DISSERTATION

COPPER TRANSPORT INTO THE CHLOROPLAST AND ITS IMPLICATIONS FOR COPPER HOMEOSTASIS IN *ARABIDOPSIS THALIANA*

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Wiebke Tapken

Department of Biology

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Doctoral Committee:

Advisor: Marinus Pilon

Stephen Chisholm

Elizabeth Pilon-Smits

Anireddy S.N. Reddy

ABSTRACT

COPPER TRANSPORT INTO THE CHLOROPLAST AND ITS IMPLICATIONS FOR COPPER HOMEOSTASIS IN *ARABIDOPSIS THALIANA*

Copper (Cu) is an essential micronutrient for most aerobic organisms including plants. It is present as Cu^{+} or Cu^{2+}, which makes it an ideal cofactor for enzymes involved in processes such as photosynthesis and respiration. Plant cuproproteins are almost ubiquitously found in every cell compartment. The blue Cu protein plastocyanin (PC) is believed to bind the majority of Cu ions in green tissues and is essential for higher plants. Cu reaches the thylakoid lumen through the activity of two P_{1B}-type ATPases called PAA1/HMA6 and PAA2/HMA8 (P-type ATPase of *Arabidopsis*/Heavy-metal ATPase), which are located in the inner chloroplast envelope and the thylakoid lumen respectively. Under Cu limiting conditions, plants have been suggested to prioritize cellular Cu to PC to ensure adequate photosynthesis. This process involves the post-transcriptional down-regulation of seemingly less essential cuproproteins through the activity of a single transcription factor called SPL7 (SQUAMOSA promoter binding protein-like7).

The first chapter reviews Cu homeostasis in plants. The research presented in the three experimental chapters of this dissertation is aimed to determine the role of the chloroplast in Cu homeostasis of *Arabidopsis thaliana*. I report a novel SPL7-independent and chloroplast-specific regulation of the thylakoid-localized Cu transporter PAA2/HMA8. The transporter is most abundant in the absence of Cu and is turned over at higher chloroplastic Cu concentrations. PAA2/HMA8 abundance in Cu deficiency is furthermore controlled by the presence of PC, because in a *pc* mutant PAA2/HMA8 abundance is always low. The regulation of the transporter likely serves as a checkpoint for the Cu requirements of the thylakoid lumen. I identified two components of the stroma-localized Clp protease (Caseinolytic peptidase) which are involved in PAA2/HMA8 turnover. The Cu status of these mutants is not affected, decreasing the likelihood of a secondary affect of Cu on PAA2/HMA8 in these plants. In the

last experimental chapter I summarize relevant results that further describe and characterize PAA1 and PAA2. Most notably, *Arabidopsis* encodes for a splice-form of PAA1. This much smaller fragment is expressed with a chloroplast targeting sequence and could potentially function as a stromal Cu chaperone.

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CHAPTER 1

Introduction and Scope of the Dissertation[1]

Summary

Copper (Cu) is essential for plant life because of its key role in photosynthetic electron transport, respiration and perception of the plant hormone ethylene. The most abundant Cu protein in plants is plastocyanin, an electron carrier in the chloroplast thylakoid lumen and essential for photo-autotrophic growth of plants. Copper is also a cofactor of superoxide dismutase and a number of extracellular cell wall enzymes of which the biological function is not yet fully elucidated. Cellular uptake is accomplished by the COPT Cu^{+} family of transporters. COPT1 and COPT2 are especially important at the root surface, whereas COPT6 is important for Cu uptake in green cells. COPT5 serves to release Cu from vacuolar stores. Members of the large ZIP family of divalent metal transporters might add capacity for uptake of Cu^{2+}. Members of the yellow stripe-like (YSL) family are proposed to function in the transport of Cu^{2+} complexed to the chelator nicotianamine, which might be important for mobilization of Cu from vegetative tissues to developing seeds. ATP-dependent P-type ATPases of the HMA family transport Cu^{+} out of the cytosol. Of these P-type ATPases, HMA5 serves to allow Cu exit from the cell, which is required for tolerance to excess and for long distance root-to-shoot transport in the vasculature. Other HMA transporters deliver the Cu cofactor to the ethylene receptors in the endomembrane system (HMA7) or to the chloroplasts (HMA6). A fourth Cu-transporting P-Type ATPase (HMA8) delivers Cu to plastocyanin in the thylakoid lumen. Cu-specific metallochaperones have been identified in plants similarly to other eukaryotes. Under impending Cu-deficiency, plants use three mechanisms to adjust their physiology. The cell surface localized COPT transporters are up-regulated in response to low Cu availability in the cytosol via the conserved Cu-responsive transcription factor SPL7 in order to increase assimilation. In addition, plants enter a Cu economy mode by the SPL7-mediated up-regulation of a set of

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small RNA molecules called the Cu-microRNAs because they target the messenger RNAs that encode for certain apparently dispensable Cu proteins. This mechanism should ensure that enough Cu is left for essential functions such as photosynthesis. Finally, the HMA8 transporter in the thylakoid membrane undergoes turnover but low Cu availability stabilizes the transporter to ensure efficient delivery of Cu to plastocyanin. Together, these homeostatic mechanism fine tune cellular and whole plant Cu distribution and allow plants to thrive on a broad range of Cu concentrations.

1.1 Copper utilization as a micronutrient

Copper (Cu) is a transition metal and is an essential micronutrient for the majority of species of all three domains of life. In cells, Cu is present in two oxidation states, Cu^{+} and Cu^{2+}, which makes it invaluable as a cofactor for proteins involved in cellular redox reactions. Copper is utilized by a variety of enzymes that fulfill basic housekeeping functions such as respiration and photosynthesis. Before about 2.7 billion years ago, when oxygen levels in the atmosphere were still extremely low, Cu is thought to have mainly existed as an insoluble cuprous sulfide (CuS) (Bekker et al., 2004). The possibility for its utilization as a nutrient was therefore limited. In contrast, anoxic conditions favored the existence of the highly bioavailable ferrous form of iron (Fe), Fe^{2+} (Bekker et al., 2004) and consequently, Fe-containing proteins evolved more abundantly than cuproproteins. With the onset of photosynthesis and after the oxygenation of the atmosphere as a result of oxygenic photosynthesis, the availability of the two metals was reversed and Cu became increasingly bioavailable (cupric, Cu^{2+}), while the non-bioavailable ferric form (Fe^{3+}) now dominated (Kerr, 2005). These events drove utilization of Cu as a micronutrient and the evolution of cuproproteins in photosynthetic and heterotrophic organisms (Burkhead et al., 2009). The interrelationship between Cu and Fe homeostasis can still be seen in a variety of metalloenzymes with seemingly redundant functions, such as Fe superoxide dismutases (FSDs) and Cu/Zn superoxide dismutases (CSDs) (Abdel-Ghany et al., 2005). The hypothesis of Cu cofactor evolution as a consequence of oxygenation of the biosphere is substantiated by the observation that most aerobic organisms utilize Cu, while the majority of anaerobic organisms do not (Ridge et al., 2008).

1.2 Cu and soil: Deficiency and toxicity symptoms

Cu concentrations in soils range between 3 and 100 mg/kg, but only about 1-20% exists as free, readily bioavailable Cu^{2+} (Salam and Helmke, 1998; Minnich et al., 1987). The majority is bound to organic matter and present as salts such as $CuCl_2$, $CuSO_4$ and $Cu_3(PO_4)_2$ (Sauvé et al., 1996). The amount of Cu in green plant tissues typically ranges around 2-50 mg/kg dry weight (ppm) and is largely species dependent (Burkhead et al., 2009). The broad range in Cu contents suggests variable need for Cu proteins, which might be related to differences in cell wall structure or other metabolic adaptations. In the plant model species *Arabidopsis thaliana* the physiological range for Cu in leaves is considered to be between 5 and 20 ppm. At these concentrations neither deficiency nor toxicity symptoms are developed (Cohu and Pilon, 2007; Abdel-Ghany and Pilon, 2008). When grown in unfertilized soils, *Arabidopsis* is often slightly Cu deficient. This is also true when grown in standard plant growth medium for *in vitro* culture, half-strength Murashige and Skoog medium (Murashige and Skoog, 1962). It contains 0.05 µM $CuSO_4$ and needs to be supplemented for maximum photosynthetic activity (Shikanai et al., 2003; Abdel-Ghany, 2009). Because Cu is fairly immobile between cell tissues, deficiency symptoms first appear in the newly formed, younger cells and the reproductive parts (Marschner, 1995). Classic symptoms of deficiency include impaired photosynthetic electron transport, reduced respiration, stunted growth due to defects in apical meristems as well as rolling-up and wilting of leaves (Marschner, 1995). Cu-deficiency makes cereals extra sensitive to lodging. Copper deficiency in pine forests severely affects wood quality and production (Oldenkamp and Smilde, 1966; Ruiter, 1969). The wilting, lodging of cereals and deformation in trees can all be attributed to defects in lignification when Cu supply is too low (Graham, 1976; Downes and Turvey, 1990; Dell, 1994). Even if primary plant productivity is not affected, the Cu content of feedstock has large economic impact because Cu is important for animal productivity and affects key animal functions including lipid metabolism (Davis, 1987; Ward and Spears, 1997; Engle, 2011).

In some areas Cu levels have become toxic due to human activities such as mining or the over use of Cu-based fungicides in viticulture (Marschner, 1995). Toxicity results when Cu is present in the cell as a free ion. In this state it can cause peroxidation of lipids as well as functional and structural damage to DNA

and proteins. Cu^{+} furthermore participates in Fenton-like reactions in which it catalyzes the formation of highly reactive hydroxyl radicals (·OH) in the presence of hydrogen peroxide (H_2O_2). Symptoms of Cu excess in plants include chlorosis of the vegetative parts and decreased root growth. Photosynthetic activity can be affected through the replacement of the Mg^{2+} atom in the center of the chlorophyll porphyrin ring by Cu^{2+} and the inactivation of Phaeophytin a (Phaeo a) in photosystem II in processes termed "shade"- and "sun"-reactions (Küpper et al., 1996). Most plants can acclimate to limited Cu excess by tuning the expression of transporters to limit uptake and by activating sequestering mechanisms such as metallothioneins and phytochelatins (Burkhead et al., 2009). However, excess is not so common and in nature and in agriculture deficiency is often a larger problem than toxicity (Burkhead et al., 2009).

1.3 Plant Cuproproteins

At the protein level Cu is a cofactor of plastocyanin (PC, photosynthesis), cytochrome *c* oxidase (COX, respiration) and the ethylene receptors. Other abundant Cu-proteins include chloroplastic polyphenoloxidases (PPO), the Cu/Zn superoxide dismutases (CSDs) in the chloroplast, cytosol and peroxisomes and a large group of apoplastic Cu proteins: ascorbate oxidases, amine oxidases, laccases and plantacyanin (Figure 1.1); (Cohu and Pilon, 2010). Apoplastic Cu proteins are implied in cell-wall modeling and possibly lignification (Cohu and Pilon, 2010). However, it must be noted that the biological roles of most Cu proteins (except PC, COX and the ethylene receptors) are unclear (Burkhead et al., 2009). Plant cuproproteins can be found in almost every cell compartment (Figure 1.1). The function of superoxide dismutases (SODs) has been studied extensively in yeast and humans. SODs function as homodimers, disproportionating toxic superoxide (O_2^{-}) by converting it to hydrogen peroxide and water in the following net reaction:

$$2O_2^{-} + 2H^{+} \longrightarrow H_2O_2 + O_2$$

Superoxide is a reactive oxygen species (ROS) and is mainly produced in the light-dependent reactions of the chloroplast through the reduction of O_2 at photosystem I (PSI), but can also be the by-product of other oxygenic reactions and respiration (Asada, 2006). It can be destructive to lipids and DNA and must

therefore be readily removed. In humans, the lack of SOD-1 activity has been implicated in neuronal diseases such as amyotrophic lateral sclerosis (ALS) (Liscic and Breljak, 2011; Deng et al., 1993; Rosen et al., 1993) and SOD-2 in Alzheimer's disease (Shaw et al., 1998; Massaad et al., 2009).

It is of interest to further elaborate on the possible functions of Cu-proteins that are regulated via small RNAs called microRNAs (1.7 Cu homeostasis). These proteins include CSDs, PPO, apoplastic plantacyanin and several laccases. CSDs have been implied in oxidative stress protection. However, an extensive study of an *Arabidopsis* mutant that lacks the copper chaperone for SOD and which has virtually undetectable CSD levels shows no phenotype, not even under stress (Cohu et al., 2009). Apparently plants have redundant anti-oxidant systems and CSDs may be largely dispensable, at least the most abundant forms in the cytosol (CSD1) and chloroplasts (CSD2).

The first discovered Cu enzyme in plant plastids was polyphenol oxidase (PPO) or tyrosinase (Arnon 1949). PPO is found in the thylakoid lumen and contains a dinuclear Cu center. Plant PPO catalyzes the conversion of monophenols to ortho-diphenols and ortho-dihydroxyphenols to ortho-quinones, resulting in black or brown pigment deposits (Mayer, 2006). Stresses such as wounding or pathogen, and herbivore attack have been shown to induce PPO activity in different plant species (Mayer, 2006). These observations suggest a role for PPO in plant resistance to stress and pathogens (Constabel et al., 2000). PPO is not ubiquitous and there is no homolog for PPO in *Arabidopsis*.

Plantacyanin is an apoplastic small blue copper protein of the phytocyanin protein family (Nersissian et al., 1998). The biological role of plantacyanins is unclear. Recent microarray data suggest that plantacyanin is a stress-related protein and may be involved in plant defense responses (Hampton et al., 2004). Other reports suggest that plantacyanins function as signaling molecules in the transmitting tract of the pistil (Dong *et al.*, 2005). Consistent with this notion, plantacyanin in *Arabidopsis* is highly expressed in flowers but it is also found in roots (Abdel-Ghany and Pilon, 2008).

Laccases are multi copper oxidases, which contain four Cu ions per polypeptide. Laccases use oxygen to catalyze the oxidation of a wide variety of aromatic or phenolic substrate molecules to reactive radicals with the production of water and oligomers (Riva, 2006). In one cycle, one dioxygen molecule is reduced

to two water molecules as four substrates are oxidized. Proposed biological functions of laccases in plants include roles in lignin synthesis, wound healing, iron acquisition, response to stress and maintenance of cell wall structure and integrity, including the formation of flavonoid derived pigments (Sterjiades et al., 1992; Bao et al., 1993; Dean and Eriksson, 1994; Ranocha et al., 2002; Hoopes and Dean, 2004; Pourcel et al., 2005; Liang et al., 2006). Fungal laccases have roles in lignin breakdown and can be virulence factors (Riva, 2006). In plants, laccase is a multi-gene family with members expressed differentially in different organs and at different developmental stages of growth. There are 17 Laccases encoded in the Arabidopsis genome whereas as many as 39 may be present in Poplar (McCaig et al., 2005; Weng et al., 2010; Weng and Chapple, 2010). In *Arabidopsis* the laccases can be grouped into six major branches based on sequence similarity (McCaig et al., 2005).

Lignin is an important structural component of plant cell walls and in abundance it is only second to cellulose (Davin and Lewis, 2005; Weng and Chapple, 2010). Lignin formation involves the oxidative coupling of phenolics called hydroxylcinnamyl alcohols (also known as coniferyl-type alcohols) that are ultimately derived from phenylalanine (Weng and Chapple, 2010). There is evidence that lignin formation is highly regulated and that assembly, or primary structure formation, requires proteins that interact with radical subunits (Davin and Lewis, 2005). In the angiosperms three types of lignin can be distinguished depending on which precursor is used (Weng et al., 2010). H lignin derives from p-coumaryl alcohol; G lignin from coniferyl alcohol; S lignin from sinapyl alcohol (Weng and Chapple, 2010). Several reports link Cu-deficiency to defects in lignification. Problems in lignification explain the deformation of pine trees, the rolling, desiccation and wilting of especially young leaves due to improper xylem vessel structure and lodging susceptibility of cereals (Oldenkamp and Smilde, 1966; Ruiter, 1969; Hopmans, 1990; Rahimi, 1973; Pissarek, 1974; Graham, 1976; Vetter, 1968). Lack of function for the Cu proteins laccase and amine oxidase might explain the effect of low Cu on secondary cell walls and lignification. It is thought that the oxidative combination of lignin subunits requires enzyme-mediated radical formation. Laccase is a candidate for this oxidative activity (Riva, 2006). For laccase the presence of so many isoforms, likely with overlapping functions, has made it difficult to analyze the roles individual laccases

using mutant approaches. Furthermore, laccases are difficult to extract from the cell wall, which is very inaccessible for biochemical approaches. As a consequence it is difficult to get good idea of the true *in vivo* substrates of these enzymes. Nevertheless, recent evidence shows that at least two of the 17 *Arabidopsis* laccases affect lignification, especially the formation of G-lignin (Berthet et al., 2011). It is noteworthy that the Cu-microRNAs, miR397 and miR408 target laccases including LAC4 and LAC17 in *Arabidopsis* (Abdel-Ghany and Pilon, 2008). Heme-containing peroxidases are also good candidates for a function in polymerization via radicals (Weng and Chapple, 2010). The Cu enzyme amine oxidase, which might act on abundant cell wall polyamimes may be a candidate to produce the peroxide (Frébort et al., 2000) that could be used by the cell-wall peroxidases. Therefore, Cu might affect lignification by two possible mechanisms.

For many Cu-enzymes an iron-containing alternative enzyme exists (Merchant et al., 2006). In several photosynthetic organisms (for example in *Chlamydomonas*) plastocyanin can be replaced by an iron containing cytochrome-c_6 when Cu becomes deficient. However, in plants plastocyanin is absolutely essential (Weigel et al., 2003). For CSDs an Fe-containing FeSOD alternative exists in plants. *Arabidopsis* plants without CCS and undetectable CSDs activity grown under conditions where FeSOD is also not expressed have very minor phenotypes (Cohu et al., 2009). Iron-containing cell wall-localized peroxidases could be alternatives for the Cu-containing laccases (Weng and Chapple, 2010). Systemic Cu deficiency will affect both photosynthetic carbon fixation and the expression of Cu-enzymes such as CSDs, plantacyanin and laccases. Carbon fixation provides the subunits for biomass formation and a large pool of reducing equivalents to cope with oxidative stress. Therefore, unless we uncouple their expression from Cu-availability it will be hard to assess the roles of CSDs in ROS metabolism and the roles of apoplastic laccases and plantacyanin in cell-wall modeling and lignification.

1.4 Plastocyanin the blue Cu protein

Most of the Cu ions in green parts of plants are thought to be bound by Plastocyanin (PC) (Marschner, 1995; Burkhead et al., 2009). PC is a small (10 kDa) blue Cu protein and it is essential for the light-

dependent reactions of photosynthesis in higher plants (Weigel et al., 2003). PC is the most abundant protein in the thylakoid lumen (Kieselbach et al., 1998). It transports one electron at a time between the cytochrome b_6f complex and P700, the reaction center of PSI, in the z-scheme of photosynthesis. PC is encoded in the nucleus and contains a bipartite targeting signal which ensures its translocation to the chloroplast stroma by the Toc/Tic machinery and further into the thylakoid lumen (Smeekens et al., 1986). PC is translocated into the thylakoids by the ATP-dependent Sec-pathway as apoPC (Jarvis, 2008). Therefore PC receives its cofactor after it has reached the thylakoid lumen (Merchant and Bogorad, 1987; Li and Merchant, 1995). Several flowering plants, including *Arabidopsis*, encode for two PC isoforms (Weigel et al., 2003; Pesaresi et al., 2009; Abdel-Ghany, 2009). The expression of plastocyanin is positively regulated by the presence of light (Takabe et al., 1986; Bichler and Herrmann, 1990; Last and Gray, 1990; Vorst et al., 1993). The coupling between the availability of light and PC expression likely serves as a means to regulate the photosynthetic activity in response to varying environmental stimuli and nutritional statuses (Schütze et al., 2008). In *Arabidopsis*, the *pc2* transcript is about 10-times more abundant than that of *pc1* (Pesaresi et al., 2009). PC2 protein abundance is furthermore positively regulated by Cu (Abdel-Ghany, 2009). Interestingly, *Arabidopsis* seems to accumulate more PC than required to support photosynthesis, suggesting a redox-buffering role in the thylakoid lumen (Pesaresi et al., 2009; Abdel-Ghany, 2009).

When either PC isoform is knocked out individually, no drastic loss of photosynthetic activity can be detected, which points to a redundant function of the two proteins (Weigel et al., 2003; Abdel-Ghany, 2009). *Arabidopsis pc1* mutants even survive in Cu deficient conditions, in which the abundance of PC2 is additionally decreased (Abdel-Ghany, 2009).This however results in a loss of photosynthetic activity as well as the ability to dissipate excess excitation energy via non-photochemical quenching (NPQ). Notably, a double-mutant *pc1*x*pc2* is seedling lethal (Weigel et al., 2003). The phenotype can only be partially rescued *in vitro* by growing the mutants in a sucrose-supplemented medium, in which photosynthetic requirements are diminished.

In the absence of Cu, apoPC is readily degraded in the unicellular algae *Chlamydomonas reinhardtii* (Li and Merchant, 1995). Interestingly, some unicellular photosynthetic organisms have a back-up system for PC in case Cu becomes so limiting that PC cannot obtain the cofactor and photosynthesis cannot be supported (Merchant et al., 1991). Under these circumstances *Chlamydomonas* can functionally replace PC by a heam-containing protein called cytochrome-c_6 (Cytc_6) (Merchant et al., 1991; Clarke and Campbell, 1996). Cytc_6 is found in some unicellular photosynthetic organisms (De la Cerda et al., 1999; Hippler et al., 1999). It was thought to be evolutionarily lost in higher plants, until genomic evidence of a modified version of Cytc_6 in multiple species, including *Arabidopsis*, Rice (*Oryza sativa*) and wheat (*Triticum aestivum),* emerged (Wastl et al., 2002). *In vitro* and RNAi studies suggested that the *Arabidopsis* Cytc_6-like protein Atc6 can functionally replace PC (Gupta et al., 2002). However, biophysical analyses of the protein indicate that its midpoint redox potential is not sufficient to be able to oxidize cytochrome *f* and that it is about 100-times less effective in donating Cu to PSI (Molina-Heredia et al., 2003). Therefore, the function of Atc6 *in vivo* is still elusive, but is not likely to serve as a backup system for PC in Cu deficiency conditions. Thus, PC activity is essential in higher plants.

1.5 Metallochaperones

Because Cu is so reactive it has to be bound to proteins in a controlled manner. In yeast cells it has been estimated that the concentration of free Cu ions is lower than 10^{-18}M, which is equivalent to less than one atom per cell (Rae et al., 1999). Cu ions are therefore thought to be bound by low molecular weight metal-delivery proteins called metallochaperones, which insert Cu into the active site of Cu-dependent proteins (O'Halloran and Culotta, 2000). So far, the function of 7 Cu-chaperones in *Arabidopsis* has been investigated: Copper Chaperone for SOD (CCS), Anti-oxidant 1 (ATX1), Cu Chaperone (CCH), Cytochrome c Oxidase11 and 17 (COX17), HCC1 and 2 homologue of the Cu chaperone Sco1 (SCO).The Cu-chaperones can be divided into three classes according to their interaction partners and function: Interaction with Cu-transporting ATPases (ATX1 and CCH), interaction with SODs (CCS) and function in mitochondrial respiration (HCC1, HCC2 and COX11, COX17) (Attallah et al., 2007; Attallah

et al., 2011; Steinebrunner et al., 2011; Balandin and Castresana, 2002; Chu et al., 2005; Shin et al., 2012; Himelblau et al., 1998; Puig et al., 2007). ATX1 and CCH are both homologs of yeast ATX1 with a distinct MxCxxC Cu-binding motif (O'Halloran and Culotta, 2000). They are thought to be involved in symplastic Cu distribution (Puig et al., 2007). The over-expression of ATX1 in *Arabidopsis* was recently reported to confer Cu resistance as well as an increased tolerance towards Cu deficiency (Shin et al., 2012). Notably, *cch* mutants did not exhibit an increased sensitivity to Cu in the same study. COX17 is located in the mitochondria, where it delivers Cu to cytochrome *c* oxidase (Balandin and Castresana, 2002; Attallah et al., 2007). HCC1 is similar to the yeast Sco1 and was recently reported to be involved in COX biogenesis (Attallah et al., 2011). In *Arabidopsis* the CCS sequence contains two in-frame ATG start codons (Chu et al., 2005; Cohu et al., 2009). Two mRNA species, a shorter and a longer version, can be formed due to the use of alternative transcriptional start sites (Cohu et al., 2009). The shorter mRNA encodes the cytosolic CCS and utilizes the second translational start site. The longer version of the mRNA allows use of the first ATG and encodes the longer isoform, which contains a functional chloroplast transit sequence for localization to the chloroplast stroma (Chu et al., 2005). So far three targets of CCS have been identified (Chu et al., 2005). The Cu/Zn superoxide dismutase 1 (CSD1), located in the cytoplasm, CSD2 in the chloroplast stroma and CSD3 in the peroxisome (Kliebenstein et al., 1998). CCS is a 34 kDa protein with three distinct domains. From studies in yeast we know that the CCS N-terminus (domain one) can bind Cu^{+} through a conserved MxCxxC in its ATX1-like *βαββαβ*-fold (Robinson and Winge, 2010), while domain two is structurally related to SODs. Domain two and three interact with the nascent apo-protein. Domain three contains a second Cu-binding motif CxC, which can bind an additional Cu^{+} atom. While yeast SODs are strictly dependent on Ccs1 for their activation, the SODs of mice and humans have alternative activation pathways (Wong et al., 2000; Carroll et al., 2004). Notably, the SOD activity in the nematode *Caenorhabditis elegans* is completely independent of CCS (Jensen and Culotta, 2005). CCS-independent activation pathways are not fully understood, but reduced glutathione (GSH) has been implicated as possible Cu-donor in yeast, humans and nematodes (Ciriolo et al., 1990; Carroll et al., 2004; Jensen and Culotta, 2005). Recently, Huang and others demonstrated for

the first time that also the *Arabidopsis* CSD1 can be activated through a CCS-independent pathway, but that the stromal CSD2 activity strictly depends on the presence of CCS (Huang et al., 2012). The authors hypothesized that a yet unknown stromal factor inhibits the activation in the absence of CCS. In this study, the presence of GSH was required for CCS-independent activation of CSD1 as well, together with a yet unidentified cellular factor (Huang et al., 2012).

