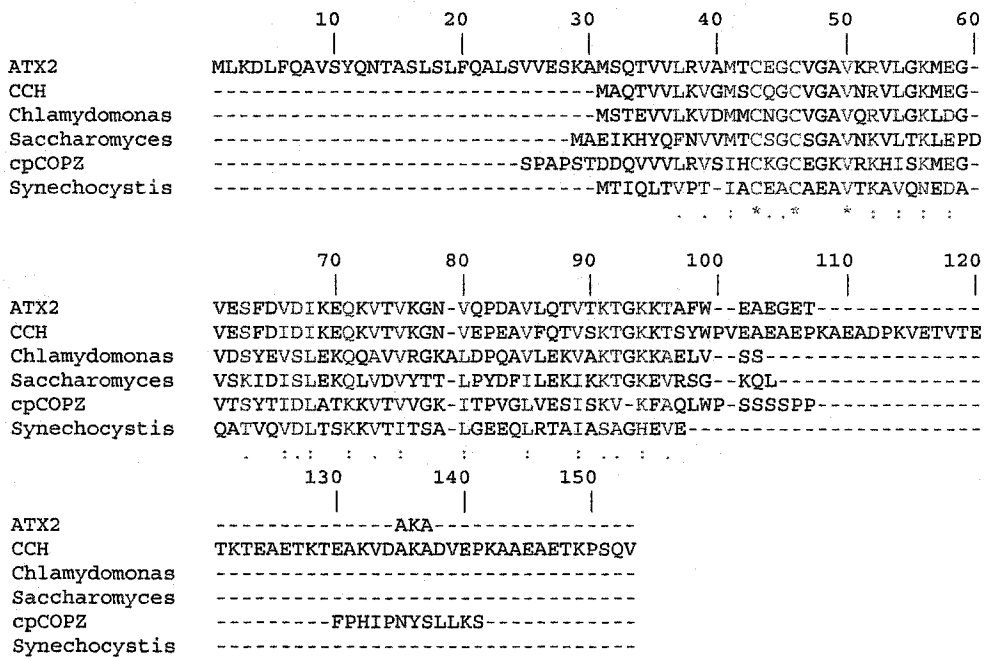


Figure 1a. Alignment of ATX2, cpCOPZ, and ATX1-like proteins identifed in algae, *Arabidopsis*, yeast, and cyanobacteria. Identical residues are noted with an asterisk (*); dots denote similar residues.