1.6 Copper transporters

Cellular Cu ions are thought to be bound by metallochaperones and metallothioneins most of the time. Consequently, when Cu ions need to cross membranes, they have to be delivered by the chaperones to transmembrane transporters, which then actively or passively facilitate Cu passage. Cu transport in plants is mediated by four types of Cu-transporters: The COPT-transporters (Copper transporters), YSL (yellow stripe-like), and ZRT- IRT-like transporters (ZIP) are ATP-independent; the P_{1B}-type ATPases are ATP-dependent (Pilon, 2011). With some exceptions, P_{1B}-type ATPases are generally involved in Cu export (transport away from the cytosol), while the others are Cu importers (transport towards the cytosol). The spatial localization of the Cu-transporters in *Arabidopsis* is depicted in Figure 1.2. The COPT family belongs to the Ctr family of yeast Cu transporters and was first identified through functional complementation of a yeast Ctr1 mutant (Kampfenkel et al., 1995; Sancenón et al., 2003). *Arabidopsis* has 6 putative COPT family members (Peñarrubia et al., 2010). Ctr transporters work as homo-trimers, where each subunit consists of three transmembrane domains with conserved Cu^{+}-binding Methionine (Met) and Histidine (His) residues at the amino (N) -terminus and carboxy (C) -terminus (Puig et al., 2002). COPT1 and COPT2 are plasmamembrane transporters and are positively regulated by the Cu-responsive transcription factor SPL7 (SQUAMOSA promoter protein-like 7) in response to Cu deficiency (Sancenón et al., 2003; Yamasaki et al., 2009; Bernal et al., 2012; Jung et al., 2012). COPT5 is localized at the tonoplast and has been suggested to aid in interorganellar distribution of Cu under deficiency (Klaumann et al., 2011). COPT3 is likely an intracellular transporter (Sancenón et al., 2003). COPT4 is the only member that does not functionally complement the yeast *ctr1Δ*mutants and lacks the conserved

Met-rich regions (Puig et al., 2007). COPT6 has recently been shown to be localized to the plasmamembrane and to be regulated by SPL7 (Figure 1.2); (Jung et al., 2012).

The function of the ZIP and YSL transporters in the distribution of Cu is less understood. A transcriptome study implicated ZIP transporters, since some members are up-regulated by Cu deficiency (Wintz et al., 2003; DiDonato et al., 2004). YSL transporters are best known for their involvement in Fe uptake and homeostasis. YSL transporters belong to the oligopeptide transporter subfamily and the *Arabidopsis* genome encodes for 8 members (Curie and Briat, 2003). YS-transporters were first described in maize, where they transport Fe-phytosiderophore complexes (Curie et al., 2001). Non-graminaceous species do not produce phytosiderophores, but instead utilize nicotianamine (NA), a non-proteinogenic amino acid precursor derived from Methionine. In *Arabidopsis*, YSL-transporters have been suggested to transport NA-Fe and NA-Cu complexes. The YS1-transporter of maize was shown to complement a yeast *Ctr1* mutant when Cu was presented as NA-Cu^{2+}, but conflicting results for the *Arabidopsis* YSL1 exist (Schaaf et al., 2005; DiDonato et al., 2004). YSL1 and YSL3 have been implicated in the remobilization of Cu from senescing leaves and the Cu-loading of seeds (Waters et al., 2006; Curie et al., 2009). *YSL2* expression in *Arabidopsis* is induced by the presence of Fe and Cu in the medium (DiDonato et al., 2004). Expression patterns of this transporter demonstrated that it is active in roots and shoots alike, with a concentration of protein expression around the vasculature. YSL2 therefore likely serves in the lateral distribution of Fe and Cu through the plant. ZIP transporters are broad-range divalent metal transporters (Zn, Cu, Fe, Mn, Cd).The *Arabidopsis* genome encodes for 14 ZIP members. IRT1 and IRT2 (Iron Regulated Transporter 1 and 2) of the ZIP-transporter family have an important role in Fe-uptake in roots (Vert et al., 2002). Interestingly, in Cu deficient conditions, the expression of the main Fe transporter in roots IRT1, which belongs to the ZIP family, is increased about 1.8-fold through SPL7-mediated transcriptional activation (Bernal et al., 2012). ZIP2 is suggested to transport Cu in roots and ZIP2 and ZIP4 were able to complement a Cu transporter mutant in yeast (Wintz et al., 2003). Furthermore their expression is induced in the absence of Cu and suppressed when Cu becomes more available.

P_{1B}-type ATPases are heavy metal transporting ATPases and present in all three domains of life. They form a phosphorylated intermediate, hence "P"-type, in the metal translocation cycle (Rensing et al., 1999). There are two classes of P_{1B}-type ATPases: those transporting monovalent cations (Cu^+ and Ag^+) and divalent cation transporters (Cd^{2+}, Pb^{2+}, Zn^{2+}, Co^{2+}) (Rensing et al., 1999). The latter class is only found in prokaryotes and photosynthetic eukaryotes (Williams and Mills, 2005). P_{1B}-type ATPases contain 8 transmembrane domains (TM), with key features located in the cytosolic loops of the proteins. The cytosolic N-terminus contains a putative heavy-metal binding domain (HMB) with a conserved MxCxxC motif (Baxter et al., 2003; Williams and Mills, 2005). The small cytosolic loop (between TM4 and TM5) harbours the phosphatase domain and the actuator domain. The actuator domain interacts with the phosphorylation domain during enzyme phosphorylation/dephosphorylation (Williams and Mills, 2005; Argüello et al., 2007). TM6 contains a specific CPx motif that has been suggested to coordinate the Cu^+ ion for transport (Argüello, 2003). The ATP-binding domain, a nucleotide-binding domain (DKTGT) and the phosphorylation domain are located in the large cytosolic loop between TM6 and TM7. In the archae *Archaeoglobus fulgidus* the ion transduction domains of the Cu-transporting P_{1B}-type ATPase CopA are located between TM6-TM7 and TM7-TM8 (González-Guerrero and Argüello, 2008). The general mechanism of Cu^+-transport follows the E1/E2 Albers-Post mechanism (Argüello et al., 2007). The steps can be divided as follows: (i) Catalytic enzyme phosphorylation upon ATP-binding to the ATP-binding domain, (ii) Metal binding to the metal binding motif in TM6 (CPx), (iii) Upon phosphorylation, Cu^+ is occluded within TM6 which leads to a (iv) conformational change and release of Cu^+ into the other cell compartment. Lastly, (v) the P_{1B}-type ATPase is dephosphorylated (Raimunda et al., 2011). The transmembrane transport rate was estimated to be <10 ions/second (Argüello et al., 2012). In contrast, other ion pumps such as the sodium/potassium have a turnover rate of about 200 ions/sec (Palmgren and Nissen, 2011). The slow transport rate for Cu is attributed to the tight metal binding capacities of P_{1B}-type ATPases (Argüello et al., 2012).

Plants have evolved more P_{1B}-type ATPases than any other species and half of them are involved in Cu transport (Williams and Mills, 2005). *Arabidopsis* encodes for 8 members, named HMA1-8 (Heavy-metal

associated, nomenclature after Baxter (Baxter et al., 2003). The first P_{1B}-type ATPase to be cloned from *Arabidopsis* was HMA6/PAA1 (P-type ATPase of *Arabidopsis* 1); (Tabata et al., 1997). It was later localized to the inner chloroplast envelope (Shikanai et al., 2003). Together with the thylakoid-localized HMA8/PAA2, HMA6/PAA1 transports Cu^+ from the cytosol to the thylakoid lumen to supply PC with its cofactor (Shikanai et al., 2003; Abdel-Ghany et al., 2005). HMA6 and HMA8 are the only known chloroplastic Cu-transporters. HMA1 was previously thought to be a third Cu transporter in the inner chloroplast envelope, but was later suggested to be involved in Zn^{2+} transport from the chloroplast to the cytosol (Seigneurin-Berny et al., 2006; Kim et al., 2009). Of the other two Cu-transporting P_{1B}-type ATPases, HMA5 resides most likely in the plasmamembrane and is the only transporter of this family to be induced by Cu on a transcriptional level (Andrés-Colás et al., 2006; del Pozo et al., 2010). *hma5* mutants are hypersensitive to the presence of Cu, which suggests a function in Cu detoxification (Andrés-Colás et al., 2006; Kobayashi et al., 2008). HMA5 interacts with the Cu chaperones CCH and ATX1 in a yeast-2-hybrid assay (Andrés-Colás et al., 2006). Notably, the C-terminal extension of CCH, which is unique to plants, seems to have a negative effect on the interaction with the transporter. The function of this extension however, is still unknown. The five Cu-dependent ethylene receptors are located in the membranes of the endoplasmic reticulum (Chen et al., 2002; Ma et al., 2006; Dong et al., 2008; Grefen et al., 2008). These receptors depend on Cu delivery from HMA7/RAN1, which is localized to post-Golgi vesicles (Hirayama et al., 1999; Woeste and Kieber, 2000). The Zn-transporting HMA2, HMA3 and HMA4 are closely related in sequence and have been suggested to have evolved through gene duplication events in *Arabidopsis* (Hussain et al., 2004). HMA2 and HMA4 are located in the plasmamembrane and are likely Zn^{2+} and Cd^{2+} transporters with a function in root-to-shoot translocation (Hussain et al., 2004; Gravot et al., 2004; Wong et al., 2009; Craciun et al., 2012). Notably, the *Arabidopsis HMA3* isoform in the Col-0 ecotype likely expresses a truncated version of the protein, as a result of a frame-shift mutation (Hussain et al., 2004). However, functional HMA3 is encoded in other *Arabidopsis* ecotypes (Morel et al., 2009). When expressed in *Saccharomyces cerevisiae*, HMA3 localizes to the intracellular membrane system and is able to complement the Cd and Pb-sensitive strain Δ*ycf1* (Gravot et al., 2004). GFP fusion

experiments *in planta* localized HMA3 to the tonoplast, where it likely aids in the storage of Zn, Cd, Co and Pb (Morel et al., 2009).

1.7 Copper homeostasis

The amount of Cu in the cell has to be distributed between cuproproteins such as CSDs, cytochrome *c* oxidase and PC. Therefore, when Cu is limiting, the cell should prioritize its Cu pool to the most important proteins. Kinetic experiments in Poplar recently demonstrated that PC is the preferred target under Cu-limiting conditions (Ravet et al., 2011). We obtained some of our knowledge about plant Cu homeostasis through work in *Chlamydomonas*. In Cu deficient conditions, homeostasis is centrally controlled by a single transcription factor called Cu response regulator 1(CRR1) (Quinn and Merchant, 1995; Eriksson et al., 2004; Kropat et al., 2005). CRR1 belongs to the family of SBP-domain containing transcription factors which can be exclusively found in plants and photosynthetic unicellular algae (SQUAMOSA-promoter binding proteins; (Klein et al., 1996; Birkenbihl et al., 2005). The SBP-domain of CRR1 recognizes a Cu-responsive element (CuRE) in promoters of its target genes. The CuRE DNA motif contains the palindromic core sequence GTAC in the 5'-UTR and/or promoter region and can be repeated multiple times in this region (Quinn and Merchant, 1995; Sommer et al., 2010; Quinn et al., 1999; Quinn et al., 2000). In Cu deficiency CRR1 ensures photosynthetic activity by mediating the functional replacement of PC by Cytc_6 as well as the degradation of apoPC. RNA-seq analysis of *Chlamydomonas* grown in the presence and absence of Cu recently revealed a total of 63 CRR1 targets and an additional 86 other Cu-regulated genes (Castruita et al., 2011).

Arabidopsis encodes for a functional CRR1 homologue called SPL7 (SQUAMOSA promoter binding protein-like 7) and it has been reported to be the central regulator of Cu homeostasis (Yamasaki et al., 2009). In Cu-limited conditions, SPL7 down-regulates seemingly non-essential proteins such as CCS, CSD1, CSD2 as well as multiple laccases, while up-regulating root and shoot Cu importers (Figure 1.3); (Yamasaki et al., 2009; Cohu et al., 2009; Abdel-Ghany and Pilon, 2008; Bernal et al., 2012; Jung et al., 2012). SPL7 down-regulates the expression of these proteins through post-transcriptional turnover of

mRNA. In low Cu conditions the transcription factor activates four so-called Cu-miRNAs, *miR397*, *miR398*, *miR408* and *miR857* (Burkhead et al., 2009). Notably, *miR398*, *miR397* and *miR408* are among the most conserved miRNA families in plant species (Cuperus et al., 2011). MiRNA genes encode for short 21nt long single-stranded RNA molecules (Ambros, 2001). Plant mRNA targets usually have only a single complementary site in their sequence and mostly exhibit a nearly perfect complementary base-pairing (Kidner and Martienssen, 2005; Zhang et al., 2006). After target binding, the double-stranded RNA molecule is subsequently degraded through the RISC-mediated silencing mechanism (Bartel, 2004). SPL7 is only active in the absence of Cu and quickly shuts off when Cu is present in the cell. The concerted regulation of so many Cu-dependent proteins and transporters has likely evolved in higher plants because they lack a functional homolog for Cytc_6 and entirely depend on PC for their photosynthetic activity. Decreasing the abundance of less essential cuproproteins results in an enhanced usable Cu-pool for the utilization by PC (Burkhead et al., 2009).The Cu-sensing mechanism of SPL7 has not been identified in *Arabidopsis*, but in *Chlamydomonas* the current model involves the SBP-domain of CRR1, which is also present in SPL7. In the absence of Cu, the SBP-domain binds to the double-stranded DNA helix by two Zn finger-like domains (Yamasaki et al., 2004). It was shown that Cu^{+}, but not Cu^{2+} can displace the Zn^{2+} in the Zn finger-like domain *in vitro* (Sommer et al., 2010). Consequently, this would lead to the inhibition of Cu-miRNA expression by suppressing the binding of CRR1 to the DNA *in vivo*.

1.8 Regulation of P_{1B}-type ATPases

Metal transporting P_{1B}-type ATPases have been found to be regulated on multiple levels and by different mechanisms. The transcript abundance of the Zn-transporter HMA4 from *Arabidopsis* is positively regulated by the presence of Zn^{2+} and Mn^{2+} and down-regulated by the addition of Cd^{2+} (Mills et al., 2003; Gravot et al., 2004). Cd is a toxic element for the yeast *Saccharomyces cerevisiae*. In anticipation of metal over-exposure, the cells constitutively express a Cd-exporter called Pca1 (Adle and Lee, 2008). Therefore, if cells should encounter Cd, they will already be primed for detoxification. However, in the

absence of cytosolic Cd, the Pca1 protein is readily degraded by the proteasome, possibly to avoid the unspecific extrusion of other micronutrients (Adle and Lee, 2008). The human ATP7A and ATP7B have an important function in intracellular Cu distribution and when inactive can lead to the development of Menke's and Wilson's disease respectively (Lutsenko et al., 2007). At low intracellular Cu concentrations ATP7A and ATP7B reside at the trans-Golgi network for metalation of cuproproteins of the secretory pathway (La Fontaine and Mercer, 2007). If Cu content in the cell increases, both transporters move to the vesicular compartment in close proximity to the basolateral (ATP7A) and apical (ATP7B) surface respectively, now pumping Cu out of the cell (Petris et al., 1996; Roelofsen et al., 2000).Their activity is partly regulated by a mechanism involving glutaredoxin (GRX) and glutathione (GSH) (Singleton et al., 2010). In the absence of cytosolic Cu, ATP7A/B are glutathionylated at the Cysteine (Cys) residues of a conserved N-terminal CxxC-motif. GSH functions as a place holder that allows only Cu to occupy the specific Cys residues in the transporter. Once Cu becomes available, GSH-dependent glutaredoxin 1 (GRX1) interacts with the N-terminal domain of ATP7A/B, releases GSH and frees up the Cu-binding sites. Only then can Cu^{+} be bound by the two Cys residues. GRX1 and oxidized GSH (GSSG) are subsequently released. Interestingly, in the absence of GSH, trafficking of ATP7A/B is inhibited, implicating its involvement in their response to the Cu status of the cell (Singleton et al., 2010). The mechanics behind this effect are still under investigation. Recently, the post-translational regulation of the Cu-transporting HMA8/PAA2 was reported in *Arabidopsis* (Tapken et al., 2012). This thylakoid-localized transporter pumps Cu into the thylakoid lumen for use by PC for photosynthesis. In Cu-deficient conditions, PAA2 protein is the most abundant, possibly aiding in the prioritization of Cu flow to PC. When Cu becomes available, the chloroplastic Cu pool directly affects the stability of the protein, leading to its turnover. Notably, in *pc2* mutants, PAA2 abundance is always low, pointing towards a regulatory affect of PC.

1.9 Scope of this Dissertation

PC is thought to be the main target for Cu in green plant tissues under deficiency conditions. Cu is prioritized to the thylakoid lumen through the activity of the Cu-responsive transcription factor SPL7. The chloroplast is therefore an integral part of plant Cu homeostasis. Because the organelle harbours a significant portion of the cellular Cu, it is conceivable that it also takes part in its homeostatic regulation. At this point our knowledge concerning such involvement is minimal.

I propose that the two chloroplastic Cu transporters HMA6/PAA1 and HMA8/PAA2 could be ideal Cu sensors and regulators of metal transport. As transmembrane proteins they are in contact with three of the chloroplastic compartments (intermembrane space, stroma and thylakoid lumen) at all times. In this dissertation I aim to understand the physiological role of the chloroplast in Cu homeostasis and distribution with special emphasis on the involvement of HMA6/PAA1 and HMA8/PAA2.

Chapter two entitled "*Plastocyanin controls the stabilization of the thylakoid Cu-transporting P-type ATPase PAA2/HMA8 in response to low Cu*" reports the effect of chloroplastic Cu on the abundance of the PAA2/HMA8 protein. The regulation of this transporter was demonstrated to be SPL7-independent and is the first report of a posttranslational regulation of a P_{1B}-type ATPase that is mediated by its own substrate.

Chapter three follows up on the mechanisms involved in the down-regulation of PAA2/HMA8 abundance. In this process the Clp protease was identified as the likely candidate for mediating PAA2/HMA8 turnover. The Clp protease is an intricate oligomeric stroma-localized protein complex. When two of its components are knocked-down individually, plants show a significant up-regulation of PAA2/HMA8 in Cu deficient and sufficient conditions. Biochemical analysis of these mutants seem to indicate that their Cu status is not altered and therefore a secondary effect of Cu seems unlikely.

Chapter four summarizes additional observations that were obtained regarding PAA1/HMA6 and PAA2/HMA8 and describes new techniques that are potentially helpful in shedding more light on the mechanisms underlying PAA2/HMA8 turnover. I furthermore describe the presence of a small splice-

form of PAA1, and discuss its possible implications in Cu chelation and transport in the chloroplast stroma.

Following the experimental chapters is a *Summarizing discussion* in which the broader impacts of the here presented results are addressed and future experiments on Cu homeostasis are suggested. Lastly, the *Appendix* contains a detailed description of the methods I have developed as well as other preliminary results that were obtained. The latter should be useful for future research on Cu homeostasis. Furthermore, additional preliminary results of the Clp proteases are shown which imply a role of both Clp components in the coping with toxic Cu concentrations *in planta*.

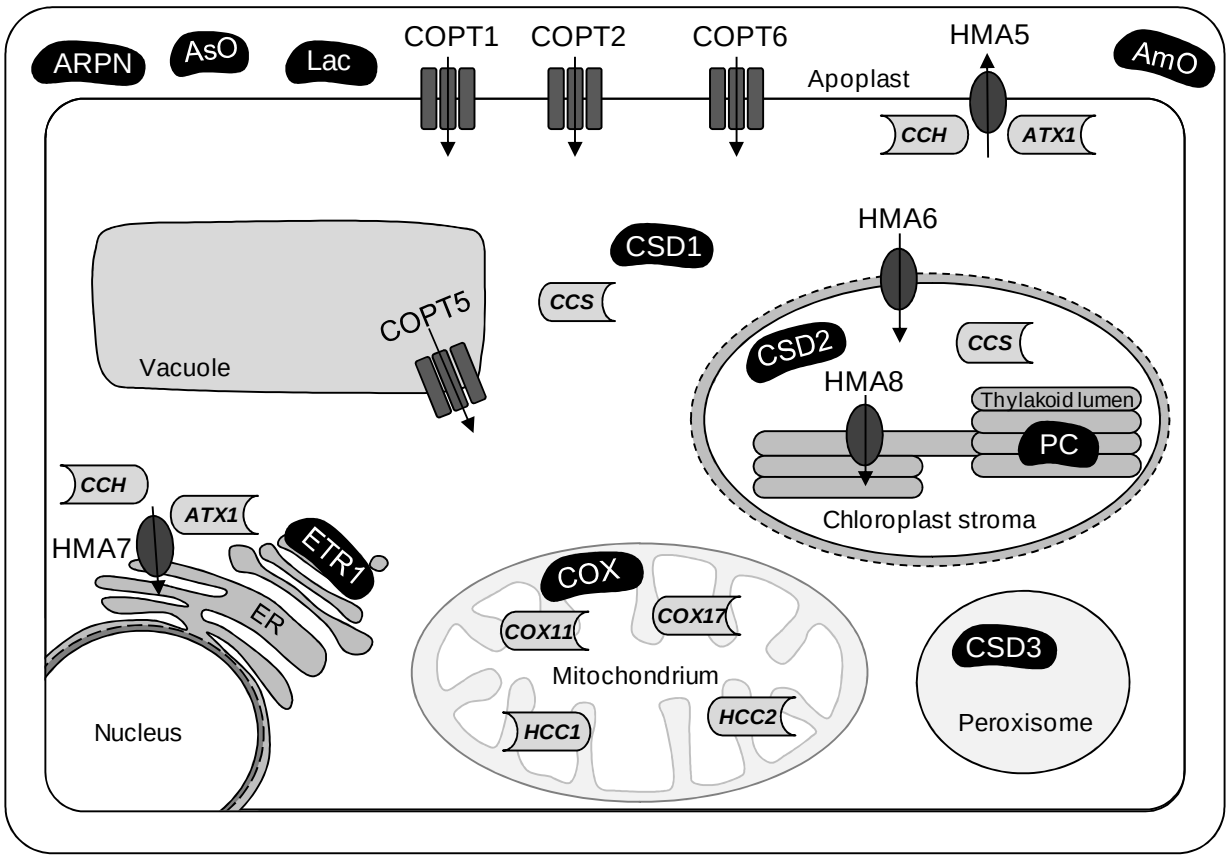


Figure 1.1: Verified cuproproteins and transporters in *Arabidopsis*

The diagram depicts the localization of verified cellular Cu-transporters and -proteins. Copper transporters (COPT) are represented as three rectangles and HMA (heavy-metal associated (P-type ATPase) are oval. Black arrows indicate the proposed direction of Cu^{+}-transport. Cuproproteins are shown with white letters on black background and the names of metallochaperones are depicted in bold and italic. The cellular compartments are labeled. AsO, ascorbate oxidase; AmO, amine oxidase; ARPN, plantacyanin; ATX1, Anti-oxidant 1; ETR1, ethylene-response 1; CCH, Cu chaperone; CCS, Cu chaperone for superoxide dismutase; COX, cytochrome *c* oxidase; CSD, Cu/Zn superoxide dismutase; HCC1, homologue of the Cu chaperone Sco1; Lac, Laccases; PC, plastocyanin.

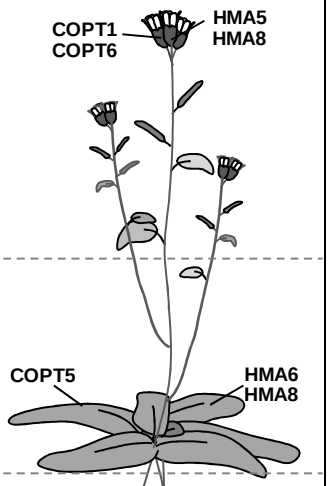
	Plasmamembrane				Intracellular		Main Cu-transporters
Reproduction	***COPT1** Pollen grains	**YSL1** Sepals, pedicel, petals, filament, siliques	**ZIP4** Flowers	**HMA5** Flowers	**COPT5^{V}** Siliques	**HMA8** Flower	
	COPT6 Stigma, ovary, pollen grains filaments	**YSL2^{V}** Flowers and siliques, Vasculature of sepals, petals, anthers				**HMA6** Flower	
		YSL3^{V} Anthers and pollen grains					
Vegetative	**COPT3?** Stems	**YSL1^{V}** Veins of leaves	**ZIP4** Shoots		**COPT3** Mainly in stems	**HMA6** Leaves	
	COPT5^{V} Siliques Tonoplast	**YSL2^{V}** Mature leaves, mainly vasculature			**COPT5^{V}** Low in leaf vasculature	**HMA7** Leaves	
	***COPT6^{V}** Trichomes Tricome base cells	**YSL3^{V}** Primarily in veins of leaves				**HMA8** Leaves	
Root	***COPT1** Root tips	**YSL1^{V}** Vascular cylinder of roots	**ZIP2** Roots	**HMA5** Roots	**COPT5^{V}** Primary root, root hairs, endodermis, vascular tissue	**HMA6^{V}** Vasculature	
	***COPT2** Roots	**YSL2^{V}** Roots, apical end of elongation zone					
	COPT5^{V} Root hairs						
	***COPT6^{V}** Lateral roots	**YSL3^{V}** Vascular cylinder of roots					

Figure 1.2: Spatial expression of *Arabidopsis* Cu-transporters

Localization of Arabidopsis Cu transporters separated into plasmamembrane and intracellular transporters. The main Cu transporters in the tissues are shown on the right. COPT, copper transporter; HMA, heavy-metal associated (P-type ATPase); YSL, yellow stripe-like; ZIP, ZRT,IRT-like protein. * indicates the regulation via SPL7. ^{V} indicates the clustering of expression in the vasculature of the respective organs and tissues.

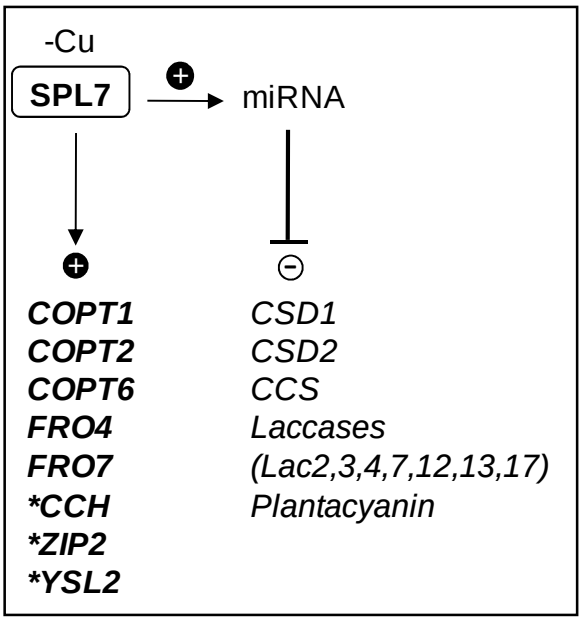


Figure 1.3: Regulation of Cu factor gene expression via SPL7 in Cu deficient conditions

In Cu deficient conditions, the Cu-responsive transcription factor SPL7 mediates the up-regulation of Cu transporters (+, bold) as well as the four Cu-miRNAs. Those in turn down-regulate seemingly non-essential cuproproteins through post-transcriptional degradation (-). * indicates the possible regulation by SPL7, as suggested through RNA-Seq analysis. Those targets have not been verified.

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CHAPTER 2

Plastocyanin Controls the Stabilization of the Thylakoid Cu-Transporting P-type ATPase PAA2/HMA8 in Response to Low Copper in *Arabidopsis*[2]

Summary

PAA2/HMA8 (P-type ATPase of *Arabidopsis*/ Heavy-metal-associated 8) is a thylakoid located copper (Cu)-transporter in *Arabidopsis thaliana*. In tandem with PAA1/HMA6, which is located in the inner chloroplast envelope, it supplies Cu to plastocyanin (PC), an essential cuproenzyme of the photosynthetic machinery. We investigated if the chloroplast Cu transporters are affected by Cu addition to the growth media. Immunoblots showed that PAA2 protein abundance decreased significantly and specifically when Cu in the media was increased, while PAA1 remained unaffected. The function of SPL7, the transcriptional regulator of Cu homeostasis, was not required for this regulation of PAA2 protein abundance and Cu addition did not affect *PAA2* transcript levels, as determined by qRT-PCR. We used the translational inhibitor cycloheximide to analyze turnover and observed that the stability of the PAA2 protein was decreased in plants grown with elevated Cu. Interestingly, PAA2 protein abundance was significantly increased in *paa1* mutants, in which the Cu content in the chloroplast is half of that of the wild-type, due to impaired Cu import into the organelle. In contrast in a *pc2* insertion mutant, which has strongly reduced plastocyanin expression, the PAA2 protein levels were low regardless of Cu addition to the growth media. Together, these data indicate that plastid Cu levels control PAA2 stability and that plastocyanin, which is the target of PAA2 mediated Cu delivery in thylakoids, is a major determinant of this regulatory mechanism.

Copper (Cu) is utilized as a cofactor by the majority of organisms . In higher plants cuproproteins can be found in most cell compartments including the apoplast, cytosol, ER, peroxisome, mitochondrion and chloroplast (for review see (Burkhead et al., 2009)). Copper proteins are part of fundamental redox

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Tapken, W., Ravet K., Pilon, M. (2012): Plastocyanin controls the stabilization of the thylakoid Cu-transporting P-type ATPase PAA2/HMA8 in response to low copper in *Arabidopsis*. *J Biol Chem*, 287 (22): 18544 – 18550

pathways such as respiration (in the form of cytochrome *c* oxidase), reactive oxygen species metabolism and photosynthesis. The majority of Cu ions in plant leaves are thought to be bound by plastocyanin (PC; (Burkhead et al., 2009)). PC is an electron carrier for the photosynthetic machinery and located in the thylakoid lumen of the chloroplast. See Figure 2.1 for an overview of the major Cu proteins and transporters. The *Arabidopsis* genome encodes for two PC isoforms (*PC1* and *PC2*; (Kieselbach et al., 2000)). PC2 accumulation is positively correlated with Cu abundance in the growth media, whereas PC1 is not affected by Cu (Abdel-Ghany, 2009). In a *pc2* mutant, Cu abundance in the thylakoids is reduced to less than 20% of that of the wild-type (Pesaresi et al., 2009). Other abundant cuproproteins are Cu/Zn superoxide dismutases (CSDs), which convert reactive superoxide (O_2^-) to hydrogen peroxide (H_2O_2) (McCord and Fridovich, 1969). The major isoforms in *Arabidopsis* leaves are CSD1 in the cytosol and CSD2 in the stroma (Kliebenstein et al., 1998). Both CSDs receive their cofactor through protein-protein interaction with the copper chaperone for superoxide dismutase (CCS), which is dually targeted to the cytosol and chloroplast stroma (Chu et al., 2005).

PC and CSD2 are translated in the cytosol and subsequently translocated to their respective chloroplastic locations (Jarvis, 2008). In order for PC to fully mature, Cu must be delivered to the thylakoids separately. This transport is mediated by two P-type ATPases. PAA1/HMA6 (P-type ATPase of *Arabidopsis* 1/Heavy-metal-associated 6) is a copper transporter located in the inner chloroplast envelope (Shikanai et al., 2003; Catty et al., 2011) and PAA2/HMA8 is located in the thylakoid membrane, see Figure 2.1 (Shikanai et al., 2003; Abdel-Ghany et al., 2005). The *paa1* and *paa2* mutant lines exhibit phenotypes that are directly correlated with a lack of Cu in the chloroplast. Both, *paa1* and *paa2* have decreased photosynthetic activity, which is attributed to a decrease in PC abundance (Shikanai et al., 2003; Abdel-Ghany et al., 2005). In addition, *paa1* mutants lack CSD2 activity and show slow growth in low Cu conditions (Shikanai et al., 2003; Abdel-Ghany et al., 2005).

In *Arabidopsis*, moderate Cu deficiency manifests similarly to mutations in *paa1* and *paa2*, resulting in the reduction of photosynthetic efficiency and stunted growth (Abdel-Ghany, 2009; Abdel-Ghany and Pilon, 2008). The same chemical properties of Cu that are utilized by cuproproteins can be

harmful if Cu is present as a free ion in the cell (Burkhead et al., 2009). Toxicity phenotypically manifests in chlorosis of the vegetative parts and a reduction of photosynthetic activity, possibly through the impairment of chlorophyll and photosystem II, as reported for aquatic algae (Küpper et al., 2003). Photosynthetic organisms have evolved a central Cu homeostatic machinery that allows a concerted response to low Cu through the Cu-responsive transcription factor SPL7 (SQUAMOSA promoter-binding protein-like 7; (Yamasaki et al., 2009)). SPL7 activates the transcription of the root plasma membrane transporter COPT1 (Sancenón et al., 2003), as well as multiple Cu-miRNAs that down-regulate seemingly dispensable cuproproteins through transcript degradation. *CSD1*, *CSD2* and *CCS* are targeted by *miR398*, while several apoplastic laccases and plantacyanin are targeted by *miR408* (Abdel-Ghany and Pilon, 2008; Yamasaki et al., 2007; Cohu et al., 2009). Intracellular Cu transporters such as PAA1 and PAA2 would be ideal control points for cellular Cu homeostasis, but thus far it has not been investigated if these transporters are affected by SPL7 or the Cu status of the plant. We now observed the stabilization of PAA2 protein on low Cu, a process which depends on PC and not SPL7. PAA1 abundance was not altered in response to the Cu status. In two *paa1* mutant lines in which Cu transport into the chloroplast is decreased, we observe a significant increase in PAA2 abundance, suggesting that Cu affects PAA2 protein turnover within the chloroplast.

Materials and Methods

Plant material, growth conditions and plant treatments- The ecotype background for *paa1-1* is Ler and Col-0 for *paa1-3* (Shikanai et al., 2003). The background for *paa2-1* is Col-3 *gl1* (Abdel-Ghany et al., 2005). The mutant lines *ccs*, *pc1* and *pc2* have been described previously (Weigel et al., 2003; Chu et al., 2005) and all have a Col-0 background. A *miR408* T-DNA insertion line was obtained from the Arabidopsis Biological Resource Center (ABRC) (Columbus, OH; SALK_023586.22.40.x). A homozygous *miR408* knock-out line was isolated through selfing. The presence and localization of the T-DNA was confirmed using gene-specific primers and the T-DNA-specific primer LBb1.3 (Appendix, Table A1).

For *in vitro* plant growth, seeds were surface sterilized by three consecutive 4 min. rinses with 70 %, 90 % and 70 % ethanol respectively and air dried prior to stratification for three days at 4 ºC. Plants were grown on solidified half-strength MS medium ((Murashige and Skoog, 1962); Caisson Laboratories, North Logan, UT; containing 0.05 µM $CuSO_4$) with 1 % sucrose (Sigma-Aldrich, St. Louis, MO), 0.6 % agar (Sigma-Aldrich, St. Louis, MO) and additions, as indicated for each experiment. Plants were grown for 18 days at a photon density of 120 µmol m^{-2} s^{-1}, in a 12-h light/12-h dark cycle at 23 ºC, unless specified otherwise. For time courses of PAA2 protein turnover, Col-0 was grown in liquid half-strength MS with 1% sucrose for 10 days in continuous light (120 µmol m^{-2} s^{-1}) and agitation in the presence of the indicated Cu concentrations. Plants were then treated for the indicated time periods with 100 µM cycloheximide (MP Biomedicals, Solon, OH), added from a 100 mM stock in 100 % ethanol (Arnaud et al., 2006).

Protoplast and chloroplast isolation- Protoplasts were isolated according to (Yoo et al., 2007) with the following modifications. Rosette leaves were cut into 1 mm strips and vacuum infiltrated for 5 min in 10 ml enzyme solution before overnight digestion in the dark. All subsequent handling was done in the dark and on ice. The next day, protoplasts were diluted with an equal volume of 2 mM MES/KOH (pH 5.7), 154 mM NaCl, 125 mM $CaCl_2$ and 5 mM KCl and filtered through a 215 µm nylon mesh (Component Supply Co., Fort Meade, FL). The flow through was centrifuged in a 50 ml conical tube for 2 min at 200 X g at 4 ºC in a pre-cooled Allegra™ 21R centrifuge using the S4180 swing-out rotor (Beckman Coulter Inc., Brea, CA). Pelleted protoplasts were then washed with 10 ml of 11 mM MES/KOH (pH 5.7), 77 mM NaCl, 63 mM $CaCl_2$, 2 mM KCl and 200 mM mannitol and again pelleted as before. Protoplasts were then taken up to a concentration of $1x10^6$ intact protoplasts/ml in the same solution. At this step protoplast samples for metal ion determination and immunoblotting were taken. The remaining protoplasts were used for subsequent chloroplast isolation according to (Fitzpatrick and Keegstra, 2001), with few modifications. Protoplasts were pelleted as before and taken up in 800 µl chloroplast isolation buffer (CIB; 400 mM mannitol, 5 mM EGTA, 5 mM EDTA, 20 mM HEPES/KOH pH 8, 0.1% BSA and 10 mM $NaHCO_3$) and subsequently mechanically lysed by forcing them through a

18 µm nylon mesh (Component Supply Co., Fort Meade, FL) held by a syringe filter holder (Pall Life Sciences, Ann Arbor, MI) into a round-bottom 2 ml centrifugation tube. Chloroplasts were then pelleted by a 2 min centrifugation step at 1125 X g. The pellet was resuspended in 400 µl CIB and loaded onto a 40% Percoll™ cushion (Amersham Biosciences AB, Uppsala, Sweden) in CIB. Intact chloroplasts were recovered after centrifugation for 5 min at 1620 X g in the bottom of the tube and resuspended in CIB containing no BSA. Chlorophyll content of protoplasts and chloroplasts was determined according to (Bruinsma, 1961), and all samples were concentrated to 1 mg chlorophyll/ ml in CIB containing no BSA. On average 50 - 60% of the chloroplasts were recovered from protoplasts.

Cu content measurements- Protoplast and chloroplast samples equivalent to 40 µg of chlorophyll were dried for 4 hours at 70 ºC and subsequently digested with 20 µl of trace metal grade nitric acid over night in a sand bath at 95 ºC. Then, samples were diluted with twice distilled water to a concentration of 10% nitric acid. 60 µl of each sample was further diluted in 540 µl of water and used for metal ion analysis on a Dionex ion chromatography system (Sunnyvale, CA) as described (Shikanai et al., 2003).

Plant sampling, protein extraction and immunoblot analysis- Arabidopsis shoots were frozen in liquid nitrogen and stored at -80 ºC until use. For protein extraction, plant tissue was ground with mortar and pestle in liquid nitrogen to a fine powder and its weight was determined while frozen. One part of tissue was homogenized in three parts of sodium dodecyl sulfate (SDS) sample buffer (250 mM Tris/HCl pH 6.8, 20% glycerol, 4% SDS (w/v), 80 mM dithiothreitol, 0.1 % (w/v) bromophenol-blue) in a microcentrifuge tube with the aid of a small pestle. The homogenate was immediately heated to 95 ºC for 5 min and the insoluble material removed by centrifugation at 16,000 X *g* for 15 min at 4 ºC. Preliminary experiments showed that flash freezing and preparation of the samples as described above yields the highest extraction of PAA1 and PAA2. The supernatant was fractionated on 10 % SDS-PAGE and transferred to nitrocellulose membrane (Trans-Blot® Pure Nitrocellulose, Bio-Rad, Hercules, CA) by electroblotting. Proteins were immunodetected using the primary antibodies as described for each experiment and a secondary antibody coupled to alkaline phosphatase. *Protein quantification-* For quantification of the immunoblots, signal intensities were analyzed using ImageJ software (NIH,

Bethesda, MD) and regression curves were obtained using dilution series on the corresponding membrane. Values are given as averages.

Antibodies- The sequences for the heavy metal binding domains of *PAA1* and *PAA2* were amplified using the primers listed in Appendix, Table A1 and cloned as *Nco*l/*Bam*Hl fragments into the expression vector pCAL-c. The fusion proteins were purified from *E. coli* strain BL21 (DE3) extracts by chromatography on Calmodulin Sepharose 4B (Amersham Pharmacia, Uppsala, Sweden) according to manufacturer's instructions. The calmodulin binding peptide tag was cleaved using thrombin (Novagen, Madison, WI) and the cleaved products were further purified by ion exchange chromatography on Q-Sepharose (Amersham Pharmacia). Purified proteins were used to raise antibodies in rabbits as described (Abdel-Ghany et al., 2005). Antibodies were subsequently affinity purified with the same peptides they were raised against using AminoLink® Coupling Resin (Pierce Biotechnology, Rockford, IL) and concentrated by centrifugation (Amicon® Ultra Centrifugal Filters, Millipore, Carrigtohill Co., Cork, Ireland). Antibody for cFBPase, a constitutively expressed protein, was purchased from Agrisera (Vännäs, Sweden). CCS antibody was described previously (Cohu et al., 2009).

RNA extraction, quantitative real-time PCR (qRT-PCR) analysis and mature miRNA stem-loop qRT-PCR- Total RNA was extracted from frozen leaf tissue with TRIzol® reagent (Invitrogen, Carlsbad, CA). Manufacturer's instructions for all enzymes and kits were followed if not otherwise specified. Twenty µg RNA were treated with DNase l (Fermentas, Hanover, MD) in order to remove genomic DNA from the extract. Prior to reverse-transcription, DNase l was removed by phenol-chloroform extraction and subsequent precipitation of mRNA (over night at -20°C in 100% EtOH with 3M NaOAc, pH 5.2). Total RNA concentration was determined and equal amounts per sample were reverse transcribed using the First Strand cDNA Synthesis Kit from Fermentas (Hanover, MD) and random hexamer primers from Promega (Madison, WI). Quantitative PCR and quality control of primers was performed on a Light Cycler® 480 with the Light Cycler SYBR Green l master mix (Roche Applied Science, Indianapolis, IN). Samples without template were used as negative controls. *PAA2* and *PAA1* transcript abundance was analyzed in biological triplicates and technical duplicates using gene-specific primer pairs (see Appendix,

Table A1). For normalization of the *PAA2* transcript level we used the two control genes yellow-leaf-specific 8 (*YLS8*) and the gene of a SAND family protein (see Appendix Table A1). Neither transcript is affected by Cu in *Arabidopsis* (Remans et al., 2008). The result for *YLS8* is presented in Fig. 2.2, *B*. Comparable results were obtained using *SAND* for normalization (data not shown). The quantitative PCR results were analyzed using the Light Cycler® 480 software from Roche. Relative transcript levels ($2^{-\Delta Ct}$) were calculated as the difference between the threshold cycle (C_t) of the target gene and the C_t of the reference gene for each respective template (Arrivault et al., 2006).

For the mature miRNA detection, stem-loop pulsed reverse transcription was performed after isolating total RNA as described above. However, ethanol washes were avoided and nucleic acid precipitation steps were performed using 1:1 (v/v) isopropanol and 1:10 (v/v) sodium acetate 3 M pH 5.2 in order to optimize small RNA molecule retrieval. The stem-loop pulsed reverse transcription and the miRNA qRT-PCR were performed as previously described (Varkonyi-Gasic et al., 2007), using the primers listed in Appendix, Table A1. Analysis of the data was performed as described above. The relative transcript levels of the mature miRNA were monitored for biological triplicates and technical duplicates, and results were standardized using miR167 expression. Similar results were obtained using miR156 as a reference (data not shown).

Statistical analysis- JMP software (version 9.0.2; SAS Institute) was used for statistical analysis. Figures and data represent average and SD values based on sampling from the indicated amount of biological and technical replicates. The number of total samples is given when appropriate. Student's t-test was used to calculate significant differences ($P < 0.05$), which is reported in the text or figures where appropriate.

Results

2.1 PAA2 protein abundance is affected by Cu

We raised antibodies to the N-terminal domains of PAA1 and PAA2. Immunoblots using total protein extracted from 18 day-old wild-type, *paa1-1* and *paa2-1* mutant plants indicated that the affinity-purified antibodies recognize their respective antigen (Appendix, Fig. A.1, *A* and *B*). The cytosolic fructose-1,6-bisphosphatase (cFBPase) was probed as a loading control in this and all following blots presented.

To investigate if PAA1 and PAA2 protein levels are affected by Cu, wild-type plants were grown *in vitro* on agar-solidified half-strength MS media in the presence of $CuSO_4$, ranging from 0.05 µM to 5 µM. The treatments clearly affected the Cu status of the plants as indicated by the abundance of CCS (Fig. 2.2, *A*). Protein abundance of PAA2 was highest in low Cu and decreased to one-third at 5 µM (Fig. 2.2, *A*; top panel). In contrast, PAA1 abundance was not affected by Cu.

To test if the abundance of PAA2 could also be affected by other metals, wild-type plants were cultured *in vitro* in the presence of various metals. To avoid too strong toxicity we used the metals at half the concentration that had been determined to give a 50% root length inhibition in *Brassica juncea* (Wangeline et al., 2004). As a control we quantified the effect of metal ion addition on root length and found that only cadmium reduced root length to more than 50% (Appendix, Fig. A.2). Importantly, only Cu affected PAA2 protein accumulation (Fig. 2.2, *B*). We conclude that the effect of Cu on PAA2 abundance is specific.

2.2 PAA2 is post-transcriptionally regulated

Sequence alignment revealed a potential *miR408* targeting site within the *PAA2* coding sequence (Appendix, Fig. A.3, *A*). *miR408* is one of the Cu-miRNAs induced by SPL7 in response to low Cu (Abdel-Ghany and Pilon, 2008). To analyze if *miR408* affects the PAA2 protein abundance we characterized a T-DNA knock-out line (Appendix Fig. A.3). The T-DNA is inserted within the mature *miR408* sequence (Appendix, Fig. A.3, *A* and *B*). The mature *miR408* was quantified by stem loop qRT-PCR for plants grown with 0.05 µM or 5 µM $CuSO_4$. In the wild-type, the mature miR408 was only

detectable on low Cu. In the knock-out line, mature *miR408* was at the detection limit which confirms that the T-DNA disrupts *miR408* (Appendix, Fig. A.3, *C*). In the *miR408* knock-out line, the PAA2 protein abundance still responded to Cu as in the wild-type and therefore *miR408* does not affect PAA2 (Fig. 2.3, *A*; top panel). To examine *miR408*-independent SPL7 involvement in the regulation of PAA2 abundance, an *spl7* mutant (Yamasaki et al., 2009) was grown *in vitro* on 0.05 µM and 5 µM $CuSO_4$ and then subjected to immunoblot analysis (Fig. 2.3, *A*). As expected, CCS was deregulated, but PAA2 protein abundance still decreased on high Cu in this mutant as it did in the wild-type. In addition, we performed transcript analysis of wild-type plants grown in low and high Cu using qRT-PCR (Fig. 2.3, *B*). Indeed, the transcript level of *PAA2* was the same in either Cu condition. The data indicate a post-transcriptional mechanism for the effect of Cu on PAA2.

2.3 Cu affects PAA2 protein turnover

Since Cu does not affect *PAA2* mRNA levels it must affect protein accumulation either through protein synthesis or turnover. We analyzed the effect of Cu on PAA2 protein turnover by inhibition of cytosolic protein synthesis with cycloheximide (CHX) (Fig. 2.4). Wild-type plants were cultured in liquid half-strength MS at 0.05 µM or 5 µM $CuSO_4$ for 10 days and then treated with 100 µM CHX for 24 hours. On low Cu, CHX treatment resulted only in about a 40% reduction of PAA2 abundance. In contrast, for plants grown on 5 µM $CuSO_4$ PAA2 abundance was reduced by 80% after 24 h of CHX treatment. Therefore, Cu strongly affects PAA2 protein stability.

2.4 Cu content in the chloroplast and plastocyanin abundance determine the effect on PAA2

To investigate if the Cu status within the chloroplast is important for the determination of PAA2 abundance, we analyzed *paa1-1* and *paa1-3* mutants, which are reported to have a lower chloroplastic Cu concentration (Shikanai et al., 2003). *paa1-1*, *paa1-3* and their respective isogenic wild-type were grown *in vitro* at 0.05 µM and 5 µM $CuSO_4$ and probed for PAA2 abundance by immunoblot. Both *paa1* alleles showed a significant increase in PAA2 compared to their respective wild-types (Fig. 2.5, *A*), while a

decrease in PAA2 protein is still seen in response to increasing Cu concentration in all lines. The data indicate that Cu levels in the chloroplast affect PAA2 protein accumulation. Known Cu binding proteins in the chloroplast are the two plastocyanin isoforms, CCS and CSD2. Of the two plastocyanin isoforms, PC1 is expressed at a low but constitutive level whereas PC2 accumulates especially on elevated Cu and is the most abundant plastocyanin isoform in most conditions except under deficiency (Abdel-Ghany, 2009). There is no mutant available for *CSD2*, but CSD2 activity and accumulation is severely disrupted in a *ccs* mutant (Cohu et al., 2009). To investigate if the abundance of any of the Cu binding proteins within the chloroplast could modulate the regulation of PAA2 accumulation in response to Cu, *pc1*, *pc2* and *ccs* mutants were grown *in vitro* at 0.05 µM and 5 µM $CuSO_4$ and probed for PAA2 protein. Interestingly, in *pc2* mutants the PAA2 protein abundance was reduced by about 75 % compared to the wild-type when grown in the presence of low Cu and stayed low at high Cu (Fig. 2.5, *B*; *top panel*). In contrast, the PAA2 protein abundance was comparable to the wild-type in *pc1* mutants, which still accumulate the PC2 protein when Cu levels are increased. In the *ccs* mutant, the PAA2 abundance was slightly elevated on low Cu when compared to the wild-type. Importantly, the regulation of PAA2 in response to Cu was maintained in this *ccs* line. These data indicate that among the chloroplast Cu proteins, PC2 expression is needed to maintain PAA2 protein levels.

We isolated protoplasts and chloroplasts from *in vitro* grown wild-type, *ccs* and *paa1-3* plants. Abundance of CCS and PC isoforms were verified as controls (Appendix, Fig. A.4). The cellular Cu levels were very similar between lines and clearly affected by Cu feeding (Appendix, Table A2). No significant change was seen in chloroplast Cu contents in the *ccs* mutant relative to the wild-type. However, the fraction of cellular Cu that was in the chloroplast in *paa1-3* plants was half of the wild-type level, which is in agreement with Shikanai *et al*., 2003 (Shikanai et al., 2003).

Discussion

The *Arabidopsis* P_{1B}-type ATPase PAA2 is a thylakoid located Cu transporter that supplies Cu to plastocyanin (PC), an essential protein for photosynthetic activity in higher plants (Weigel et al., 2003).

Here we demonstrate that the PAA2 protein is stabilized under Cu limiting growth conditions, leading to its accumulation in the thylakoids (Fig. 2.2, *A*). We show that PAA2 accumulation is modulated by the Cu levels within the chloroplast (Fig. 2.5, *A* and Appendix, Table A2). Importantly, this regulation is independent of SPL7, the master transcription regulator which controls other responses to Cu deficiency (Burkhead et al., 2009; Yamasaki et al., 2009). In contrast, it appeared that PC, the sink for Cu in the thylakoid lumen, ultimately controls this regulation (Fig. 2.5, *B*).

The mRNA expression level of *PAA2* is very low (Abdel-Ghany et al., 2005); (Fig. 2.3, *B*). Affinity purification of the antibodies was required for specific detection and allowed us to observe changes in PAA2 protein abundance. The effect of Cu on PAA2 abundance was not only observed for plants on agar media but was also observed for mature plants grown hydroponically (not shown). Only Cu and no other metal affected PAA2 (Fig. 2.2, *B*). Additional observations underscore the qualitative and quantitative effect of Cu on the transporter abundance. We noted that PAA2 protein accumulated more in the *spl7* mutant on low Cu when compared to the wild-type (Fig. 2.3, *A*). The *spl7* mutant phenotype is most evident on low Cu because the SPL7 transcription factor is required to up-regulate Cu transporters under impending deficiency (Yamasaki et al., 2009). Thus *spl7* is specifically deficient for Cu and therefore limited in its capacity to provide Cu for PAA2 and PC in the ½ MS growth conditions (0.05 µM $CuSO_4$) in which wild-type plants show no deficiency symptoms. Addition of 5 µM Cu largely rescues *spl7* phenotypes and under this condition, PAA2 protein abundance is the same as in the wild-type (Fig. 2.3, *A*). In *paa1* mutants, Cu also has limited access to PAA2 and PC but compared to *spl7* much higher and even toxic Cu levels are required for full rescue of the phenotype (Shikanai et al., 2003). Indeed we observed that the abundance of PAA2 was higher in *paa1* lines when compared to the wild-type on both regular ½ MS (0.05 µM Cu) and ½ MS with 5 µM $CuSO_4$ present (Fig. 2.5, *A*). The protein accumulation data in Fig. 2.5, *A* together with the chloroplast Cu levels reported in Appendix, Table A2 support the notion that the regulation of PAA2 is controlled by the Cu content in the chloroplast.