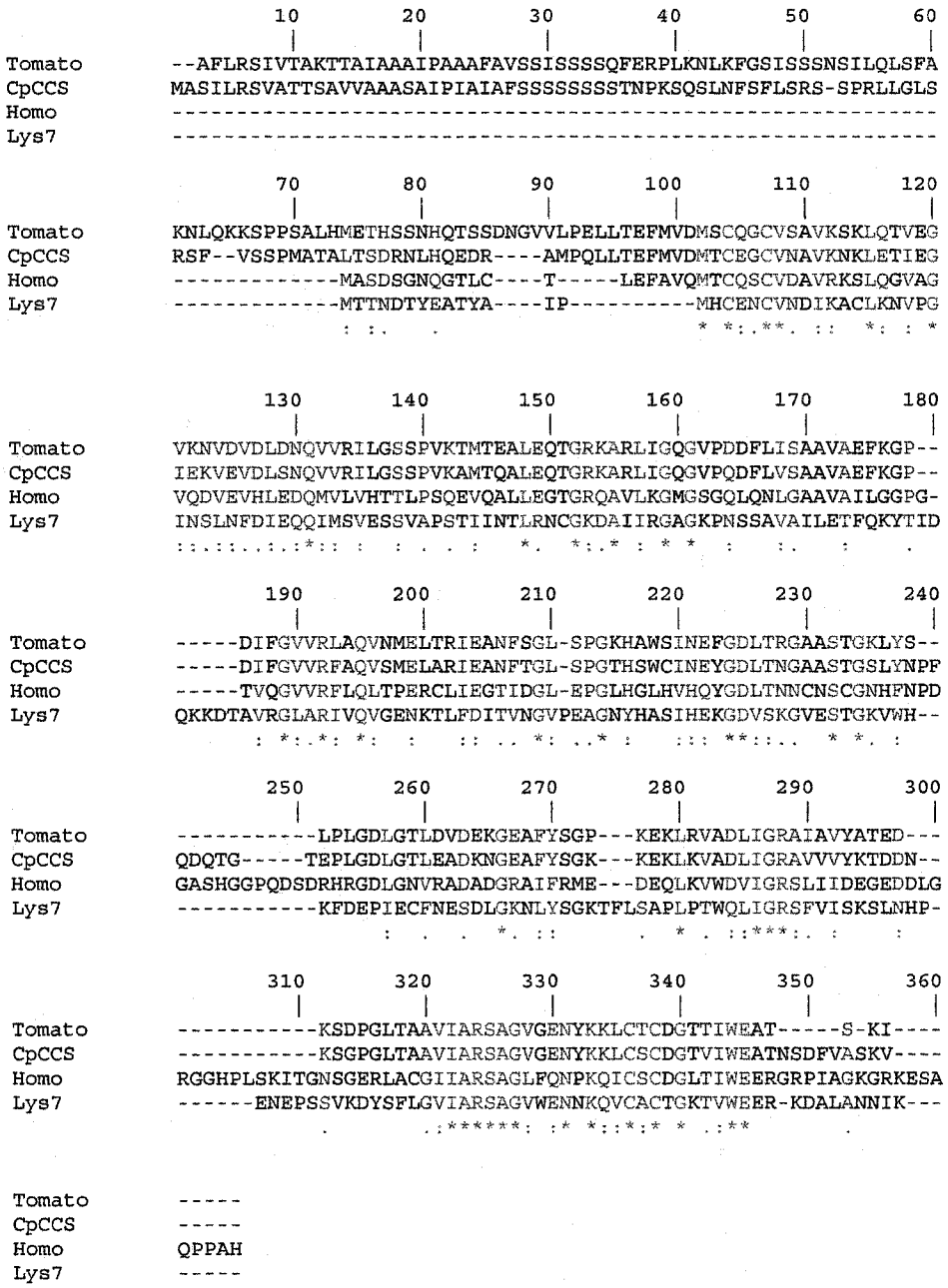


Figure 1b. CpCCSA aligned with identified tomato, human, and yeast copper chaperones for CuZnSOD. Identical residues are noted with an asterisk (*); dots denote similar residues.

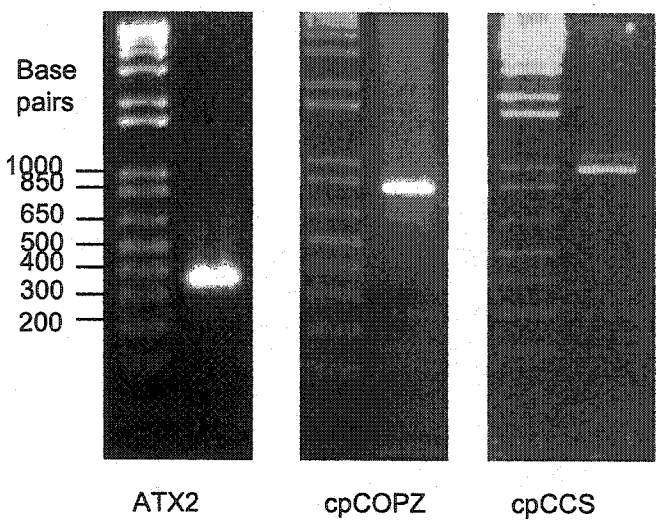


Figure 2. PCR cloning of *ATX2*, *cpCOPZ*, and *cpCCS* from *Arabidopsis* cDNA.