How does PAA2 protein regulation compare to regulation of other metal transporters of the P_{1B}-type class? Post-translational Cu dependent and independent modifications such as phosphorylation and

glutathionylation among others have been shown in mammalian cells to modify the activity of the P_{1B}-type Cu transporters ATP7A and ATP7B (Vanderwerf et al., 2001; Vanderwerf and Lutsenko, 2002; La Fontaine et al., 2010). In addition, ATP7A and ATP7B influence Cu homeostasis by being differentially sorted between the *trans* Golgi network and vesicles (La Fontaine and Mercer, 2007). Perhaps most reminiscent of PAA2 is the regulation of the *Saccharomyces cerevisiae* cadmium-exporting P_{1B}-type ATPase Pca1. This transporter is conditionally stabilized in the presence of its substrate, allowing the cell to avoid the cytotoxic effects of cadmium (Adle and Lee, 2008; Adle et al., 2009).

It is unlikely that PAA2 protein turnover represents an adaptation to toxic Cu excess because the effect is already seen at 0.5 µM $CuSO_4$ (Fig. 2.2, *A*), which is 40 times lower than the toxicity threshold (Burkhead et al., 2009). Instead, the stabilization of PAA2 occurs at very low Cu concentrations similar to those that activate the SPL7 pathway (see CCS abundance in Fig. 2.2, *A*). Therefore, we propose that the post-translational control of PAA2 contributes to optimal Cu use within the plant. When Cu becomes limiting, SPL7 up-regulates plant Cu acquisition and controls the Cu-microRNA mediated down-regulation of certain cuproproteins. In the chloroplast, CCS and CSD2 are down-regulated so that the Cu pool in this compartment might now be used in "economy mode" for only the most essential functions. In this context, we recently reported that, when Cu is resupplied to Cu-starved *Populus trichocarpa*, Cu is preferentially allocated to PC and the photosynthetic activity is quickly recovered (Ravet et al., 2011). By contrast CCS and CSD2 recovery in the stroma was delayed (Ravet et al., 2011). The stabilization of PAA2 is likely an additional regulatory mechanism, acting collectively with the SPL7-mediated responses, in order to facilitate the flow of Cu ions into the thylakoid lumen. On the other hand, the destabilization of PAA2 protein on elevated Cu may be part of a feedback mechanism that could allow the plant to quickly adjust to varying Cu statuses that are specific to the chloroplast sub-compartment. On elevated Cu more than enough PC may be active so that it is no longer a priority for cofactor delivery (Abdel-Ghany, 2009). Here, low abundance of PAA2 would retain Cu in the stroma and cytosol for use by cuproproteins in these locations.

The mechanism by which PAA2 is stabilized under Cu limiting conditions represents a future challenge in this research area. Here, three important observations that have bearing on this mechanism are presented. Our data show that the process is not mediated by SPL7 or miRNA408 and therefore involves a new Cu dependent signaling pathway. The *paa1* mutants that have less Cu in the chloroplast, show increased PAA2 accumulation (Appendix, Table A2 and Fig. 2.5, *A*). Finally, our finding that PAA2 is not stabilized under Cu limiting conditions in a *pc2* mutant brings genetic evidence for a crucial role of PC in the control of this regulation. PC2 is very abundant in the lumen and thus represents most of the luminal sink for Cu. As suggested above, a feed-back regulatory mechanism may serve to signal that Cu is not preferentially allocated to PC at higher Cu concentrations, resulting in the destabilization of PAA2. This model agrees with the observed roughly 80% reduction of Cu levels in the thylakoid lumen of *pc2* mutants compared to the wild-type (Pesaresi et al., 2009).

In summary, this study shows an exciting new regulation for a P_{1B}-type ATPase transporter and reveals another layer of Cu homeostasis control in *Arabidopsis*. Low Cu levels in the nucleus and cytosol activate the economy mode via SPL7. This in turn affects cuproproteins, increasing Cu availability for PC in the thylakoid lumen. Our work reveals that intra-organellar Cu pools can also participate in this control. More specifically, plastidial Cu regulates its own transport into the thylakoid lumen by acting on PAA2 stability, a process requiring PC, the ultimate target of Cu delivery.

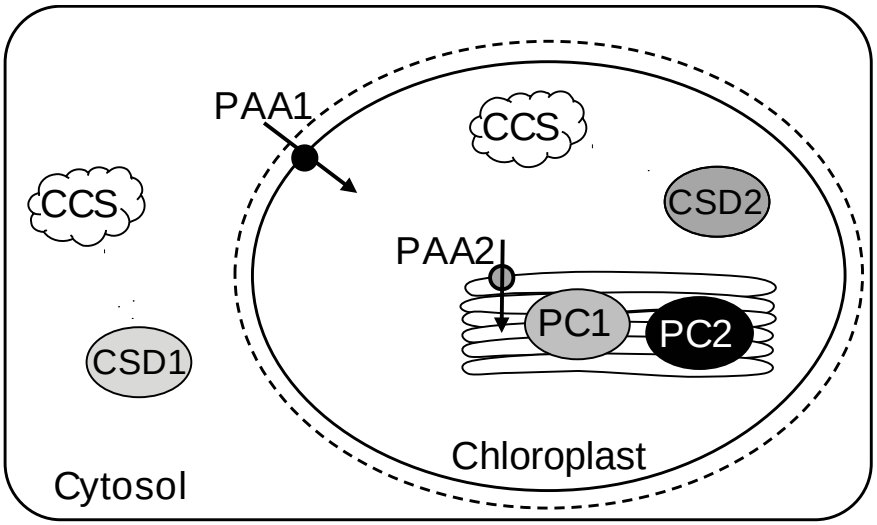

Figure 2.1: Intracellular localization of cuproproteins

The figure schematically shows the location of major cuproproteins under consideration in this study. CCS isoforms are located in the cytosol and the chloroplast stroma, where they supply Cu to CSD1 and CSD2 respectively. PC1 and PC2 are located in the thylakoid lumen. PAA1 and PAA2 transport Cu over the inner chloroplast envelope and into the thylakoid lumen respectively, as indicated by the black arrows.

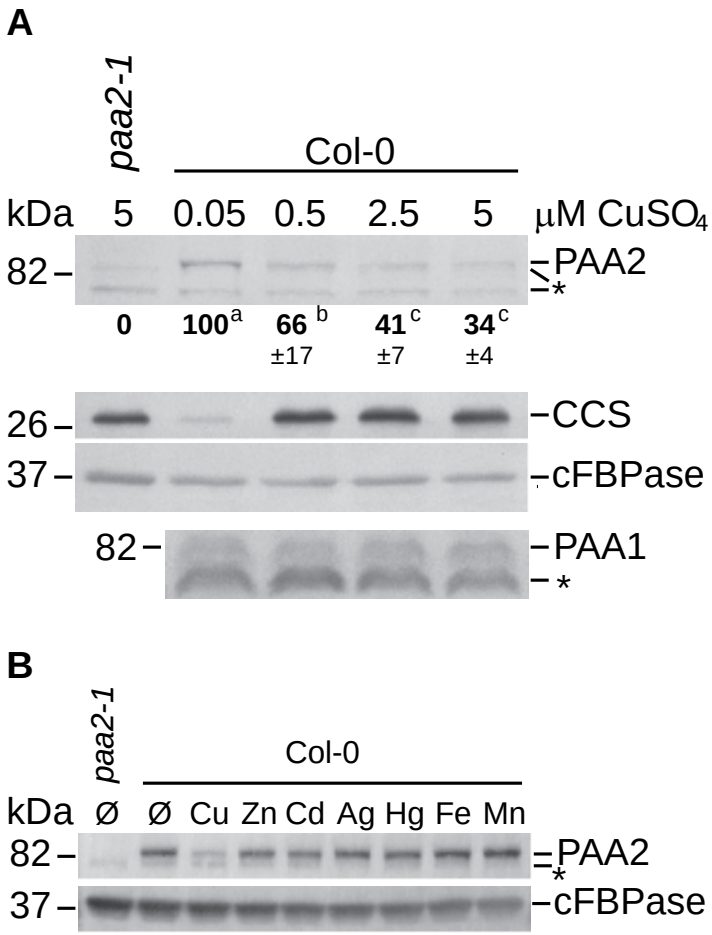

Figure 2.2: PAA2 protein abundance is modulated by Cu

A. Immunoblot analysis of PAA1 and PAA2 in rosette leaves of wild-type (Col-0) and *paa2-1* (Col-0) seedlings grown on agar media in the presence of increasing $CuSO_4$ concentrations. From here on out bold numbers below the PAA2 panels indicate relative protein abundance determined using ImageJ software as described in experimental procedures. PAA2 abundance in the wild-type grown on 0.05 µM $CuSO_4$ was arbitrarily set at 100%. Numbers in this figure represent an average of 4 replicates ± SD. Superscripts indicate statistically significant groups (Student's t-test; $P < 0.05$). In this and following figures, CCS antibody was used as an indicator for the Cu status of the plants and cFBPase as a loading control. *****indicates an unspecific band. B. Immunoblot of PAA2 and cFBPase in rosette leaves of wild-type (Col-0) plants cultured for 18 days in the presence of $CuSO_4$ (15 µM), $ZnSO_4$ (150 µM), $CdSO_4$ (20 µM), $AgNO_3$ (30 µM), $HgCl_2$ (10 µM), Fe-EDTA (100 µM) and $MnCl_2$ (300 µM) respectively. *paa2-1* seedlings grown on regular ½ MS served as a control. The result is representative of 3 separate experiments. *****indicates an unspecific band.

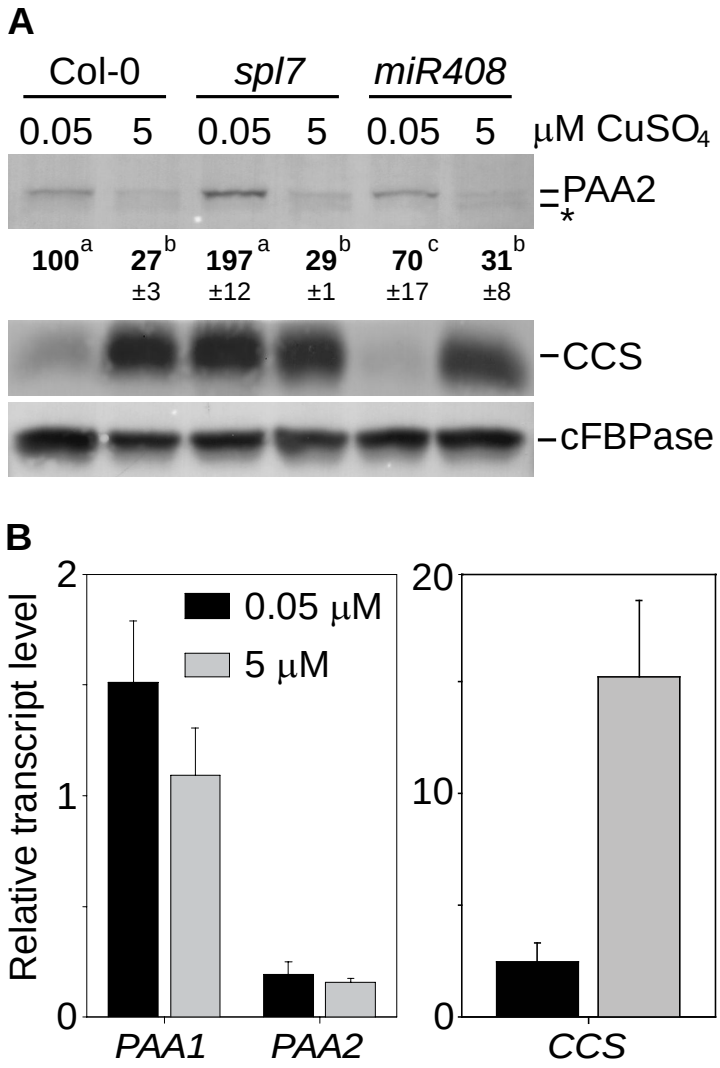

Figure 2.3: PAA2 is not regulated on a transcriptional level through SPL7

A. Immunoblot analysis of PAA2, CCS and cFBPase in rosette leaves of wild-type (Col-0), *spl7* and *miR408* knock-out lines grown on agar media. The immunoblot is representative of 3 separate experiments. Quantification of band intensities represent an average of 3 replicates ± SD. B. mRNA expression levels measured by qRT-PCR for *PAA1*, *PAA2* and *CCS* in wild-type (Col-0) rosette leaves in the presence of 0.05 μM and 5 μM $CuSO_4$. Shown are the average values of three biological replicates with two technical repeats each. Data are normalized to *YLS8* expression. Values are given as averages ± SD.

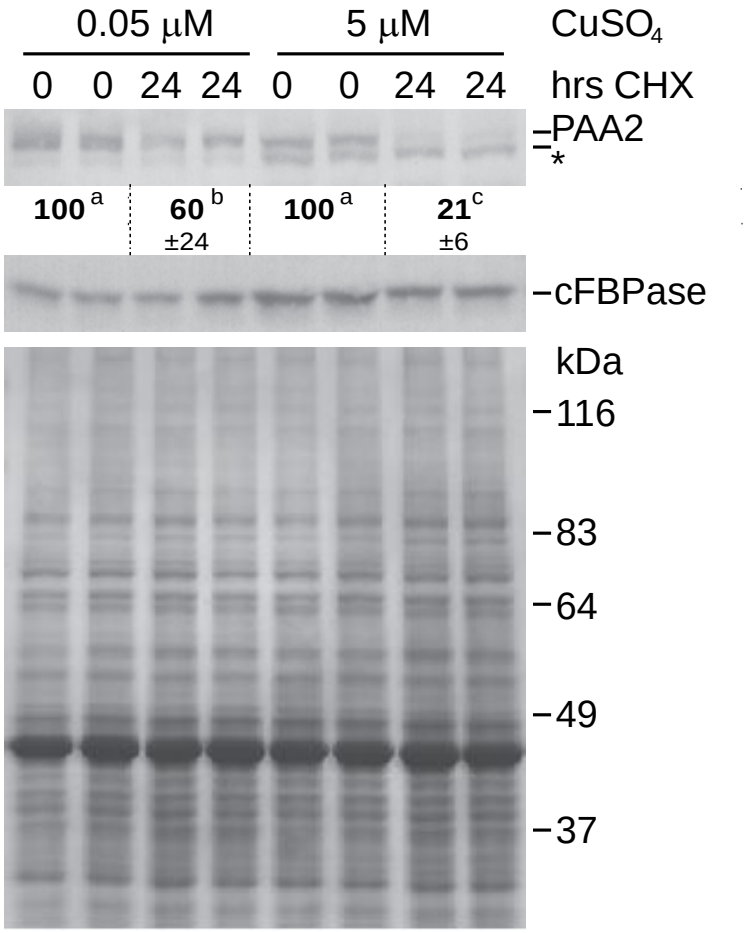

Figure 2.4: Cu affects PAA2 stability

(Top panel) Immunoblot of PAA2 in wild-type (Col-0) plants grown in liquid half-strength MS in the presence of 0.05 μM $CuSO_4$ and 5 μM $CuSO_4$. 10 day-old plants were treated with 100 μM CHX for the indicated times. Shown are two biological replicates for each time point. In order to compensate for the reduced abundance of PAA2 in the presence of 5 μM $CuSO_4$, twice the amount of protein extract was loaded in each lane for these samples. Results are representative of 4 separate biological replicates. Quantification of band intensities represent an average of 4 replicates ± SD.*****indicates an unspecific band. (Lower panel) Coomassie Brilliant Blue stained 10% SDS-PAGE with the same arrangement of samples shown in the top panel; equal amounts of protein were loaded.

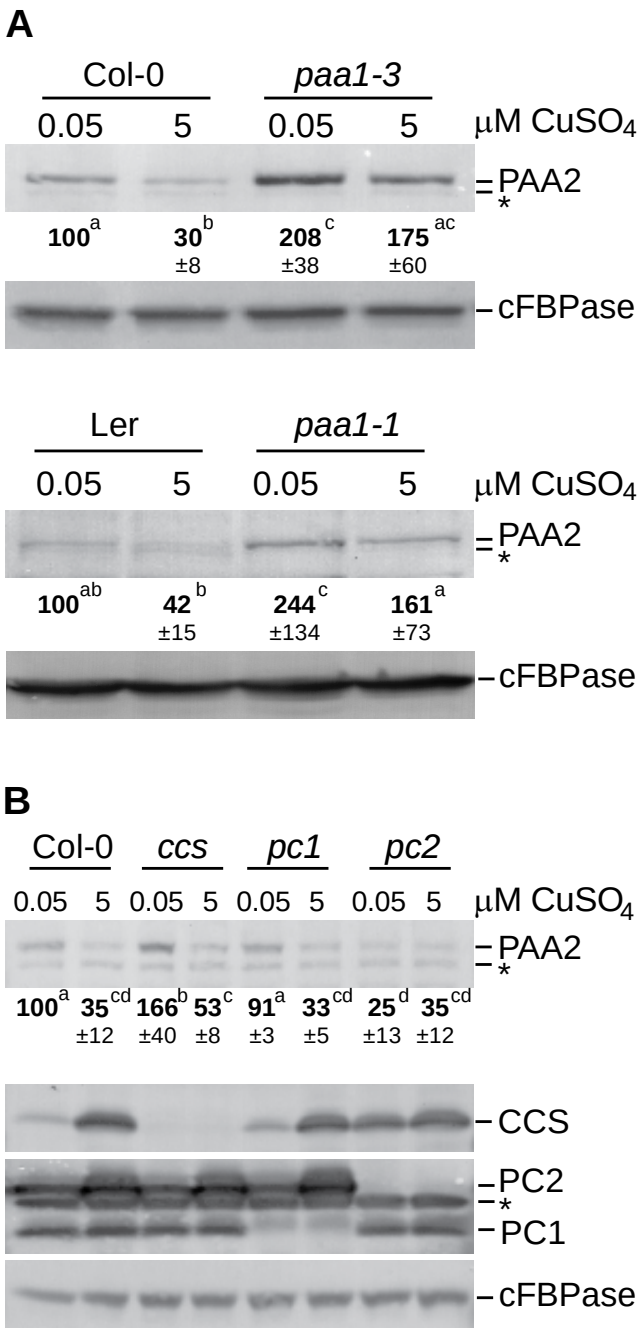

Figure 2.5: PAA2 protein abundance is affected by Cu in the chloroplast and by PC2

(A) Immunoblot analysis of PAA2 and cFBPase control in *paa1-3* (Col-0; top panel), *paa1-1* (Ler background; lower panel). In order to compensate for the reduced abundance of PAA2 in the Ler background, twice the amount of protein extract was loaded in each lane for Ler and *paa1-1* in both Cu conditions. (B) Immunoblot analysis of PAA2, CCS, PC and the cFBPase control in Col-0 wild-type, *ccs*, *pc1* and *pc2* (all Col-0 background) mutant seedlings grown on agar media in the presence of 0.05 µM and 5 µM $CuSO_4$. Results are representative of 4 separate biological replicates. Quantification of band intensities represent an average of 4 replicates ± SD. *****indicates an unspecific band.

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CHAPTER 3

The Clp Protease is Involved in PAA2 Turnover

Summary

The copper-transporting P-type ATPase PAA2/HMA6 is active in the thylakoid membrane and serves to deliver copper to plastocyanin. Cu availability to the plant affects PAA2 abundance via regulated protein turnover in *Arabidopsis*. The PAA2 protein is degraded when plants are grown on media containing high Cu. In contrast, the transporter is stabilized when chloroplast Cu levels are low. In order to identify the proteolytic components responsible for this regulation by Cu we screened a large number of mutants defective in chloroplast protease subunits. We found that mutants in ClpC1 and ClpR2 accumulated PAA2 protein on both low and high Cu, suggesting that the Clp proteolytic machinery mediates PAA2 turnover. In contrast PAA1 protein levels were not affected in these Clp mutants. The regulation of expression and activity of both cytosolic and chloroplast localized Cu/Zn superoxide dismutases was not altered in the Clp mutants, indicating that the Clp system does not affect other aspects of cellular Cu homeostasis. We propose that the Clp proteolytic system mediates PAA2 turnover in response to chloroplast Cu availability.

Introduction

Copper (Cu) is utilized by the vast majority of aerobic organisms as an enzyme cofactor. In higher plants, cuproproteins are present in most cellular compartments, such as mitochondria (cytochrome *c* oxidase), endoplasmic reticulum (ethylene receptor), apoplast (laccases), peroxisome (Cu/Zn superoxide dismutase 3 (CSD3)), chloroplast (plastocyanin and Cu/Zn superoxide dismutase 2 (CSD2)) and the cytosol (Cu/Zn superoxide dismutase 1 (CSD1)). Of the Cu-binding proteins, it is believed that plastocyanin (PC) binds the majority of Cu ions within the cell (Burkhead et al., 2009). PC is a thylakoid-located blue Cu protein that transports electrons between the cytochrome b_6f complex and photosystem I (PS I) in the z-scheme of the photosynthetic apparatus. The *Arabidopsis* genome encodes for two PC isoforms (PC1 and PC2; (Weigel et al., 2003)). The expression of PC2 is positively regulated by the presence of light (Takabe,

1986; Bichler and Herrmann, 1990). The protein abundance of PC2 is furthermore regulated by Cu abundance in *Arabidopsis* (Abdel-Ghany and Pilon, 2008). Studies in poplar (*Populus trichocarpa*) have shown that Cu is preferentially allocated to PC under limiting conditions, which was hypothesized to allow the plant to remain photosynthetically active under Cu-depleted conditions (Ravet et al., 2011). Prioritization is achieved through the concerted transcriptional down-regulation of seemingly non-essential cuproproteins. Among these are CSD1, CSD2, CCS and multiple laccases. The mechanism involves the expression of four Cu-miRNAs (Burkhead et al., 2009) by a single Cu-responsive transcription factor called SPL7 (SQUAMOSA promoter binding protein-like7; (Yamasaki et al., 2009)).

Both PC isoforms are encoded in the nucleus and are synthesized with a bipartite targeting sequence in the cytosol. They are transported as apoproteins into the thylakoid lumen by the bacterial Sec-pathway (Jarvis, 2008). Thus, Cu has to be transported into the chloroplast stroma and further into the thylakoid lumen in a separate pathway. In *Arabidopsis*, two P_{1B}-type ATPases called PAA1/HMA6 and PAA2/HMA8 fulfill this function. PAA1/HMA6 and PAA2/HMA8 are the only identified Cu-transporters in the chloroplast and are localized to the inner chloroplast envelope (PAA1/HMA6) and the thylakoid membrane (PAA2/HMA8) respectively (Shikanai et al., 2003; Abdel-Ghany et al., 2005). We have recently observed that PAA2/HMA8 is involved in the homeostatic regulation of Cu allocation in the chloroplast in an SPL7-independent manner (Tapken et al., 2012). PAA2 protein abundance is highest under Cu-limiting conditions and decreases when Cu becomes available. We hypothesized that this post-translational regulation functions in Cu sufficient conditions to aid in the redirection of Cu to cuproproteins other than PC. Interestingly, PAA1 protein abundance was not altered in response to Cu availability.

Using the protein synthesis inhibitor cycloheximide, we previously demonstrated that chloroplastic Cu affects PAA2/HMA8 stability and that the induced turnover occurs within 24 hours (Tapken et al., 2012). These observations imply the involvement of a plastid protease. We here report that PAA2/HMA8 protein is significantly increased in the two Clp protease mutants *clpc1-1* and *clpr2-1*. While the regulation of PAA2/HMA8 is conserved in the *clpc1-1* background, it is absent in *clpr2-1*. A bioinformatics analysis

showed no transcriptional regulation of the two proteases in response to Cu. Interestingly, although both protease mutants show a chlorotic phenotype, the Cu concentration within the chloroplast does not seem to be altered compared to the wild-type. We propose that ClpC1 and ClpR2 are involved in the regulation of PAA2/HMA8.

Material and Methods

Plant growth conditions and treatments Plants were grown for the indicated time periods on half-strength Murashige and Skoog (MS) medium (Murashige and Skoog, 1962); Caisson Laboratories, North Logan, UT; containing 0.05 µM $CuSO_4$), supplemented with 1% sucrose (Sigma-Aldrich, MO) and 0.6% Phytagel™ (Sigma-Aldrich, MO). For *in vitro* growth, seeds were surface sterilized according to Tapken *et al*., 2012. Plants were grown at a photon density of 120 µmol $m^{-2}s^{-1}$, in a 12-h light/12-h dark cycle at 23 °C, unless specified otherwise. Cu concentrations in the media are indicated for each experiment. Col-0 was used as a wild-type control and all mutants have been described previously: *clpc1-1* (Sjögren et al., 2004), *clpc2-1* (Park and Rodermel, 2004), *clpd* (Syngenta, reviewed in (Olinares et al., 2011), *clpr2* (Rudella et al., 2006; Kim et al., 2009), *sppA* (Wetzel et al., 2009), *var2/ftsH2* (Sakamoto et al., 2004), *prep1xprep2* (Nilsson Cederholm et al., 2009), *deg2*, *deg5*, *deg7*, *deg8* (Sun et al., 2010), *paa1-1* (Shikanai et al., 2003) and *paa2-1* (Abdel-Ghany et al., 2005).

Protein extraction, immunoblot analysis and enzyme activity assay Proteins for denaturing SDS-polyacrylamide gel analysis were extracted as described (Tapken et al., 2012). Equal loading was ensured by probing for cytosolic-1,6-bisphosphatase (cFBPase), a constitutively expressed protein which is not affected by the Cu status of the plant. The antibody for cFBPase was purchased from Agrisera (Vännäs, Sweden). CSD2 abundance is dependent on Cu availability and was used as a control for the Cu status of the plants. PAA1, PAA2 as well as the CSD2 antibodies were described before (Tapken et al., 2012; Kliebenstein et al., 1998). After fractionation on SDS-PAGE, proteins were electroblotted on a nitrocellulose membrane (Trans-Blot®Pure Nitrocellulose, 0.2 µm, Bio-Rad, Hercules, CA). For the

enzymatic activity of SODs, total leaf protein was extracted under non-denaturing conditions as described (Abdel-Ghany et al., 2005). Protein amounts were determined according to Bradford, using bovine serum albumin as a standard (Bradford, 1976). For in-gel SOD activity, 40 µg of protein per sample was loaded on a 15% non-denaturing poly-acrylamide gel. The gel was run at 4 ºC for 4.5 hours at 90 V. The staining was performed as described (Beauchamp and Fridovich, 1971). Immunoblots and in-gel SOD activity assays have been performed at a minimum of three biological replicates. Representative blots are shown.

Fluorescence analysis and biomass determination of Cu-treated plants Col-0, *clpc1-1* and *clpr2-1* were grown for 18 days on half-strength Murashige and Skoog (MS) medium (Caisson Laboratories, North Logan, UT; containing 0.05µM $CuSO_4$), supplemented with 1% sucrose (Sigma-Aldrich, MO) and 0.6% Phytagel™ (Sigma-Aldrich, MO). The Cu concentrations ranged from 0.05 µM to 30 µM $CuSO_4$. Chlorophyll fluorescence analysis was performed on a FluorCam 701MF with version 5 software (Photon Systems Instruments, Brno, Czech Republic). Plants were dark-adapted for 30 min prior to analysis. The Fv/Fm was calculated as described (Maxwell and Johnson, 2000). Plant biomass was determined by weighing freshly cut rosettes of 18 day old plants.

Quantification of band intensities and statistical analysis For quantification of the immunoblot signals, the bands were analyzed using the software ImageJ (NIH, Bethesda, MD). PAA2 abundance was normalized using cFBPase abundance as reference. Signal regression curves were obtained using a dilution series. The values given are in relation to the PAA2 signal in the Col-0 wild-type when grown in the presence of 0.05 µM Cu (half-strength MS), which was arbitrarily set to 100 % PAA2 abundance. Values represent averages and the standard deviation is given. Statistical analysis was performed by Student's *t*-test using the software JMP (Version 9.0.2; SAS Institute). Significant differences are reported where appropriate and represent values of $p < 0.05$.

Results

3.1 Bioinformatics screen for Cu-regulated chloroplastic proteases

The proposed topology of the thylakoid Cu-transporter PAA2 suggests that the protein is in contact with the chloroplast stroma and the thylakoid lumen. Therefore it is conceivable that the degradation can be initiated by a protease located in either plastid compartment. The *Arabidopsis* chloroplast contains more than 50 experimentally verified proteases, with some members having been verified as homologs of bacterial and other plant proteases (Olinares et al., 2011). In order to narrow the field of putative candidates for PAA2 degradation, I searched publicly available whole transcriptome shotgun sequencing data (RNA-Seq) from *Arabidopsis* grown in Cu deficient and sufficient conditions (Bernal et al., 2012). *Arabidopsis* was grown hydroponically in the presence of Cu for 6 weeks (Cu sufficient) or first grown in the presence of Cu for three weeks and then depleted of Cu for an additional 3 weeks before the transcriptome was analyzed (-Cu). I searched the database for the abundance of a total of 34 chloroplast-localized proteases that belong to three distinct protease families and proteases of other function: FtsH (Zn-metallo protease family), Deg (ATP-independent serine-type protease family), Clp (ATP-dependent serine-type protease family), protein processing and other proteases (Figure 3.1). None of the transcripts were altered significantly (more than two-fold) with respect to the Cu treatments and this candidate approach was therefore dismissed.

3.2 PAA2 protein abundance in different chloroplast protease mutants

In Cu sufficient conditions PAA2 abundance is about 65% lower than in Cu deficiency (Tapken et al., 2012). In this study we also showed the direct effect of Cu on the stability of the protein. We hypothesize that the PAA2 protein will be more abundant in a protease mutant that is involved in its turnover. Therefore we tested a total of 11 available and viable *Arabidopsis* protease mutants for their PAA2 abundance in low and sufficient Cu concentrations (Figure 3.3 and 3.4). None of the protease mutants showed a Cu dependent phenotype *in vitro* under these non-toxic Cu concentrations, although some of the mutants were chlorotic (Figure 3.3). The treatments affected the Cu status of all plants as indicated by

CSD2 abundance (Figure 3.4). Interestingly, in *var2* CSD2 is present in Cu-deficient conditions, possibly reflecting an increase of Cu abundance in the cytosol. PAA2/HMA8 protein abundance was comparable to the wild-type in all lines except for *clpc1-1* and *clpr2-1*. In *clpc1-1* PAA2/HMA8 abundance is significantly increased in either Cu condition, but is still relatively less abundant in the presence of Cu (Figure 3.4 and 3.5). PAA2/HMA8 abundance in the *clpr2-1* background is also significantly increased, but in addition, the regulation in Cu sufficient conditions seems to be less pronounced. I conclude that ClpC1 and ClpR2 are involved in PAA2 turnover.

3.3 The Cu status of the chloroplast is not altered in *clpc1-1* and *clpr2-1*

For the regulation of PAA2, we demonstrated that the Cu content in the chloroplast is responsible for the turnover of the protein (Tapken et al., 2012); (Chapter 2). PAA1 and PAA2 are the only known Cu transporters in the chloroplast (Shikanai et al., 2003; Abdel-Ghany et al., 2005). It is possible to envision that the loss of either ClpC1 or ClpR2 could lead to an altered PAA1 abundance, and thus I verified PAA1 abundance in the two mutants. Immunoblot analysis of PAA1 in both mutants show that the protein abundance is not affected (Figure 3.7).

A decrease in Cu abundance in the chloroplast of the two mutants could explain the increase in PAA2 abundance in the Clp lines. Both CSD1 and CSD2 mRNA levels are controlled by SPL7 via miRNA398, which responds to Cu availability in the cytosol. Furthermore, CSD2 protein activity is affected by plastid Cu levels (Abdel-Ghany and Pilon, 2008). In order to test if the Cu concentration in the cytoplasm and chloroplasts of *clpc1-1* and *clpr2-1* mutants is altered, we determined the activity of CSD1 and CSD2 in these lines (Figure 3.6). We expected a positive correlation between the activity of CSD2 and the Cu abundance in the chloroplast, because CSD2 activity is dependent on Cu availability in the organelle. However, the activity of the CSDs in *clpc1-1* and *clpr2-1* are comparable to the wild-type. Therefore the increase in PAA2 protein in the two mutants is a direct result of the loss of Clp function and not due to low Cu concentrations.

Discussion

PAA2 is a Cu-transporting P_{1B}-type ATPase located in the thylakoid membrane of chloroplasts. We have previously shown that the transporter is negatively regulated by the presence of its substrate and thus likely participates in the homeostatic regulation of Cu in the cell (Tapken et al., 2012); (Chapter 2). We observed that the turnover of the protein occurs within 24 hours upon Cu exposure. Protein turnover and processing in the chloroplast is mediated by multiple classes of proteases. We now analyzed the involvement of chloroplast-localized proteases, stromal as well as luminal, in the turnover of PAA2. Of the 11 tested viable protease mutant lines, only *clpc1-1* and *clpr2-1* showed a significant increase of PAA2 protein accumulation. ClpC1 and ClpR2 are nuclear encoded proteins. They are subunits of the Clp protease, which is located in the chloroplast stroma (Figure 3.2).

The Clp protease system is of bacterial origin and has been extensively studied in *Escherichia coli* and *Bacillus subtilis* (Olinares et al., 2011). The Clp subunits ClpP and ClpR form a barrel-shaped oligomeric protein complex in which the proteolytic site is occluded within the center. Proteins enter the complex through the axial pores, are processed and 7-8 amino acid long peptides then diffuse out (Choi and Licht, 2005). The central proteolytic core consists of two types of proteins; the catalytically active ClpPs (ClpP1-5) and non-catalytic ClpRs (ClpR1-4) (Clarke, 2012). The opening of the Clp core is thought to be too narrow for larger proteins to enter unaided, so that only small proteins and linear peptides are processed (Wang et al., 1997; Thompson et al., 1994; Grimaud et al., 1998; Weber-Ban et al., 1999). Larger proteins therefore need to first be recognized for degradation, unfolded and actively threaded through the axial pores. In *Arabidopsis* plastids, this process is dependent on three putative ATP-dependent Clp chaperones ClpC1, ClpC2 and ClpD (Clarke, 2012). How Clp chaperones recognize their substrate is still poorly understood in plants (Olinares et al., 2011). An example of a ClpC1 target in plants is the membrane-bound chlorophyllide *a* oxygenase, which converts chlorophyll *a* to chlorophyll *b* (Eggink et al., 2004; Nakagawara et al., 2007; Sakuraba et al., 2009). While most of the homozygous ClpP and ClpR mutants are seedling lethal or are unable to set seed, *clpc1-1* and *clpr2-1* are still viable and able to grow and reproduce on soil.

The *clpc1-1*mutant line has a T-DNA insertion in its fourth exon and is a knock-out line (Sjögren et al., 2004). The absence of ClpC1 results in smaller plants with chlorotic leaves. While chloroplast ultrastructure and number is not affected, a significant reduction in the photosynthetic parameters Fv/Fm and electron transport rate (ETR) is observed. All five ClpP paralogs as well as ClpR2 and ClpR4 are significantly increased in *clpc1-1* (Sjögren et al., 2004). Notably, PAA2 regulation in Cu sufficient conditions is still conserved in *clpc1-1*, which could be explained by the increased accumulation in ClpPs and ClpRs in this line. The *clpr2-1* mutant exhibits a stronger chlorotic phenotype than *clpc1-1* (Rudella et al., 2006). It has a T-DNA insertion 7 bp upstream of the translational start codon of the gene, resulting in a 50 % reduction of its mRNA levels (Rudella et al., 2006). *clpr2-1* has smaller chloroplasts and fewer thylakoids than the wild-type. Plants are viable on soil, but they are developmentally delayed. The total thylakoid proteome is reduced about 2-fold in young plants. Especially proteins of the oxygen evolving complex are affected. Furthermore, several plastid enzymes and envelope transporters are upregulated, including the ATP/ADP translocator.

We aimed to determine at which level PAA2 regulation is altered in *clpc1-1* and *clpr2-1*. Analyses of publicly available RNAseq data predict that neither ClpC1 nor ClpR2 are regulated on a transcriptional level in response to Cu (Figure 3.1); (Bernal et al., 2012). Future work will have to verify this result for our specific experimental conditions by quantitative Real-Time PCR analysis. Although it is very unlikely, we cannot exclude secondary effects of the protease mutants on PAA2 expression. In the future we will quantify *paa2* transcript abundance in *clpc1-1* and *clpr2-1* as well. We expect however that Clp affects the post-translational regulation of PAA2 (Tapken et al., 2012); (Chapter 2). To test this directly, we have to grow *clpc1-1* and *clpr2-1* in liquid culture and protein synthesis will be arrested using cycloheximide. PAA2 abundance will then be followed over time. We expect to see an increased stability of PAA2 protein in the two mutant backgrounds.

PAA2 abundance in *clpc1-1* and *clpr2-1* resembles that of a low Cu status in the wild-type chloroplasts. Thus, if the Cu concentration in these lines is altered, we expect a lower Cu accumulation compared to the wild-type. We investigated if the Cu status in leaves of the Clp mutants is affected, by determining the

CSD2 protein abundance and the CSD activity in these lines (Figure 3.6 and 3.7). *csd2* transcript is targeted by miR398 in Cu-deficient conditions and CSD2 activity relies on the presence of Cu (Burkhead et al., 2009). Notably, neither the protein abundance nor activity of CSD2 seems to be affected. Thus, at this time we do not expect any differences in the Cu content in these lines. These results will be verified by directly measuring the Cu content of *clpc1-1* and *clpr2-1* leaves by inductively coupled plasma mass spectrometry (ICP-MS, at USDA Robert W. Holley Center, Cornell University). Interestingly, PAA1 protein abundance is not affected in either Clp mutant and therefore Cu transport into the chloroplast is not expected to be restricted (Figure 3.7).

A Cu-dependent conformational change of PAA2 may be the signal for Clp-mediated proteolysis. In the absence of PC or the presence of high Cu, PAA2 would be more likely in a Cu bound state, when Cu cannot be donated efficiently to the thylakoid lumen. Conformational changes in P_{1B}-type ATPases have been suggested and are largely based on the biochemical and biophysical analyses of other ion pumping P-type ATPases such as the Na, K-ATPase and Ca^{2+}ATPase Jorgensen et al., 2003; Demeis and Vianna, 1979). Like these transporters, the Cu-transporting P_{1B}-type ATPase CopA of *Archaeoglobus fulgidus* is postulated to follow the E1/E2 Albers-Post model for transport (Arguello et al., 2007). Here, transport is initiated upon Cu^{+} and ATP-binding to CopA. Conformational changes occur, following protein phosphorylation. Other conformational changes that are not primarily associated with the Cu translocation have been reported as well. In humans, parts of the heavy metal binding domain of ATP7A, a functional homolog of PAA2, can interact with its ATP-binding domain in a Cu-dependent manner (Tsivkovskii et al., 2001). The interaction is favored in the absence of Cu and the N-terminal domain is released upon Cu binding.

Clp proteases are involved in protein homeostasis in the chloroplast and play an integral role in chloroplast development. Clp has been shown to be involved in the degradation of thylakoid-located transmembrane proteins such as photosystem II (PSII) and cytochrome b_6f complex in *Chlamydomonas reinhardtti* (Majeran et al., 2000; Majeran et al., 2001). We now report the Cu-transporter PAA2 as a possible novel target of the stromal Clp protease in *Arabidopsis*. The thylakoid lumen-localized

plastocyanin is thought to be the primary recipient of Cu in deficient conditions. Cu is cytotoxic in its ionic form and plants need an efficient system to avoid Cu overloading into the thylakoids to avoid damages to the photosynthetic apparatus. Therefore, the chloroplast needs a reliable mechanism to decrease ion flow to this compartment when Cu levels increase. The conformational changes in PAA2 in a Cu-dependent manner could be a simple and direct gauge for the Cu status of the thylakoids. Reactive oxygen species could possibly function as an indirect signal upon the presence of thylakoid overloading with Cu. However, their presence might in turn lead to cellular damage, while degradation of PAA2 would prevent Cu-overloading altogether. However, we cannot exclude the involvement of other unknown cellular factors that would mediate PAA2 degradation by Clp at this point.

This ongoing project is realized in collaboration with the group of Dr. van Wijk at Cornell University, USA. Further studies will include the investigation of physiological consequences of the absence of PAA2 regulation in *clpc1-1* and *clpr2-1*. In the future, we like to gain a more detailed mechanistic inside and investigate the possible protein-protein interaction between the Clp protease and PAA2. Substrate recognition by the plant Clp system is largely unknown. The identification of common motifs and signals for protein degradation will allow the prediction of novel proteolytic targets and with that, give more insight into their functions and regulation. Here, PAA2 could be used as a thylakoid-localized model protein.

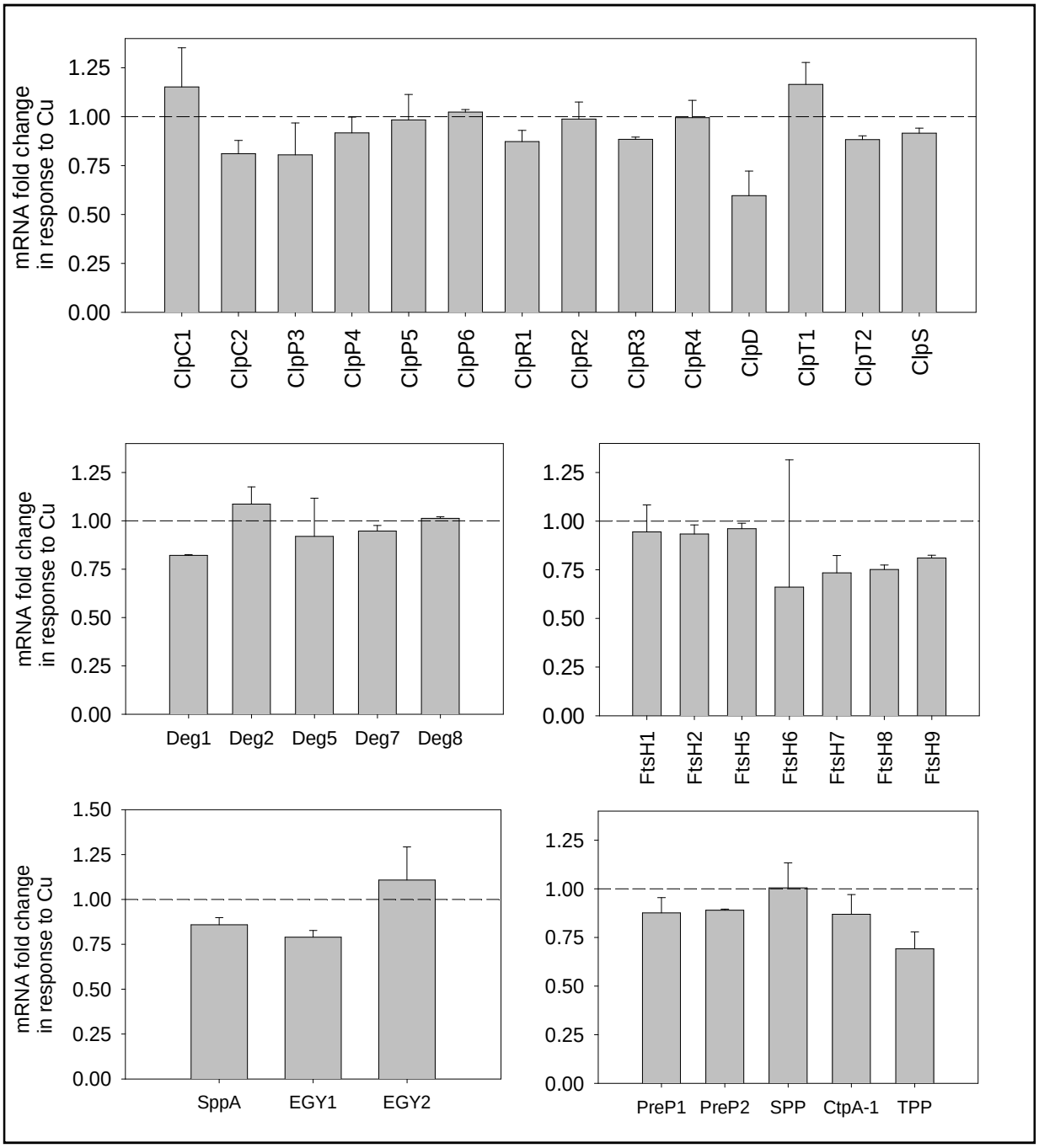

Figure 3.1: mRNA fold change of Arabidopsis grown in Cu deficient and sufficient conditions

RNA-Seq analysis of *Arabidopsis* grown hydroponically in the presence of Cu (+Cu, 0.25 µM $CuSO_4$) for 6 weeks and the absence of Cu (-Cu; 3 weeks in 0.25 µM $CuSO_4$, followed by 3 weeks without Cu addition) (Bernal et al., 2012). Represented are the ratios of the transcript abundances of the chloroplast proteases in Cu deficient and sufficient conditions. The values are averages of two biological replicates ±SD.

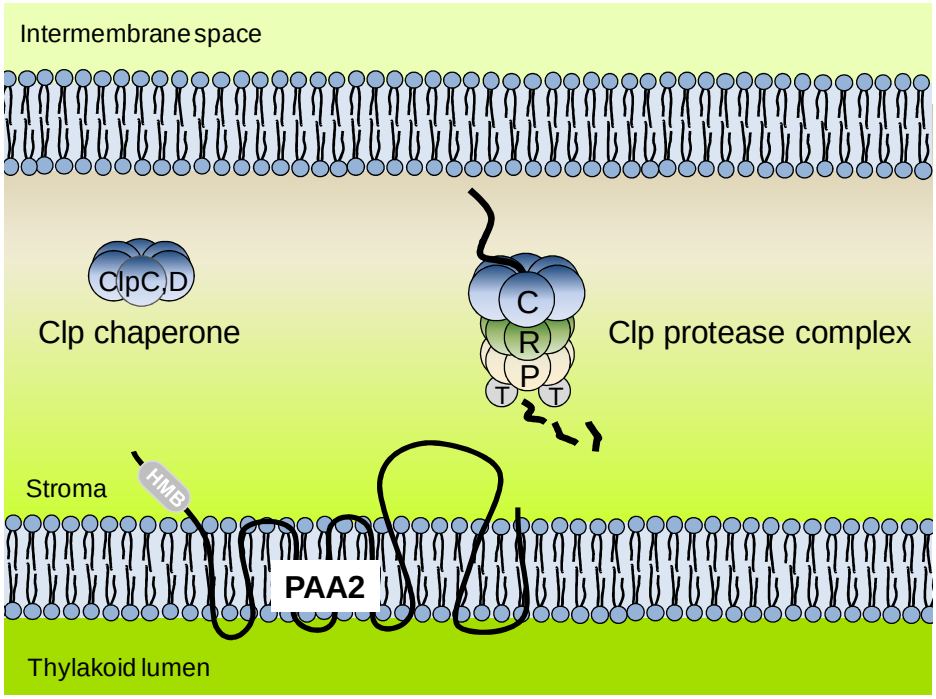

Figure 3.2: Schematic representation of chloroplast-localized Clp protease in *Arabidopsis*

Depicted is a cross section of a chloroplast showing the thylakoid lumen, stroma and intermembrane space. PAA2 is shown as a black line with its predicted structure and topology in the thylakoid membrane. The N-terminal heavy-metal binding domain (HMB) is depicted in grey. The Clp protease and its subunits are shown in the stroma. The Clp chaperones ClpC1, ClpC2 and ClpD are not always associated with the core complex. The interaction of ClpC with the proteolytic complex has been demonstrated in plants (Halperin et al., 2001). C = ClpC1,2, R = ClpR1-4, P = ClpP1-5, T = ClpT1, 2. Proteins that are being degraded are unfolded by ClpC proteins and threaded through the barrel shaped, proteolytic complex. ClpT1 and ClpT2 are specific to plant chloroplasts, but their function is still unknown.

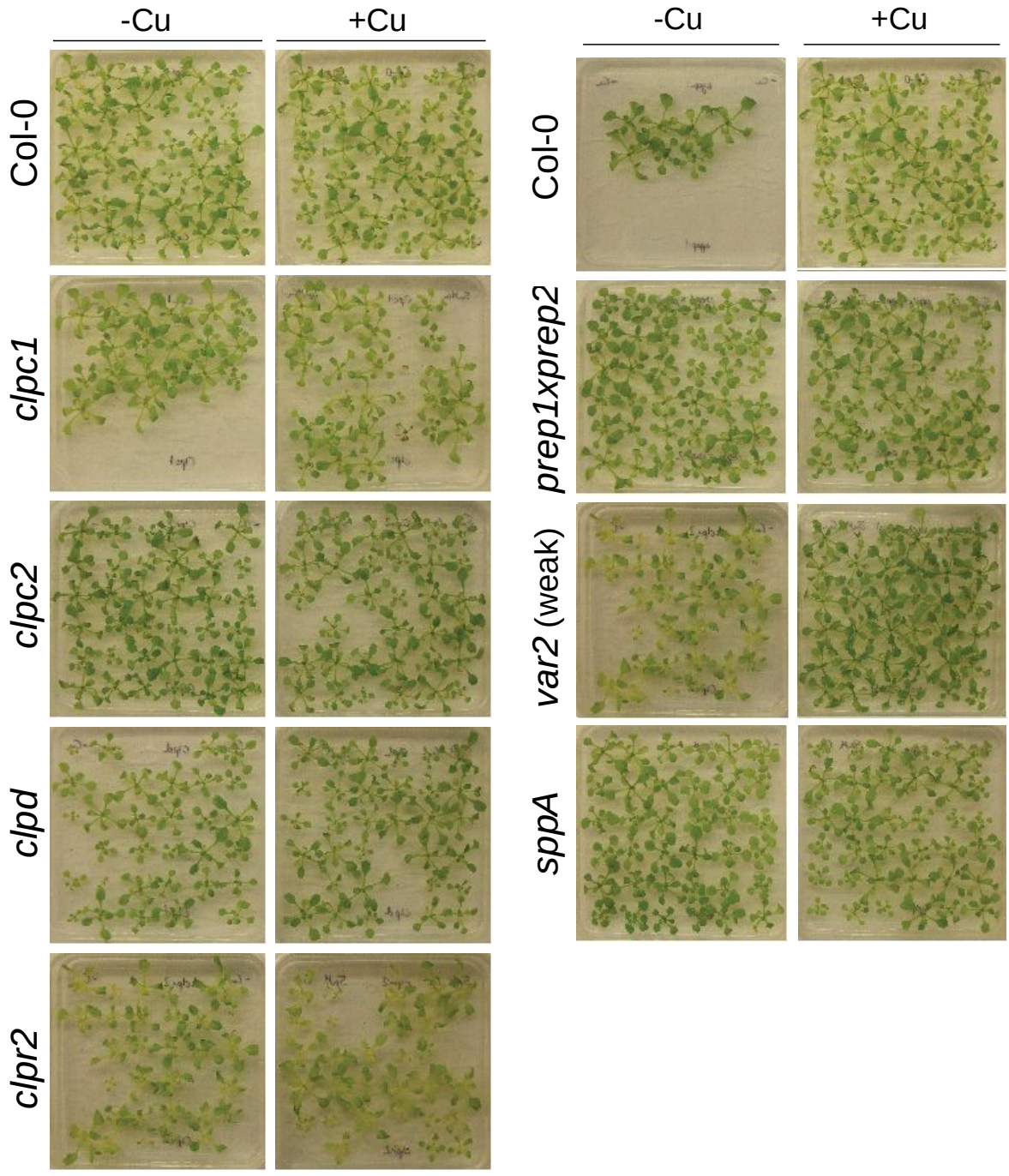

Figure 3.3: Phenotype of protease mutants on low and sufficient Cu

Pictures are representative of two biological replicates and 6 technical replicates. Plants were grown *in vitro* for 18 days before pictures and samples for immunoblot analysis were taken. Overall, most protease mutants show a clear phenotype that manifests in chlorosis. However, no differences were detected between low and sufficient Cu concentrations under these conditions.