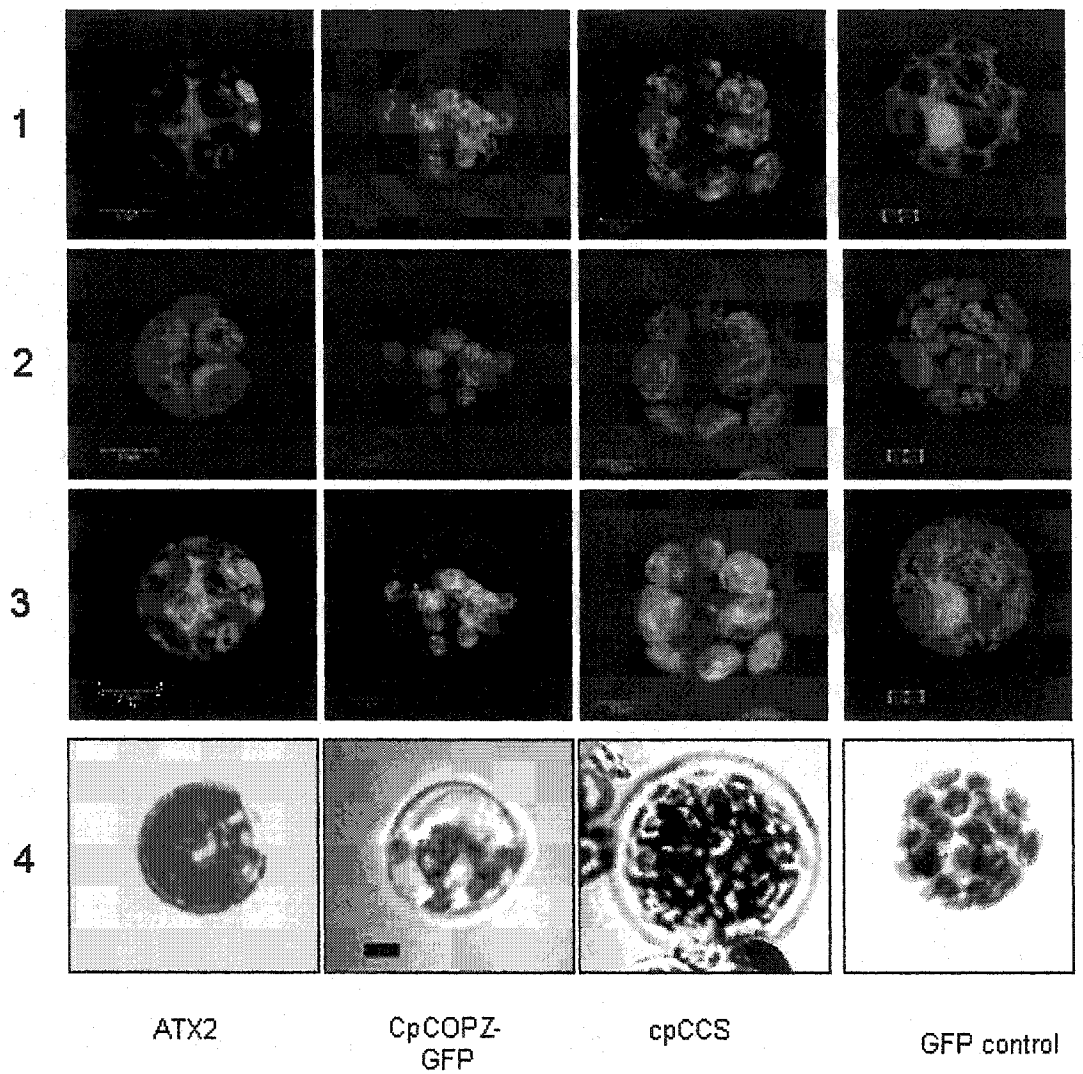


Figure 3. Expression of GFP-fusion proteins in isolated *Arabidopsis* protoplasts visualized by confocal microscopy. Row one: green fluorescence; row two: chlorophyll fluorescence (red); row three: overlay of green and red images; row four: brightfield image.

Chapter 6

Summarizing Discussion

All organisms require copper to survive. Although it has an essential role as a cofactor, its ease of oxidation and reduction also make copper dangerous to cellular structures. Cells must always retain tight control over copper, delivering it to essential locations while keeping it sequestered and preventing toxic reactions.