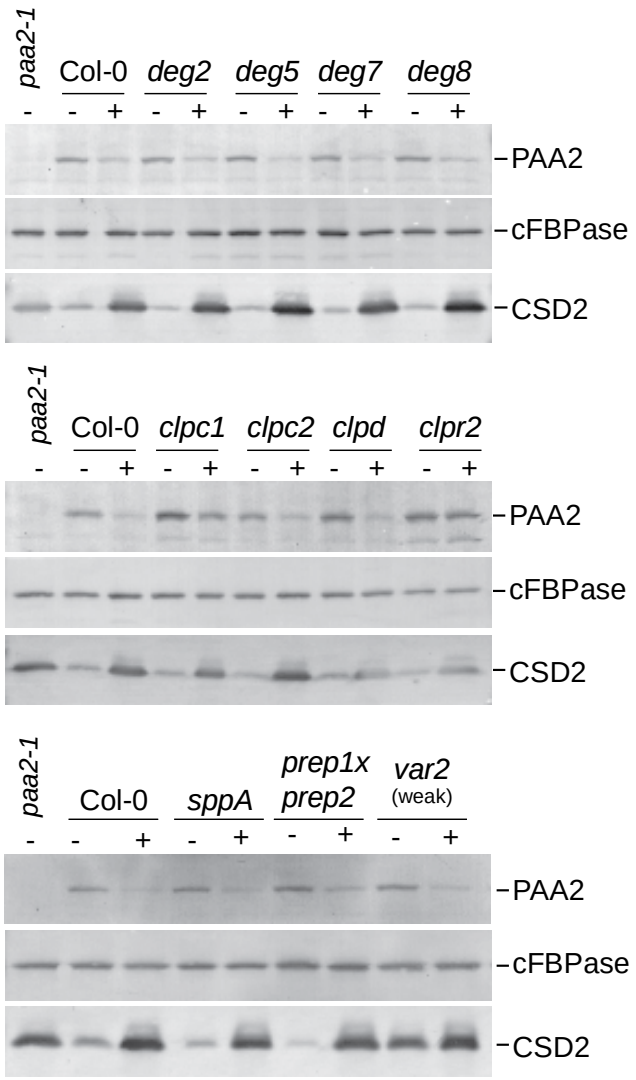

Figure 3.4: PAA2 protein abundance in different protease mutants

Immunoblot of protease mutants. cFBPase antibody was used as a loading control and CSD2 as a stromal marker for Cu abundance. Only *clpc1-1* and *clpr2-1* show a clear misregulation of PAA2 in low (-; 0.05 µM) and sufficient (+; 5 µM) Cu conditions.

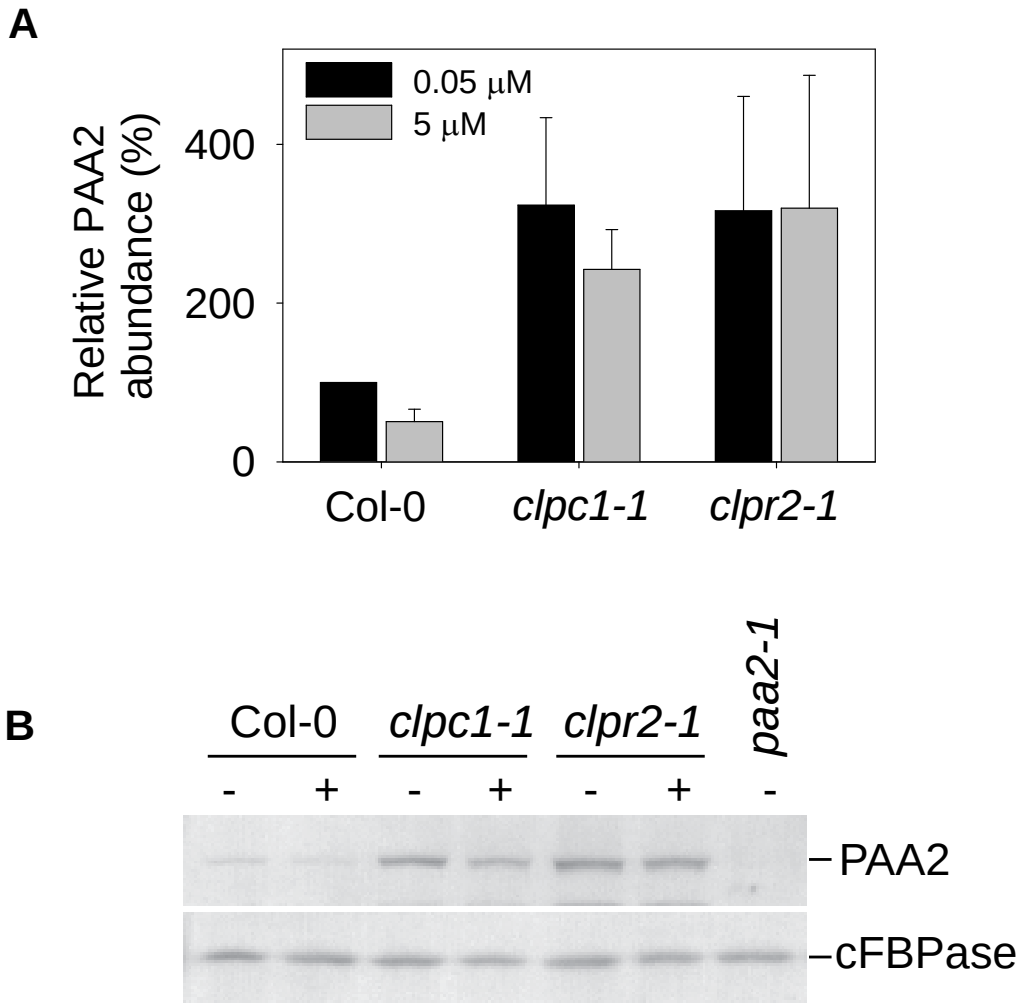

Figure 3.5: PAA2 protein abundance in *clpc1-1* and *clpr2-1*

(A) Quantification of PAA2 protein abundance in plants grown in the presence of 0.05 and 5 μM $CuSO_4$ for 18 days. Immunoblots were quantified the software ImageJ. Shown are the means ±SD of three biological replicates and at least two technical replicates using. (B) Representative immunoblot depicting the protein abundance of PAA2 in the two Clp mutants in total leaf extract.

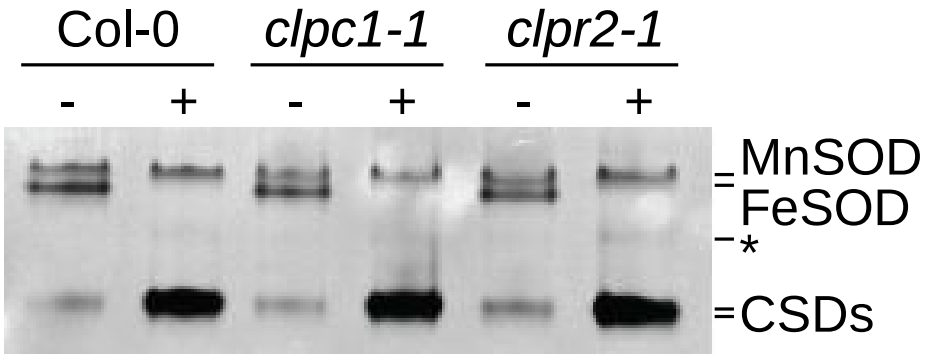

Figure 3.6: Superoxide dismutase activity in *clpc1-1* and *clpr2-1*

Shown is an inverted image of the in-gel SOD activity of Col-0, *clpc1-1* and *clpr2-1* grown in the presence of 0.05 µM (-) and 5µM (+) $CuSO_4$ for 18 days. Dark bands represent protein activity. 40 µg of protein was loaded per sample. In all genotypes, CSD activity is decreased and FeSOD increased in -Cu conditions. MnSOD is not affected by the Cu status of the plants. The activity of CSDs in the three genotype backgrounds is comparable between -Cu and +Cu.

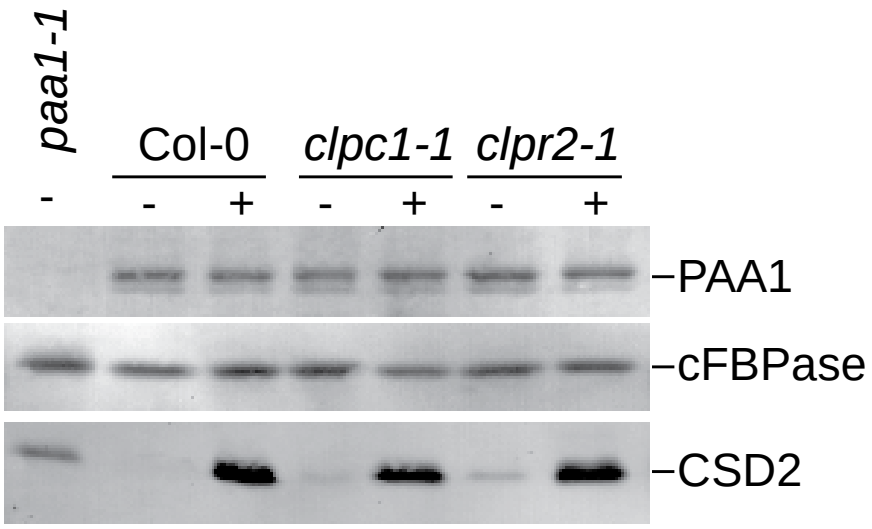

Figure 3.7: PAA1 protein abundance is not affected in *clpc1-1* and *clpr2-1*

Immunoblots of Col-0, *clpc1-1* and *clpr2-1* grown in the presence of 0.05 µM (-) and 5µM (+) $CuSO_4$ for 18 days. The Cu treatment clearly affected the Cu status of the chloroplasts in all three genotypes as indicated by CSD2 protein abundance. In contrast, neither the Cu status, nor the absence of either ClpC1 or ClpR2 changed the abundance of PAA1.

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CHAPTER 4

Further Observations and Characterization of PAA1 and PAA2

Summary

In the two previous experimental Chapters I am describing the novel regulation of the P-type ATPase PAA2 in response to chloroplastic Cu levels and plastocyanin abundance. We furthermore identified the stromal Clp protease as being responsible for the turnover of the PAA2 protein. Thus we gained valuable insight into novel mechanisms of Cu homeostasis in *Arabidopsis*. However, some questions remain that specifically concern the transport of Cu through the stroma. Central to this is the identification of the component(s) through which Cu is shuttled between PAA1 and PAA2 once it enters the chloroplast. So far no metallochaperone has been identified as a likely candidate to fulfill this function. In this chapter I am introducing the possible existence of a small PAA1 splice-form which is predicted to be a soluble, stromal protein. Furthermore, I am describing approaches that include isolated protoplasts and chloroplasts, which allow a kinetic analysis of cellular proteins in response to Cu as well as other pharmacological agents.

Introduction

In *Arabidopsis*, two Cu-transporting P_{1B}-type ATPases in the chloroplast work in tandem to deliver Cu to plastocyanin (PC), an essential cuproprotein located in the thylakoid lumen of the organelle. Remarkably, it is still unknown how Cu reaches PAA2 after it has been transported into the chloroplast, because no Cu chaperone fulfilling this function has been identified so far (Burkhead et al., 2009). We have shown that PAA2 protein abundance is negatively regulated by the presence of Cu in the chloroplast (Tapken et al., 2012). This regulation is dependent on the presence of PC2, the most abundant isoform of PC. We hypothesized that this SPL7-independent regulation aids in the intracellular redistribution of Cu and thus represents the first involvement of an organelle in Cu homeostasis. We proposed that the increase in PAA2 abundance in Cu deficient conditions facilitates the flow of Cu to PC in order to maintain photosynthetic activity of the plant. In contrast, when Cu is abundant, the decrease in PAA2 could aid in

the redistribution to other cell compartments and prevent overloading of the thylakoid lumen. I recently identified two members of the Clp family (caseinolytic proteases) as being involved in PAA2 regulation (Chapter 3). In mutants of ClpC1 and ClpR2, I detected a significant increase of the PAA2 protein in Cu deficient and sufficient conditions, compared to the wild-type. Interestingly, so far no obvious change in the Cu status was detectable in *clpc1-1* and *clpr2-1* chloroplasts, indicating that the increase in PAA2 protein is not due to a decrease in chloroplastic Cu content. These and previous observations helped me gain valuable insight into the mechanism of PAA2 regulation. However, questions about the involvement of other cellular factors, the biochemical mechanism as well as the role of PC2 in PAA2 regulation remain to be elucidated. Especially the fact that a Cu donating chaperone for PAA2 has not been identified, represents a big gap in our understanding of Cu homeostasis and PAA2 turnover. The results obtained open up new avenues regarding the PAA2 turnover mechanism and allow a better description of Cu transport and homeostasis in the chloroplast. In this chapter I addressed the following questions regarding chloroplast Cu transport: Is the predicted soluble splice form of PAA1 expressed and located to the chloroplast? How can we follow up on its possible involvement in chloroplast Cu distribution? Is the turnover of PAA2 conserved in other *Arabidopsis* ecotypes? Does an increase in PC2 abundance lead to an increased stabilization of PAA2? Does the presence of an additional Cu sink alter PAA2 abundance? Is the PAA2 turnover dependent on cytosolic factors?

Materials and Methods

Plant lines, growth conditions and genotyping Col-0 was used as the wild-type and other *Arabidopsis* ecotypes were acquired from Lehle (Lehle Seeds, Round Rock, TX). *paa1-1*, *paa2-1*, PC2-overexpressor line and the CSD2-overexpressor line have been described (Shikanai et al., 2003; Abdel-Ghany et al., 2005; Yamasaki et al., 2007; Pesaresi et al., 2009). For verification of the PC2-overexpressor line by PCR, total DNA was extracted from plants by grinding frozen leaves for 15 seconds with a small micropestle in a microcentrifuge tube. The sample was further homogenized in 500 µl shorty buffer (200 mM Tris/HCl, pH 9, 400 mM LiCl, 25 mM EDTA, 1 % sodium dodecyl sulfate (SDS)) and

then centrifuged for 5 min at 14000 rpm in a table top centrifuge at room temperature. 350 µl of the supernatant were then added to 350 µl isopropanol and thoroughly mixed. After another 10 min centrifugation step, the supernatant was discarded and the pellet washed with 350 µl 100 % ethanol. The DNA was again pelleted by another 10 min centrifugation step and the total supernatant was removed. The DNA was then dried for 30 min at room temperature. The pellet was subsequently dissolved in 90 µl water and stored at -20 ºC until the genotyping PCR reaction. For primer sequences see Table 4.1.

For *in vitro* growth, seeds were surface sterilized through three consecutive 4 min rinses in 70%, 90% and again 70% ethanol. Dried seeds were then sown on half-strength Murashige and Skoog (MS) medium ((Murashige and Skoog, 1962); Caisson Laboratories, North Logan, UT; containing 0.05 µM $CuSO_4$), supplemented with 1% sucrose (Sigma-Aldrich, MO) and 0.6% Phytagel™ (Sigma-Aldrich, MO). Cu concentrations in the medium are given for each experiment. Cuprizone was dissolved in 100% ethanol and added from a 100 mM stock solution. Seeds were then stratified for 3 days in the dark at 4 ºC to synchronize germination. Plants were grown at a photon density of 120 µmol m^{-2} s^{-1}, in a 12-h light/12-h dark cycle at 23 °C. For growth in hydroponics, plants were germinated on Cu-depleted *in vitro* medium for 10 days and then transferred to $1/10^{th}$ Hoagland solution (Hoagland and Arnon, 1938). The medium was changed every week. For growth in soil, plants were sown on once fertilized soil (Miracle-Gro®, ScottsMiracle-Gro, Marysville, OH; Fafard germination mix, Conrad Fafard, Inc., Agawam, MA) and grown in constant light. For kinetic experiments, plants were grown in liquid half-strength MS medium supplemented with 1% sucrose and the indicated Cu concentrations. Seeds were sterilized with ethanol as described above and then stratified for 3 days in the dark at 4 ºC. One sample constituted of 30 seeds in 40 ml of liquid medium in a 60 ml polystyrene jar (QORPAK, Bridgeville, PA). Plants were grown in continuous light and agitation for the indicated time periods.

Protein extraction, Immunoblot analyses and antibody pre-incubation Total leaf protein was extracted for immunoblot analysis by grinding flash frozen tissue to a fine white powder in liquid nitrogen. Leaves of plants from liquid culture were first separated from the roots and blotted dry using KimWipes®(Kimberly-Clark, Irving, TX) prior to flash freezing in liquid nitrogen and protein extraction

as described in (Tapken et al., 2012) and *Appendix*. PAA1 and PAA2 antibodies have been described before (Gogolin, PhD dissertation, (Tapken et al., 2012). The cytosolic fructose-1,6-bisphosphatase (cFBPase) was purchased from Agrisera (Vännäs, Sweden) and used as a loading control as well as a control for the purity of isolated chloroplasts. PC1, PC2, Fe superoxide dismutase 1 (FSD1) and CCS antibodies have been described (Abdel-Ghany *et al*., 2005; Kliebenstein et al., 1998; Cohu et al., 2009) et al., 2009). The antibodies for ferredoxin (FD) and Tic40 were purchased from Agrisera (Vännäs, Sweden). All antibodies were raised in rabbit. Proteins bands were visualized through the activity of alkaline phosphatase in the presence of 5-bromo-4-chloro-3'-indolyphosphate (BCIP) and nitro-blue tetrazolium (NBT) (Sigma-Aldrich, MO). Alkaline phosphatase was conjugated to the secondary antibody (anti rabbit IgG, produced in goat, Sigma-Aldrich, MO). In experiments aimed to mask specific protein bands through antigen-competition, 5 µl of the primary antibody was incubated with 5 µg of the purified antigen for 30 min at room temperature in the presence of 1x TBS (50 mM Tris/HCl, pH 7.5, 150 mM NaCl) prior to incubation with the nitrocellulose membrane.

Chloroplast isolation and Chloroplast Cu-treatment Intact chloroplasts were isolated from 8-week old soil-grown Col-0 if not specified otherwise. All chloroplast isolations were performed from isolated protoplasts as described (Tapken *et al*., 2012; see *Appendix*). Chlorophyll content was determined as described (Bruinsma, 1961). Protein content was analyzed on an SDS-polyacrylamide gel followed by immunoblotting. Proteins were loaded normalized to the chlorophyll content of the samples. For Cu-treatment of chloroplasts, isolated organelles were incubated in CIB buffer (20 mM HEPES/KOH pH 8.0, 400 mM mannitol, 5 mM EGTA, 5 mM EDTA, 10 mM $NaHCO_3$) supplemented with 1 mM dithiothreitol (DTT), 1 mM adenosine-5'-triphosphate (ATP) and 1 mM ascorbate at the indicated Cu concentrations.

RNA extraction, cDNA synthesis, quantitative Real-time PCR and PCR analysis Total RNA was extracted from frozen leaf tissue with TRIzol® reagent (Invitrogen, Carlsbad, CA). Manufacturer's instructions for all enzymes and kits were followed. 20 µg of RNA were treated with DNase l (Fermentas, Hanover, MD) to remove genomic DNA from the extract. Prior to reverse-transcription, DNase l was removed by phenol-chloroform extraction and subsequent precipitation of mRNA (overnight at -20 °C in

100% EtOH with 3 M NaOAc, pH 5.2). Total RNA concentration was determined and equal amounts per sample were reverse transcribed using the First Strand cDNA Synthesis Kit from Fermentas (Hanover, MD) and random hexamer primers from Promega (Madison, WI). The relative expression levels of the small PAA1 splice form (*spaa1*) and *paa1* were tested on Col-0 grown *in vitro* for 18 days in the presence of 2.5 µM $CuSO_4$. Primer efficiency was determined through a dilution series. The quantitative PCR results were analyzed using the Light Cycler® 480 software from Roche. Relative expression levels (REL) were calculated by normalizing to the non-Cu regulated gene YLS8 (Remans et al., 2008) as described by Arrivault and others (Arrivault et al., 2006).

Bioinformatics and protein sequence analyses Protein sequence alignments were performed using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Bioinformatics concerning sPAA1 and its predicted intracellular localization were performed utilizing the freely available database of The Arabidopsis Information Center (TAIR; http://www.arabidopsis.org/) and ARAMEMNON (http://aramemnon.botanik.uni-koeln.de/).

Results

4.1 PAA1 and PAA2 are localized to the chloroplast

The intracellular localization of PAA1 and PAA2 has been studied in some detail. Bioinformatics analysis predicts both proteins to be localized to the chloroplast (TargetP and ChloroP; Emanuelsson et al., 2000) and the transit peptide sequences of the Cu transporters have been shown to act as such *in vivo* (Shikanai et al., 2003; Abdel-Ghany et al., 2005). Furthermore, the phenotypes of both mutants are consistent with chloroplast-localized Cu-transporters. However, their native presence in leaves and chloroplasts has never been shown to this date. The affinity purification of the two antibodies now enabled me to detect PAA1 and PAA2 in isolated chloroplasts for the first time (Figure 4.1). The PAA1 protein can be easily detected with a protein amount equivalent to 4 µg of chlorophyll, while PAA2 is better detected using 8 µg in chloroplasts isolated from soil-grown plants.

4.2 The predicted small splice form of PAA1, sPAA1

Cu is very reactive and can participate in Fenton-like reactions, leading to the formation of cell destructive reactive oxygen species. Thus, in order to be able to prevent its cytotoxic effects, the vast majority of Cu ions are thought to be bound by metallochaperones and metallothioneins (Rae et al., 1999). Consequently, when Cu ions need to cross membranes, they would have to be delivered by the chaperones to their transmembrane transporters, which then actively or passively facilitate Cu passage. For instance in the archae *Archaeoglobus fulgidus*, the Cu transporting P_{1B}-type ATPase CopA receives its substrate for transport through direct protein-protein interaction with the Cu-chaperone CopZ (González-Guerrero and Argüello, 2008).

In order for PC to receive its cofactor, Cu has to cross at least two chloroplast membranes (not including the porous outer chloroplast envelope). CCS is so far the only Cu chaperone validated to be localized in the chloroplast stroma. Here, CCS interacts with CSD2 and donates the Cu for its enzymatic activity. No interactions with transporters have been reported for CCS. Furthermore, CCS mutants do not exhibit a photosynthetic phenotype, thus excluding an essential role in Cu shuttling between PAA1 and PAA2. The involvement of another Cu-binding protein called CutA, has also been excluded through mutant analyses (Burkhead et al., 2003). Therefore, it is still unknown how Cu is shuttled in the stroma.

Gogolin (a former PhD student) showed that the N-terminal domains of PAA1 and PAA2, which contain the heavy metal binding domains of the transporters, can interact in a yeast-2-hybrid assay (Gogolin, dissertation, Colorado State University). Interestingly, the Arabidopsis Information Resource (TAIR) website predicts the expression of a small splice form of PAA1, which encodes for a small protein containing the same N-terminal heavy metal binding domain and chloroplast targeting sequence as PAA1. Therefore, I hypothesized that this small protein (from now on called sPAA1), if expressed *in planta*, could act as a stroma-localized Cu chaperone with a possible involvement in Cu shuttling between PAA1 and PAA2. Here, I aim to investigate the possibility that a splice form of PAA1, sPAA1, could function as a stromal Cu chaperone. I use predictive bioinformatics software, expression analyses and immunoblot assays to set the basis for future research on sPAA1.

4.2.1 Bioinformatics analysis of sPAA1

The TAIR website predicts the existence of three *paa1* mRNA isoforms (Figure 4.2, *A*). *paa1.2* and *paa1.3* are virtually identical and only differ in their 5'UTR sequence (not shown). *paa1.1* (*spaa1*) however is 75 % shorter and does not encode for any predicted transmembrane domains (Figure 4.2, *A*). The amino acid sequence of sPAA1 is 95% identical to the N-terminal part of the full-length PAA1 and contains a variable stretch only in the last carboxy-terminal 13 amino acids (Figure 4.2, *B*). The first transmembrane domain of PAA1 starts at amino acid 251 and is thus not contained in sPAA1. sPAA1 is predicted to be localized in the chloroplast (Table 4.2). The predicted length of the transit peptide cleavage site varies with the software used and thus needs to be validated experimentally (Figure 4.2, *B* and Table 4.2). However, because PAA1 and sPAA1 are identical in their N-terminal regions, sPAA1 if expressed likely contains and uses the same chloroplast targeting sequence as PAA1. The PAA1 mutant *paa1-3* (Col-0) is the only published mutant so far in which both PAA1 and sPAA1 would be affected.

4.2.2 Expression analysis of sPAA1 in Col-0

To investigate if the predicted small splice form of PAA1 is indeed expressed, I produced cDNA from total RNA of Col-0 wild-type and aimed to detect the *spaa1* transcript in a PCR reaction with primers that were able to distinguish between *spaa1* (paa1.1) and *paa1* (paa1.3) (Figure 4.3, *A*). With the support of Dr. Karl Ravet, we were able to detect *spaa1* transcript in these samples. To verify the result, I then did a first quantitative Real-Time PCR with this *spaa1* specific primer pair and normalized the abundance of the transcripts to YLS8, a constitutively expressed, non Cu-regulated gene (Figure 4.3, *B*). Interestingly, the abundance of the short fragment seems to be higher using this more sensitive method. Most importantly, both results clearly indicate the expression of *spaa1* in Col-0. However, primer efficiencies still have to be improved and more biological replicates are required to give a quantitative statement about the relative abundances of *spaa1* and *paa1*.

4.2.3 Detection of sPAA1 using PAA1 antibodies

The N-terminal domains of sPAA1 and PAA1, including the heavy metal binding domain, are predicted to be identical (TAIR and Figure 4.2, *B*). The PAA1 antibody was raised against parts of the N-terminal domain of the protein. An alignment of the antigen with sPAA1 is shown in Figure 4.4, *A*. Their protein sequences are 95% identical. Thus it is likely that the PAA1 antibody will recognize sPAA1 in an immunoblot. The predicted size of sPAA1 with its chloroplast targeting sequence is 24 kDa (TAIR). After import into the chloroplast, the targeting sequence will be cleaved off and therefore the expected size of the mature protein is smaller. However, because the prediction software programs identify different cleavage sites, the size of the putative mature protein is unknown (Figure 4.2, *B*). With the assumption that one amino acid has an average mass of 110 Da, the expected sizes for the mature sPAA1 range from 13 - 23 kDa.