Higher plants require copper in order to carry out photosynthetic electron transport. The electron carrier plastocyanin is absolutely required for photosynthesis in *Arabidopsis* (Weigel *et al.*, 2003), and the same likely is true for other higher plants. Plastocyanin is also the primary copper protein in chloroplasts, containing approximately half of the copper in the organelle—and a large portion of the copper in plant cells (Marschner, 1995). Thus, it is of interest to understand the mechanisms and genes involved in copper homeostasis in chloroplasts.

This thesis research was undertaken to gain insight into copper homeostasis in chloroplasts. The initial strategy was to identify genes predicted to encode chloroplast-targeted proteins with sequence homology to prokaryotic proteins that

have likely involvement in copper homeostasis. The initial focus of this study was on one uncharacterized yet highly conserved gene, *AtCUTA*. As more data became available about the function and localization of copper proteins, particularly copper transporters in chloroplasts and cytosolic copper chaperones in plants and other organisms, the study was broadened to include identification of candidate copper chaperones in chloroplasts.

In Chapter 2, we presented a first analysis of *AtCUTA*. *AtCUTA* is a homologue of *Escherichia coli CUTA* (Fong *et al.*, 1995), which is thought to be involved in bacterial copper homeostasis, but its precise biological function remains unclear. Recombinant AtCutAp expressed in *E. coli* is localized to the bacterial periplasm, which can be considered evolutionarily homologous to the chloroplast envelope. In confirming chloroplast localization of AtCutAp, we also found that the protein appears to be sorted to the envelope in the intermembrane space. Determining a biological function for AtCutAp may also give insight into the function of CutAp homologues in *E. coli* and perhaps mammals, which have a homologue in an extracellular location (Navaratnam *et al.*, 2000).

We found that *AtCUTA* mRNA is expressed in all major tissues at similar levels, but the protein has low expression. Data also indicated that the mRNA is alternatively spliced, yielding a predicted truncated protein (lacking 26 C-terminal amino acids). Though difficult to detect in plants, cloning of the splice variant from an EST clone provided a useful variant compared to the fully-spliced transcript in biochemical analyses of translated proteins. We found that the full-length protein specifically binds divalent copper, while the truncated protein interacted with nickel

and zinc in addition to copper in an immobilized metal affinity chromatography test. This lent support to the premise that *AtCUTA* plays a role in copper homeostasis.

In Chapter 3, our aim was to gain information about the biological function of *AtCUTA*. We analyzed plants with altered *AtCUTA* expression levels for normal growth conditions as well as under copper stress. We found no significant differences from wild type plants under the conditions tested, but the identification and construction of plant lines with altered *AtCUTA* expression may prove useful in future experiments to more precisely ascertain its function.

Abundant mRNA expression for *AtCUTA*, coupled with low protein levels in wild type plants as noted in Chapter 2 suggests posttranscriptional regulation of *AtCUTA* expression. Our mapping of the 5'UTR for *AtCUTA* revealed a sequence with potential to form a stable secondary structure that could influence translation initiation. Information on other copper proteins such as plastocyanin (Vorst *et al.*, 1988) and *CCH* (Mira *et al.*, 2001) indicates they are largely transcriptionally regulated.