I investigated if sPAA1 is present in Col-0 and *paa1-1* using immunoblot analysis (Figure 4.4, *B*, left). Many proteins, including PAA1 were detected in the wild-type. As expected, PAA1 was not detected in *paa1-1*. One band at about 23 kDa and multiple bands between about 9 and 15 kDa are present. The mutation in *paa1-1* is downstream from *spaa1*, and thus it is possible that the small fragment is still expressed in this mutant. Because of the possibility that sPAA1 is still expressed in *paa1-1* I tried to identify the unspecific bands by removing the specific bands in the immunoblot. This was done by pre-incubating the PAA1 antibody with 5 µg of purified PAA1-HMB domain prior to incubation with the nitrocellulose membrane. While PAA1 is absent in this blot, all of the putative sPAA1 bands are still present (Figure 4.4, *B*, center). Therefore, none of the other bands is specific to PAA1. However, it is still unclear if the PAA1 antibody cannot detect sPAA1, or if sPAA1 is not present.

sPAA1 is predicted to be localized in the chloroplast. By analyzing only chloroplast-localized proteins I aimed to increase the amount of sPAA1 and to remove other unspecifically recognized proteins present in total leaf extract. Again, faint but defined bands between about 9 and 15 kDa as well as a band at 23 kDa are detected in both protoplasts and chloroplasts (Figure 4.4, *C*). However, no particular accumulation of one protein band in the chloroplast extract compared to the protoplast samples is detected.

4.2.4 Future research on sPAA1

The possibility of the identification of a novel, chloroplast-localized Cu chaperone is exciting and essential for our understanding of Cu homeostasis in *Arabidopsis*. The putative sPAA1 protein contains the same metal binding motif as PAA1 and PAA2. Thus the form of Cu bound by the protein is expected to be Cu^{+}, the substrate of the two transporters. The expression analyses clearly show that sPAA1 mRNA is expressed, but no final conclusions about the existence of the protein can be made at this time. However, in my opinion it would be valid to follow up on these preliminary but encouraging results. I propose the recombinant expression of the predicted sPAA1 fragment to test if it can indeed be identified by the existing PAA1 antibody in an immunoblot assay. The recombinant protein can then also be used to test the Cu-binding capacity of the protein *in vitro* through the methods described for CutA (Burkhead *et al*., 2003). The available PAA1 mutants in the lab as well as the recently published *gig*/*paa1* are all putatively expressing sPAA1 because their mutation is downstream of the predicted *spaa1* sequence (Shikanai *et al*., 2003; Abdel-Ghany *et al*., 2005; Lee *et al*., 2012). Furthermore, since both PAA1 isoforms are transcribed from the same gene, a knock-out of sPAA1 would likely lead to a simultaneous PAA1 knockout, creating an *spaa1xpaa1* double mutant as it would in *paa1-3*. Although this might ultimately be useful in analyzing the function of sPAA1, I propose to first create a single mutant through RNAi knock-down. Here, *spaa1* would be directly targeted through transcript degradation within the distinct region of the gene (coding sequence bases 673-714). The RNAi lines can then be analyzed for Cu-dependent phenotypes including growth in the presence of different Cu concentrations, photosynthetic activity and PAA2 accumulation. The localization of sPAA1 needs to be investigated *in vivo*, including the validation of the possible chloroplast transit peptide sequences. For this, I propose to sequence chloroplastic proteins corresponding to the predicted sizes of sPAA1 by mass spectrometry. This will not only determine whether sPAA1 is expressed, but also which chloroplast targeting cleavage site is used. An alternative would be to stably complement a mutant that lacks a stromal protein with the protein fused to the different lengths of the transit peptide sequences of sPAA1. Furthermore, if the PAA1 antibody does recognize sPAA1, cell fractionation followed by immunoblotting with the control of isolated

chloroplasts from the RNAi knock-out line, can be used to verify the localization of the protein. Lastly, to test the interaction between sPAA1 with either PAA1 or PAA2, yeast-2-hybrid studies and Split-YFP studies can be conducted.

4.3 Further characterization of PAA2 turnover

4.3.1 PAA2 turnover is conserved in 13 ecotypes

I established that the regulation of PAA2 in response to chloroplastic Cu is present in the Col-0 and Ler ecotypes (Tapken et al., 2012). It has also been determined that the lack of PAA2 in the thylakoids leads to a photosynthetic phenotype due to a lack of Cu for plastocyanin activity (Abdel-Ghany et al., 2005). However, we do not know if the regulation of PAA2 in response to Cu is essential for Cu homeostasis. Conservation of a regulatory system throughout evolution and between populations can indicate its importance to a species. Therefore, I analyzed if the regulation of PAA2 is conserved in multiple ecotype backgrounds (Figure 4.5). Indeed, in all 13 tested ecotypes, PAA2 can be detected and responds to Cu abundance in the growth medium in a comparable manner to Col-0. Photosynthetic analyses of the plants grown in the presence of different Cu amounts show no obvious correlation between PAA2 abundance and photosynthetic activity (not shown).

4.3.2 PAA2 abundance in PC2 and CSD2 overexpressor lines

4.3.2.1 PC2 overexpressor line

We have shown that in a *pc2* mutant PAA2 is not stabilized in Cu deficient conditions and hypothesized that its stabilization is dependent on the presence of the PC2 protein (Tapken et al., 2012). Thus it is conceivable that in a PC2 overexpressor line PAA2 abundance would be enhanced in Cu deficient and sufficient conditions compared to the wild-type. I obtained and confirmed the PC2 overexpressor line generated by Pesaresi and others (Pesaresi et al., 2009). As expected, PC2 protein abundance in this line is slightly higher than in the wild-type (Figure 4.6, *A*). The immunoblot shows that PC2 is more abundant in lower Cu concentrations compared to the wild-type and its maximum accumulation is higher in Cu

sufficient conditions (0.25 - 5 µM $CuSO_4$). No effect on SPL7 functions is detected, which responds to cytosolic Cu concentrations as indicated by CCS and FSD1 abundance. Furthermore, the presence of PC2 at Cu deficient concentrations (Ø and 0.05 µM) does not seem to have an additive effect on PAA2 stability. Interestingly, the presence of the Cu chelator Cuprizone (Ø) in the medium seems to have a bigger effect on PAA2 abundance then the presence of PC2. This indicates that in this PC2 overexpressor line the Cu concentration within the chloroplast is more important for PAA2 regulation than the presence of PC2.

PAA2 abundance is dependent on the presence of PC2. However, it is still unclear whether this effect is mediated by the direct interaction between the two proteins or indirect through, for instance, a feedback loop mechanism. In the latter scenario, when the Cu buffering capacity of the thylakoid lumen is exceeded, the resulting formation of reactive oxygen species could initiate PAA2 turnover. Here, the presence of PC2 is crucial for the initial buffering capacity of the thylakoid lumen (Abdel-Ghany, 2009). The absence of enhanced stabilization of PAA2 in the PC2 overexpressor line might suggest that the process of stabilization in regard to PC2 abundance could be indirect. I believe that in order to understand the involvement of PC2, we have to distinguish between the apo and holo-forms of the protein. When Cu is limiting, most of the PC2 protein is believed to be present as apoPC. It is conceivable that in this form, PC2 is able to interact with PAA2, directly receiving its cofactor from the transporter. With increasing Cu concentrations in the lumen, the amount of apoPC decreases. The lack of interaction might subsequently signal PAA2 turnover. Therefore, I propose to correlate PAA2 abundance in plants grown on a Cu range with apoPC abundance following the protocol described by Shikanai (Shikanai et al., 2003). I expect a reciprocal correlation between PAA2 and apoPC if their abundance is correlated. Furthermore, the form of PC2, apo or holo, in the PC2 overexpressor lines should also be investigated. Although the thylakoids of a PC2 overexpressor line did not show any significant increase in Cu abundance, it could still be increased in the stroma, leading to PAA2 turnover (Pesaresi et al., 2009). This would mask the actual effect of PC2 abundance in these lines.

4.3.2.2 CSD2 overexpressor line

We have shown that the Cu concentration in the chloroplast regulates PAA2 turnover. The *paa1* mutants we utilized in previous experiments alter the Cu status of the chloroplast by restricting its access to the entire organelle. Thus, we do not know which Cu pool, stromal, luminal or both, is the determining factor of PAA2 turnover. As a first step in distinguishing between the different Cu pools, I analyzed PAA2 abundance and turnover in a CSD2 overexpressor line. In these plants, Cu access to the chloroplast is not restricted, but the stroma has an increased Cu chelating capacity. If the stromal Cu pool is important for PAA2 regulation, I might detect an increase in PAA2 protein abundance in Cu deficient and sufficient conditions compared to the wild-type. The regulation of PAA2 in the CSD2 overexpressor line is comparable to the wild-type and no difference in PAA2 protein abundance can be detected (Figure 4.6, *B*). If CSD2 indeed decreased the available Cu pool in the stroma, then PAA2 turnover is not sensed in this compartment. Notably, neither the overexpression of PC2 in the thylakoids nor CSD2 in the stroma had an effect on PAA2 abundance. Therefore, in order to make a definitive statement, the validity of the system will have to be verified. Is the Cu status in chloroplasts and thylakoids of the CSD2 overexpressor line comparable to the wild-type? Can we utilize different and stronger overexpressors to affect the Cu status of the stroma and the thylakoids?

4.3.3 Inducing PAA2 turnover

All previous experiments have been conducted in plants germinated and grown in the presence of certain Cu concentrations on MS agar media. I hypothesize that if the regulation of PAA2 is important to plant Cu homeostasis, then the system should be flexible, allowing the plant to adapt to varying Cu concentrations. To test this hypothesis, I conducted a kinetic experiment in which I grew plants in the presence and absence of Cu respectively and added or removed the Cu from their growth medium when they were at the seedling stage (8 days-old). Then, I followed PAA2 abundance in these plants over an additional 6 day period. All plants that were treated longer than 3 days (starting from day 8) showed strong signs of chlorosis and were overall not healthy (not shown). Shown are representative

immunoblots of two biological replicates (Figure 4.7, *A*). The abundance of PC2 and CCS indicate the increase and decrease in Cu levels within the plants (Figure 4.7 *A* and *B*), showing that the Cu treatments were effective. After 2 days, PAA2 abundance in Cu deprived plants decreased sharply after Cu addition. Unexpectedly, similar results were obtained upon Cu starvation.

It is possible that the observed effect is one of age, rather than of treatment. Thus, future experiments have to follow PAA2 accumulation in non-treated plants to reveal a putative age effect. Notably, neither PC nor CCS abundance seems to be affected by age, but show a distinct response to the Cu treatment. I propose to start treating older plants of about 10 days of age. In these plants the amount of PAA2 should be increased because of their higher demand for Cu in photosynthesis. Furthermore, for Cu deprivation, 10 mM Cuprizone was added to the medium. This concentration is very high and might have to be adjusted. The decision to use an excess of Cuprizone in the media was to ensure that all free Cu ions would be chelated and therefore inaccessible to the plants.

4.3.4 Treating isolated chloroplasts and protoplasts with Cu

By treating isolated chloroplasts from Cu-deprived plants with Cu and following PAA2 turnover, we can test the hypothesis that a cytosolic factor or novel protein synthesis is required for PAA2 regulation. Here, I aimed to set the system for the Cu treatment of chloroplasts. Many parameters have to be considered in this experiment including temperature, light, Cu concentration and treatment time. Isolated chloroplasts are very fragile and they needed to survive the procedure intact and photosynthetically active. I isolated chloroplasts from 5 week-old plants grown in Cu-deficient concentrations *in vitro*. In a first approach isolated chloroplasts were incubated in a 21 ºC water bath in the light for up to 90 min in the absence and presence (100 nM) of Cu (Figure 4.8, *A*). All proteins detected seemed to be stable over the tested time period. However, the addition of Cu did not seem to have an effect on PC2 abundance (right). The PAA2 band was very weak and no effect of Cu can be detected during this time period. The amount of protein loaded per sample needs to be drastically increased for PAA2 detection. I propose at least protein amounts equivalent to 8 µg of chlorophyll. A second experiment in which chloroplasts of soil-grown

plants were used that we treated with different amounts of Cu was also inconclusive (Figure 4.8, *B*). It is possible that the transport of Cu needs a distinct cytosolic factor which is not present in the experiment, explaining the lack of increase in PC2 amount in Cu-treated plants. However, even in Cu-treated isolated protoplasts it is hard to detect an effect of Cu on PAA2 abundance (Figure 4.8, *C*).

Conclusions

Our understanding of the interplay between chloroplast Cu transport and cellular Cu homeostasis has made significant progress since the identification of PAA1 and PAA2. For the first time the presence and localization of native PAA1 and PAA2 has been verified *in planta*. Furthermore, we report that chloroplastic Cu has a direct effect on PAA2 stability and that stromal Clp proteases are involved in its turnover. However, some challenges in the understanding of Cu homeostasis in the chloroplast still remain to be investigated. Among the most vital is the question of how Cu transport through the stroma is mediated. Does PAA1 directly hand Cu ions to PAA2, or is a stromal Cu chelator shuttling between the two transporters? In this chapter I have explored the possible presence of a small splice form of PAA1 through bioinformatics, mRNA and protein analyses. Indeed, I observed that sPAA1 mRNA is expressed in wild-type *Arabidopsis*, thus opening up a new direction for research on this topic.

The next challenge in understanding PAA2 turnover, besides the involvement of the proteases, is to untangle the importance of the Cu in the thylakoids versus stroma as well as the involvement of PC2 in the regulation. The analysis of the PC2 and CSD2 overexpressor lines did not lead to any new definitive conclusions. More analyses, especially of their Cu content in the respective chloroplast compartments will help in understanding PAA2 abundance in these lines. Lastly, it is worth following up on the induction of PAA2 turnover in isolated chloroplasts. Although the protein amounts in the presented experiments were not enough, I observed that most chloroplastic proteins are not degraded in the conditions tested. Therefore it is possible that if Cu can reach the stroma in these chloroplasts, PAA2 turnover would be induced, proving the independence of the mechanism of any cytosolic factors.

Table 4.1: Primer sequences used to identify the small PAA1 splice-form

PCR and qRT-PCR primers			
AGI	**Gene**	**Sequence (5' to 3')**	**Sense**
AT4G33520.1	*spaa1*	GATTGGCAAAAGAGTTTAGGC	Forward
	spaa1	TAGGTTTACAATCCATTCGCC	Reverse
AT4G33520.3	*paa1*	ACGACAAAAATGTCAAACCGG	Forward
	paa1	CCCTCTCAAGACCAAGAGC	Reverse
35S:: PC2 overexpressor verification primers			
AGI	**Gene**	**Sequence (5' to 3')**	**Sense**
AT1G20340	35S	CCTTCGCAAGACCCTTCCTC	Forward
	Plastocyanin	ACATTGTGTGGGTATCCAGCG	Reverse

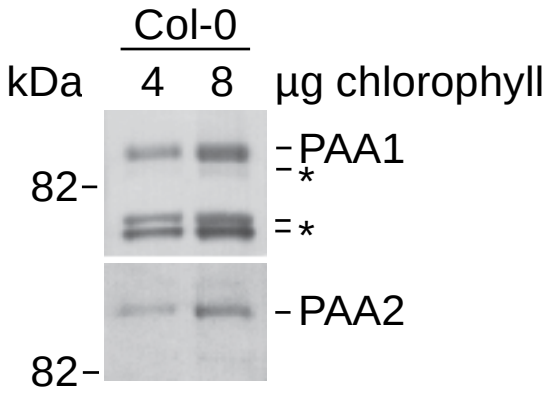

Figure 4.1: PAA1 and PAA2 detection in Col-0 chloroplasts

Immunoblot showing PAA1 and PAA2 in isolated chloroplasts. Plants were grown *in vitro* in Cu-deficient conditions for 5 weeks prior to chloroplast isolation. Chloroplast proteins were separated on a 10 % SDS-polyacrylamide gel prior to immunoblotting. *indicates unspecific bands.

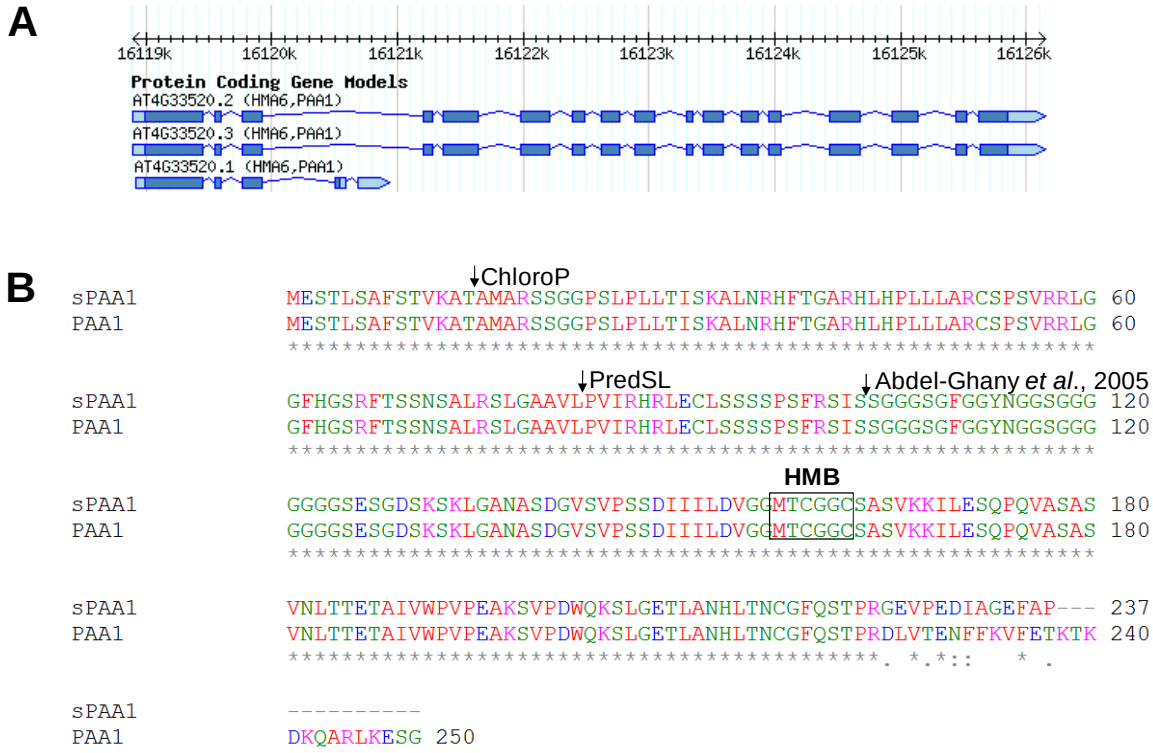

Figure 4.2: Bioinformatics analyses of the putative sPAA1 protein

The gene models for PAA1 shown in (A), were predicted by TAIR. Blue squares represent exons. The alignment between the N-terminal domains of sPAA1 and PAA1 were obtained using the software ClustalW2 (B). The arrows indicate predicted splice sites of specific programs for the chloroplast targeting sequence. Color code for amino acids: Red = small and hydrophobic, blue = acidic, magenta =basic, green = hydroxyl-, sulfhydryl- amine- group and basic, grey= others. (*) = perfect match, (:) = conserved substitution, (.) = semi-conserved substitution.

Table 4.2: Summary of bioinformatics analyses of sPAA1

Property	Result	Source
Molecular weight (with transit	24 kDa	TAIR
Length (with transit peptide)	237 amino acids	TAIR
Localization	Chloroplast	ChloroP, TargetP, PCLR, PROWLER, PredSL, SLP-local, WoLF-PSort
Length of transit peptide	14 amino acids 85 amino acids	ChloroP PredSL
Heavy metal binding domain	MTCGGC aa 157-162	TAIR, Abdel-Ghany *et al*., 2005

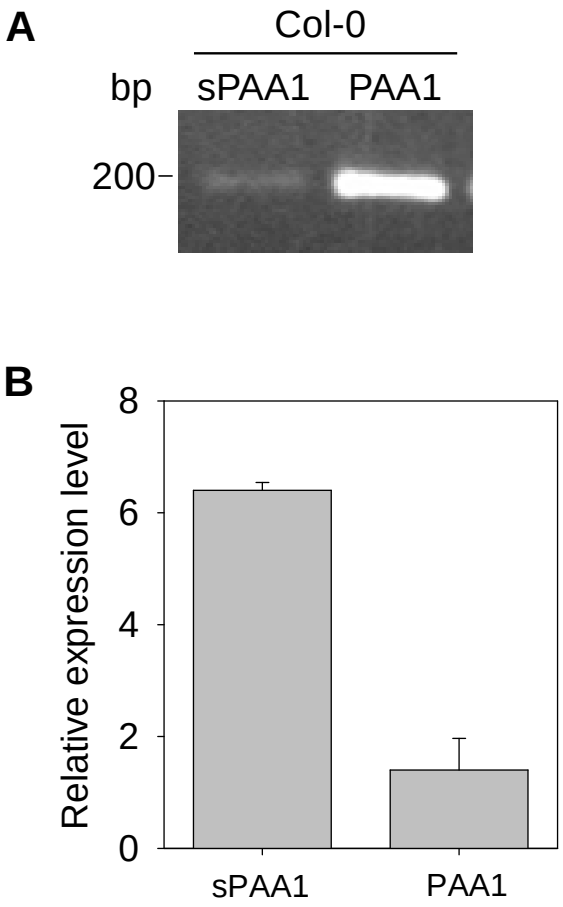

Figure 4.3: *spaa1* mRNA is expressed in Col-0

Analysis of sPAA1 and PAA1 using gene-specific primers. (A) The expression of the genes was analyzed in a regular PCR reaction using cDNA as a template and products visualized in an ethidium bromide agarose gel after 30 PCR cycles. The cDNA had been isolated from *Arabidopsis* grown *in vitro* in the presence of 5 µM $CuSO_4$. (B) Expression of sPAA1 and PAA1 from cDNA isolated from *in vitro* grown plants (2.5 µM $CuSO_4$). The relative expression level (REL) was calculated using the non-Cu regulated gene YLS8.

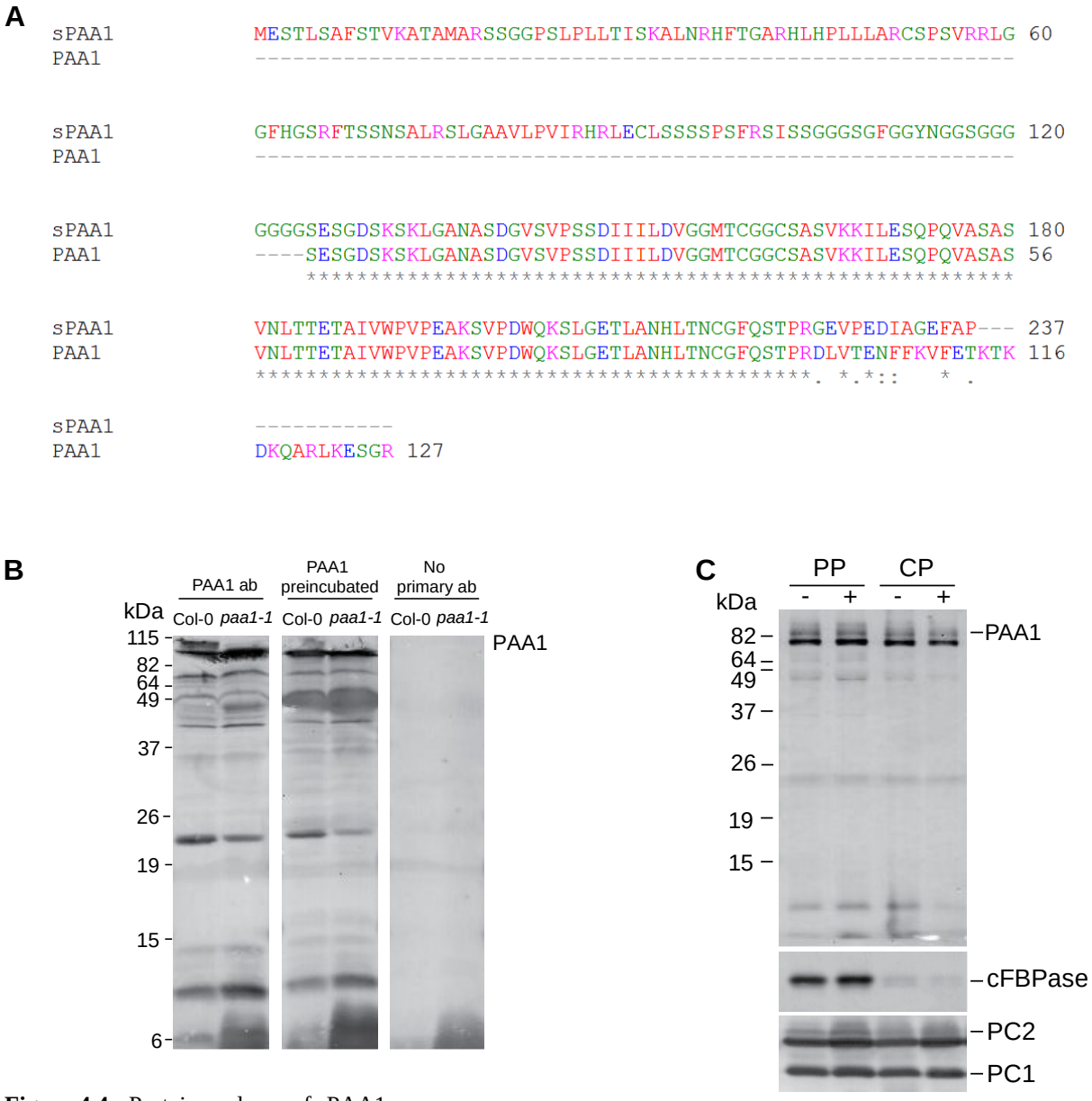

Figure 4.4: Protein analyses of sPAA1

Protein sequence alignment of sPAA1 and the antigen used to produce the PAA1 antibody (A). The alignment was obtained using ClustalW2. Color code for amino acids: Red = small and hydrophobic, blue = acidic, magenta = basic, green = hydroxyl-, sulfhydryl- amine- group and basic, grey= others. (*) = perfect match, (:) = conserved substitution, (.) = semi-conserved substitution. Immunoblot of a total leaf extract (B) and isolated protoplasts (PP) and chloroplasts (CP) (C). (B) Plants were grown in soil. 15µl (left) and 20 µl (center and right) of total leaf extract were separated on a 15 % SDS-polyacrylamide gel, immunoblotted and probed with PAA1 antibody. (C) Col-0 was grown in hydroponics in Cu sufficient (-, 50 nM $CuSO_4$) and excess (+, 5 µM $CuSO_4$) conditions for 6 weeks. Protein equivalent to 10 µg chlorophyll was loaded into each lane.