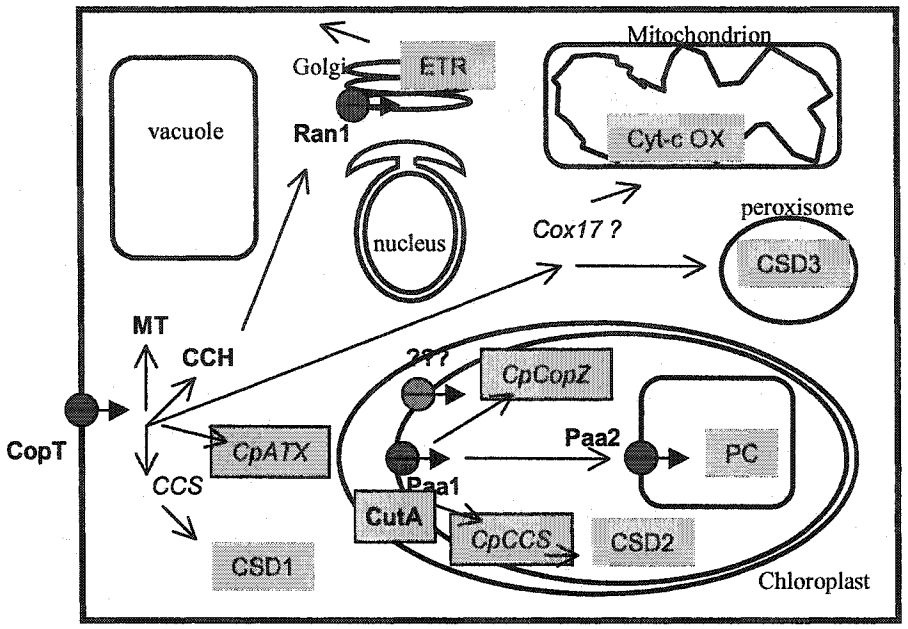
We found that AtCutAp expression was increased in wild type plants during senescence, a trait it shares with both *CCH*, a copper chaperone in *Arabidopsis* (Himelblau *et al.*, 1998; Mira, 2002), and *MT1*, a metallothionein (Mira, 2002). Further indication of *AtCUTA* involvement in copper homeostasis in chloroplasts is that the protein is expressed in chloroplast copper transporter mutants at higher levels than wild type plants grown under the same conditions. These data further substantiate the initial suggestion of a role for *AtCUTA* in chloroplast copper homeostasis.

In Chapter 4, we described biochemical characteristics of AtCutAp, AtCutAp-tr, and ten point mutants. Since *CUTA* proteins are highly conserved, information about AtCutAp may be applicable to its homologues. We found that AtCutAp is a stable protein that is largely protease resistant. Gelfiltration analysis indicated that the purified protein is a trimer. The truncated protein is purified as multiple oligomers, and is highly protease sensitive, suggesting it may not be a functional form of the protein. Single amino acid substitutions had little influence on protein folding or protease sensitivity, though replacement of two different acidic residues with alanine residues appeared to slightly reduce copper specificity in IMAC tests. AtCutA proteins with point mutations had similar copper binding capacity when compared to wild type, though some appeared to bind more copper per protein. Copper binding in AtCutAp may fall in a very precise range and leads us to speculate there may be a regulatory or sensing role for the protein—functioning only under certain conditions.

Since membrane copper transporters have recently been identified in chloroplasts (Abdel-Ghany *et al.*, unpublished; Shikanai *et al.*, 2003) and targets for copper such as CuZnSOD and plastocyanin are known, we were interested in filling in the missing soluble chaperones that may shuttle copper between transporters and targets. We identified three candidate genes encoding proteins with similarity to characterized copper chaperones and localized two of them (cpCopZp and cpCCS) to chloroplasts. CpCopZp has a unique hydrophilic domain preceding its C-terminal metal binding domain, leaving its function open to broad speculation at this point. The hydrophilic domain may not be retained, leaving an Atx1-like protein, or it may

serve an unidentified function. CpCCS is most likely a copper chaperone for CuZnSOD, filling in that piece of copper trafficking in chloroplasts.

The proteins identified in this work as likely to be involved in copper homeostasis are labeled as shaded boxes in the figure below.



Copper traffic in a green *Arabidopsis* cell. Primary copper proteins are indicated in gray boxes. Membrane transporters are filled circles with arrows. Characterized components of copper traffic are indicated in boldface. Putative copper traffic proteins are in italics.

We believe that these studies have led to a greater understanding of copper homeostasis in chloroplasts. We have identified and characterized a highly conserved protein, AtCutAp, that interacts with Cu(II). This information may lead to a discovery of the exact biological function of AtCutAp and provide insight to the function of *CUTA* genes in other organisms. We have also identified several new likely copper chaperones, two of which are positively localized in chloroplasts, increasing the pool of knowledge upon which further studies may be based.

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