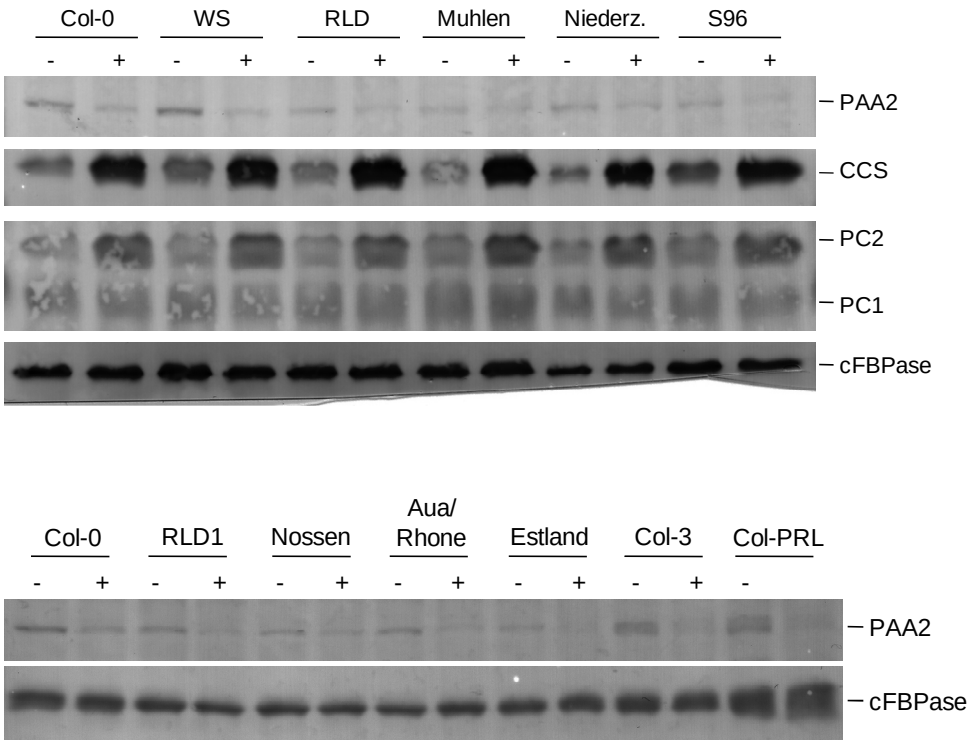

Figure 4.5: PAA2 regulation in *Arabidopsis* ecotypes

Immunoblot analysis of 13 different ecotypes grown in the presence of 0.05 µM $CuSO_4$ (-) and 5 µM $CuSO_4$ (+) for 18 days. 12 µl of total leaf protein extract was separated on a 10% SDS-polyacrylamide gel prior to immunoblotting.

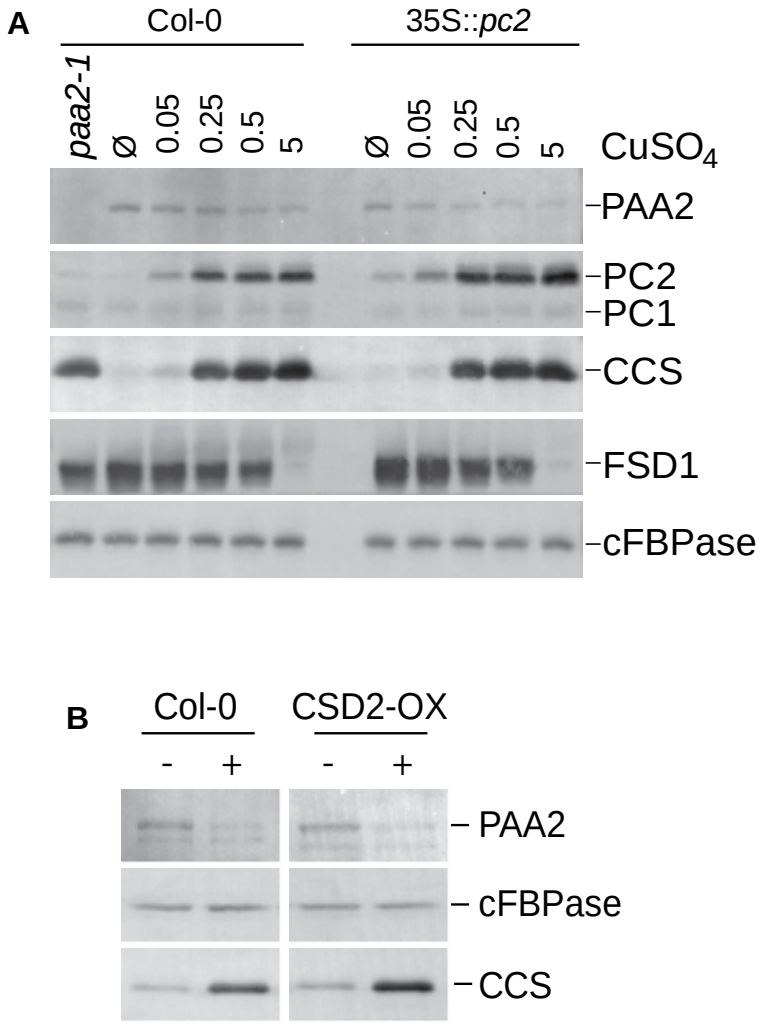

Figure 4.6: PAA2 abundance in a PC2 and a CSD2 overexpressor line

(A) Immunoblot analysis of *Arabidopsis* Col-0 and the PC2 overexpressor line, grown in the presence of different Cu concentrations. For detection of PAA2 and cFBPase 18 µl and for the other proteins 6 µl of protein extract was loaded into each lane and separated on an SDS-polyacrylamide gel.(B) PAA2 abundance was detected in a CSD2 overexpressor line grown in Cu deficient (-, 0.05 µM $CuSO_4$) and Cu sufficient (+, 5 µM $CuSO_4$) conditions. 15 µl of total leaf protein extract was separated on an SDS-polyacrylamide gel prior to immunoblotting. In both blots, proteins were separated on a 10 % (PAA2, cFBPase) and 15 % (PC1, PC2, CCS, FSD1) SDS-polyacrylamide gel.

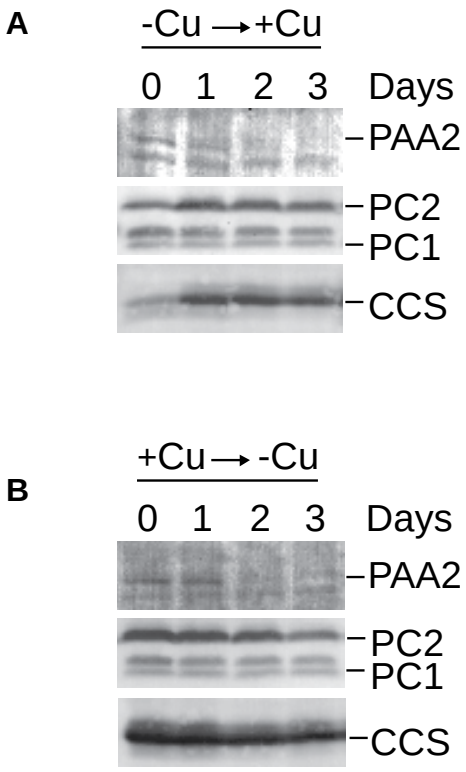

Figure 4.7: Kinetic experiment to analyze PAA2 abundance in liquid culture

(A, B) Col-0 was grown in liquid half-MS medium in the presence of 50 nM $CuSO_4$ or 500 nM CuSO4 (+) for 8 days in continuous light and agitation before switching to starvation by adding 10 mM Cuprizone and to excess by adding 10 mM $CuSO_4$. Total leaf protein was then extracted and separated on a 10 % (PAA2) and 15 % (PC2, PC1 and CCS) SDS-polyacrylamide gel before immunoblotting.

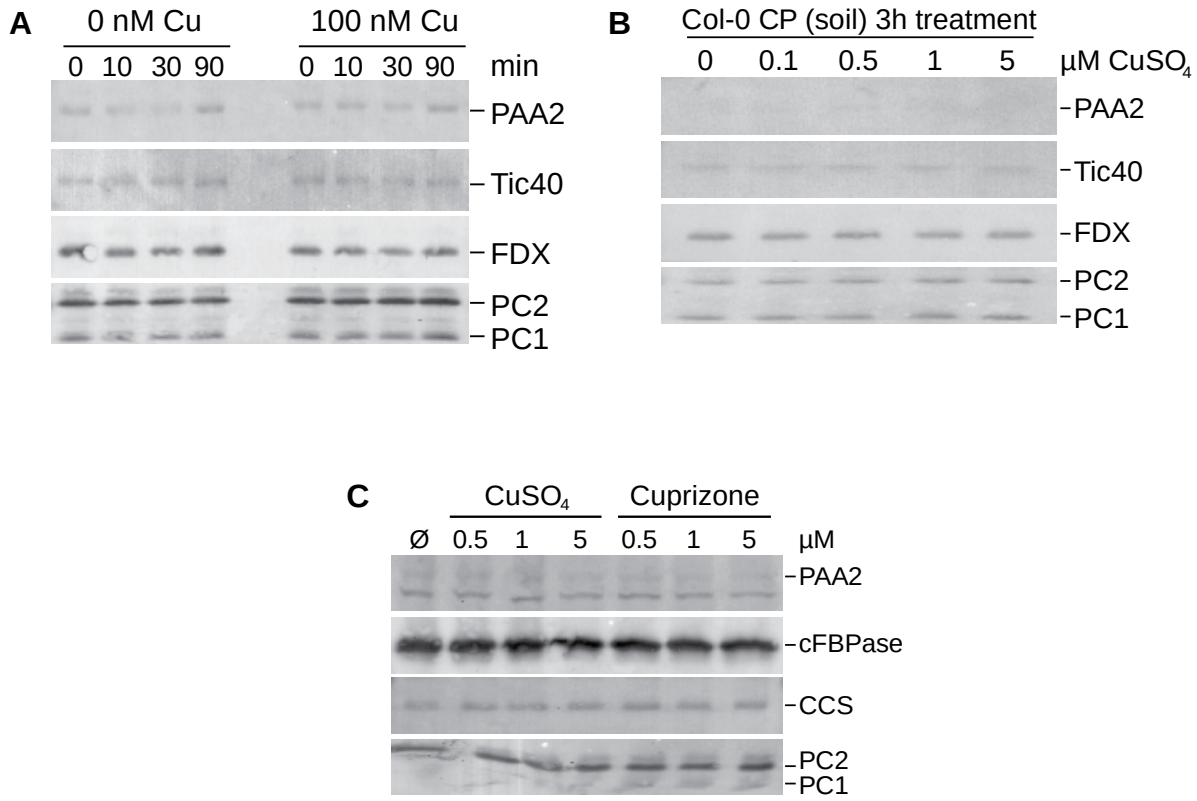

Figure 4.8: PAA2 turnover in isolated chloroplasts and protoplasts

(A) Isolated chloroplasts from Col-0 grown *in vitro* in the absence of Cu were treated for the indicated times with no added Cu and 100 nM $CuSO_4$. Protein equivalent to 4 µg of chlorophyll was loaded into each lane for detection of PAA2. For detection of the remaining proteins, Protein equivalent to 2 µg of chlorophyll was loaded. (B) Chloroplasts isolated from soil-grown Col-0 were treated for the indicated times and Cu-concentrations. Protein equivalent to 8 µg of chlorophyll was loaded into each lane for detection of all proteins. (C) Protoplasts from soil-grown Col-0 were treated with the indicated $CuSO_4$ and Cuprizone concentrations for 17 hours at room temperature in the light before protein extraction with 2x (w/v) SDS buffer. 16 µl (PAA2 and cFBPase detection) and 5 µl (CCS, PC1 and PC2 detection) were separated on an SDS-polyacrylamide gel and immunoblotted.

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CHAPTER 5

Summarizing Discussion

The cuproprotein plastocyanin (PC) is an essential electron carrier in the thylakoid lumen of *Arabidopsis* and therefore Cu is required for photoautotrophic growth. Copper is delivered to PC by two P-Type ATPases, PAA1 in the chloroplast envelope and PAA2 in the thylakoid membrane. The experiments described in this dissertation were aimed to gain more insight into the mechanisms and regulation of PAA1- and PAA2-mediated Cu transport over the chloroplast envelope and thylakoid membranes. In this chapter I summarize the findings of Chapters 2, 3 and 4 and integrate the results in a discussion in the light of recent literature.

Summary

In *Chapter two* I report an SPL7-independent regulation of a plant Cu-transporting P-type ATPase. PAA2/HMA8 (PAA2) is a thylakoid Cu transporter and its protein abundance decreases significantly in the presence of elevated Cu. The PAA2 transcript contains a putative *miR408* binding site, which we demonstrated to be non-functioning in *Arabidopsis*. Furthermore, transcript levels of *PAA2* did not vary between deficient and sufficient conditions, implying a post-transcriptional regulation. Pharmacological experiments using the protein synthesis inhibitor cycloheximide demonstrated that Cu directly affects the turnover of the transporter. PAA2 protein is stabilized in *paa1* mutants, indicating that the turnover of PAA2 protein is stimulated by chloroplastic Cu. Furthermore, plants that lack PC2, the most abundant PC isoform, accumulate only low amounts of PAA2 protein and no longer stabilize PAA2 even in Cu deficient conditions. Therefore, chloroplastic Cu and PC abundance together seem to control PAA2 protein accumulation.

In *Chapter three* I report that components of the stromal Clp protease are involved in the degradation of PAA2. Two Clp mutants *clpC1* and *clpR2* were significantly affected in PAA2 protein turnover. The mutations in these Clp components did not affect the expression and activity of cytosolic

and chloroplastic Cu/ZnSODs, both good indicators of cellular Cu and chloroplast Cu status. Therefore, the Clp machinery seems to be directly involved in PAA2 turnover.

In *Chapter four* I describe approaches that employ isolated protoplasts and chloroplasts. I confirmed the location of PAA1 and PAA2 in isolated chloroplasts directly via immunoblots. I also developed a system to allow a kinetic analysis of cellular proteins in response to Cu as well as a variety of other pharmacological agents such as methylviologen, cuprizone and cycloheximide. My results indicate that PAA2 protein levels decrease within 2 days after Cu addition to protoplasts, which were isolated from Cu-deficient plants. Chapter 4 also reports the identification of a small splice-form of PAA1 mRNA. This predicted mRNA would encode for a possible Cu chaperone within the chloroplast, however we could not definitively detect the protein encoded by the mRNA splicing variant.

5.1 A clearer picture of chloroplast Cu transport and homeostasis

The function of PAA1 and PAA2 in chloroplast Cu transport was proposed based on phenotypes of mutants and the subcellular location of GFP fusions (Shikanai et al., 2003; Abdel-Ghany et al., 2005). The location of PAA1 and PAA2 has been confirmed by proteomics studies (Ferro et al., 2010). Direct evidence for a role of the PAA1 protein in Cu transport has been provided by *in vitro* studies utilizing a bacterial expression system (Catty et al., 2011). I now detected both PAA1 and PAA2 directly in isolated chloroplasts (Chapter 4). The observation that mutations in PAA1 affect PAA2 protein accumulation in response to Cu (Chapter 2) further supports the notion that the two transporters constitute a Cu transport system in the chloroplast. Unpublished observations from our group suggested that the N-terminal domains of PAA1 and PAA2 could interact (Gogolin, Dissertation CSU). However, given that there is no metal transporting P-type ATPase described in the literature which transports Cu towards the ATP binding site and HMB domain, it is likely that the topology of full PAA1 cannot allow interaction with PAA2 via the N-terminal regions. Nevertheless, I discovered a splice variant of PAA1 which encodes for a protein that corresponds to the N-terminal Cu chaperone-like region of PAA1 and is predicted to be stromal. The mRNA for this splice form is highly expressed which suggests a significant function even if

protein has not been detected definitively. I believe that this observation opens exciting new possibilities to answer the question of how Cu reaches the thylakoids after it has been imported into the chloroplast by PAA1. In Chapter 4 I have suggested several possible experiments that could lead to the identification of the sPAA1 protein and delineate its cellular function.

5.2 The mechanism of Cu-mediated PAA2 turnover

The regulation of PAA2 by its own substrate reveals another layer of the control of plant Cu homeostasis and represents a novel mechanism. The turnover of PAA2 is different from reported mechanisms by which P_{1B}-type ATPases are regulated across species. Our results presented in Chapter 2 and 3 clearly demonstrate that the chloroplastic Cu determines PAA2 abundance and that the Clp machinery mediates PAA2 turnover. It is still unclear however, if the signal (or signals) for degradation originates from the stroma, the thylakoid lumen or even PAA2 itself. We identified PC2 as a thylakoid-localized determinant for PAA2 abundance. PC is a Cu-binding protein and this feature may be important for control of PAA2 turnover. A straight forward model can be proposed in which a conformational change of PAA2 during metal transport triggers its turnover. The more Cu is present in the stroma, the more likely it is for the transporter to be in a Cu-bound state. Likewise, if apoPC is not present to accept Cu, then PAA2 also might remain in a Cu bound state. This model is attractive because it does not invoke any additional unknown components. On the other hand, PC is an electron carrier and as such can affect the ΔpH over the thylakoid membrane, the redox state of the quinone pool or $NADPH/NADP^+$ ratios. In addition, it might be possible that apoPC is used to signal via several components that PAA2 should not be degraded by Clp. However, the electron carrying capacity of PC is also compromised in a *paa1* mutant that is defective in chloroplast Cu import (Shikanai et al., 2003) and PAA2 protein abundance in these mutants is significantly increased (Chapter 2). Similarly, in a *paa1* mutant PC is mostly present in the apo form. I therefore propose to study a *paa1*x*pc2* double mutant with respect to PAA2 turnover.

The link between metal homeostasis and Clp proteases was weak to this point. Only one report in *Arabidopsis* suggested that the Clp chaperone ClpC1 is potentially involved in Fe homeostasis in

chloroplasts, because the chlorotic phenotype of *clpc1-1* mutants was partially reversed through Fe-feeding (Wu et al., 2010). However, it was suggested that in these mutants multiple proteins, including Fe-transporters are inactive because they are misfolded (Olinares et al., 2011). Thus, the lack of functioning Fe transporters might lead to Fe deficiency in the chloroplast, explaining the reversible phenotype. PAA2 on the other hand seems to be a viable candidate for a direct Clp target. My results do not indicate a change in the Cu status of the two Clp mutants and PAA1 protein abundance is also not affected. Clp proteases have not been known to be either directly or indirectly part of Cu homeostatic regulations and thus, the possible link with PAA2 is novel and exciting. The upcoming challenges involve showing that Clp mutations affect the stability of PAA2. We can achieve this by calculating the half life of PAA2 in the absence and presence of Cu in the wild-type and the two Clp mutants. In the Appendix I present a pilot experiment that point to a possible Cu-dependent phenotype of *clpc1-1* and *clpr2-1*. If the increased protein abundance of PAA2 is present under these high Cu concentrations, we could attribute the reduction in biomass to PAA2.

The turnover of chloroplastic proteins that are unwanted or misfolded is realized by multiple chloroplast-localized proteases. Substrate recognition by plant Clp proteases is not well understood. In fact, despite the large pleiotropic phenotypic effects of most Clp mutations, PAA2 is one of the very few known direct substrates of the Clp system. The signals or motifs that determine degradation of chloroplastic proteins are largely unknown. If Cu traps PAA2 in a conformation in which Clp recognizes it as a substrate, as I propose, then this system could be explored further via structure function studies to analyze how Clp selects substrates.

5.3 The biological significance of PAA2 regulation

When Cu abundance is low, plant growth and development are in danger of being impaired and therefore plants have evolved mechanism that allow the acclimation to varying Cu availability. One response that plants have to limiting Cu is the SPL7-mediated up-regulation of genes which mediate plant Cu assimilation (Yamasaki et al., 2009; Bernal et al., 2012). In addition, SPL7 controls Cu economy via the

Cu microRNAs (Yamasaki et al., 2007; Yamasaki et al., 2009; Abdel-Ghany and Pilon, 2008; Dugas and Bartel, 2008; Cohu et al., 2009). I believe that the observed regulation of PAA2 by protein turnover is also important in this context of acclimation to low Cu. In PAA2 mutants, Cu-transport into the thylakoids is strongly decreased, which results in strongly reduced photosynthetic activity (Abdel-Ghany et al., 2005). At the same time, enough Cu is retained in the cytosol to support the presence and activity of CCS and CSD1. Thus, PAA2 is not only important for Cu transport into the thylakoids, but also affects Cu homeostasis and distribution on a cellular level. A key finding in my dissertation is that PAA2 protein abundance is dependent on the presence of Cu in the chloroplast. In Cu deficiency, PAA2 is the most abundant and decreases at the low end of Cu-sufficiency. P_{1B}-type ATPases are estimated to transport about <10 ions/second, which in comparison to other ion pumps such as sodium and potassium channels is very slow (Palmgren and Nissen, 2011; Argüello et al., 2012). In circumstances in which Cu is preferentially targeted to PC, the higher PAA2 abundance could compensate for the slow transport rate and facilitate Cu-loading into the thylakoids. If PAA2 abundance indeed affects the Cu status of the entire cell, then its down-regulation in Cu sufficient conditions might aid in the redistribution of Cu for other functions as well as prevent excessive Cu loading into the thylakoids, which could potentially lead to functional and structural damage of the photosynthetic apparatus. Therefore Clp-mediated, Cu-dependent PAA2 regulation fine tunes cellular Cu homeostasis, separately of SPL7 dependent mechanisms.

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APPENDIX I

Supplementary Figures and Tables of Chapter 2

Table A.1: Primers used for cloning of *PAA1* and *PAA2*

AGI	Gene	Sequence (5' to 3')	Sense
At4g33520	*PAA1 Nco*l	catgccatgGAGAGTGGTGATTCCAAGTCAAAACTGGG	Forward
	*PAA1 Bam*Hl	cgcggatccCTCGCGGCCACTCTCTTTTAAGCG	Reverse
At5g21930	*PAA2 Nco*l	catgccatgGAATCTTCAATCGAATCTGTGAAATCCATT	Forward
	*PAA2 Bam*Hl	cgcggatccACGGTTCCTGCTCTTAACAAGCAA	Reverse

qRT-PCR primers

AGI	Gene	Sequence (5' to 3')	Sense
At5g08290	Mitosis protein *YLS8*	TTACTGTTTCGGTTGTTCTCCATTT	Forward
		CACTGAATCATGTTCGAAGCAAGT	Reverse
At2g28390	*SAND* family	AACTCTATGCAGCATTTGATCCACT	Forward
		TGATTGCATATCTTTATCGCCATC	Reverse
At5g21930	*PAA2*	AATCTAGCGTGGGCAATTGCG	Forward
		CTCCAAAATAGATGTGGAGCCG	Reverse
At4g33520	*PAA1*	ACGACAAAAATGTCAAACCGG	Forward
		CCCTCTCAAGACCAAGAGC	Reverse
At1g12520	*CCS*	AGATCAAACTGGCACAGAGCC	Forward
		GCTCTCCCGATAAGGTCTGC	Reverse

miRNA Stem-loop qRT-PCR primers

AGI	Gene	Sequence (5' to 3')	Sense
At2g47015	*miR408* RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGCCAGG	Reverse
	miR408 SL	GTGTGATGCACTGCCTCTTC	Reverse
At3g63375	*miR167* RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACT AGATC	Reverse
	*miR167*SL	TCGCGTGAAGCTGCCAGCAT	Reverse
-	miRUniversal	CCAGTGCAGGGTCCGAGG	Forward

***miR408* mutant line verification**

AGI	Gene	Sequence (5' to 3')	Sense
At2g47015	*miR408F*	AGAGGGTTAGGAATACAGTTGG	Forward
	miR408R	CATTTGTTTCACCCACTACGC	Reverse
T-DNA	LBb1.3	ATTTTGCCGATTTCGGAAC	Forward

Table A.2: Cellular Cu content

Cu content was measured for protoplasts and chloroplasts of wild-type (Col-0), *paa1-3*, and *ccs* plants grown *in vitro* for 4 weeks in the presence of 0.05 µM or 5 µM $CuSO_4$. Values for protoplasts (cells) and chloroplasts of each line and treatment are given in ng Cu/ mg chlorophyll and represent averages of three measurements ± SD. The ratio represents the fraction of cellular Cu found in the chloroplasts.

	Col-0		*paa1-3*		*ccs*	
$CuSO_4$	0.05 µM	5 µM	0.05 µM	5 µM	0.05 µM	5 µM
Protoplast	**333** ± 70	**500** ± 98	**385** ± 68	**580** ± 162	**312** ± 63	**523** ± 145
Chloroplast	**234** ± 38	**440** ± 163	**137** ± 41	**247** ± 75	**265** ± 8	**382** ± 174
Ratio	**0.7**	**0.88**	**0.36**	**0.43**	**0.85**	**0.73**

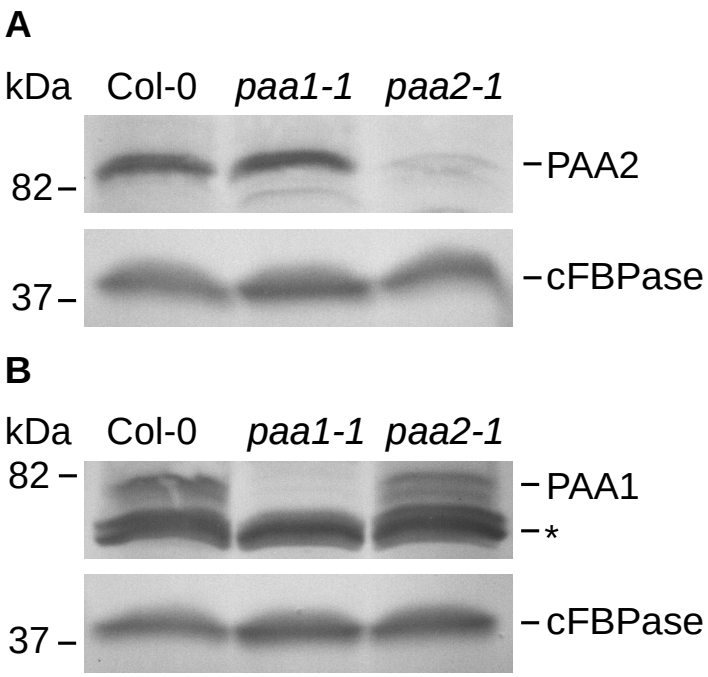

Figure A.1: PAA1 and PAA2 antibody specificity

(a,b) Immunoblot analysis of PAA2, PAA1 and cFBPase in rosette leaves of wild-type (Col-0), *paa2-1* and *paa1-1* seedlings grown *in vitro* for 18 days on agar solidified half-strength MS. *paa1-1* and *paa2-1* were used as negative controls for the respective antigens. *****indicates an unspecific band.

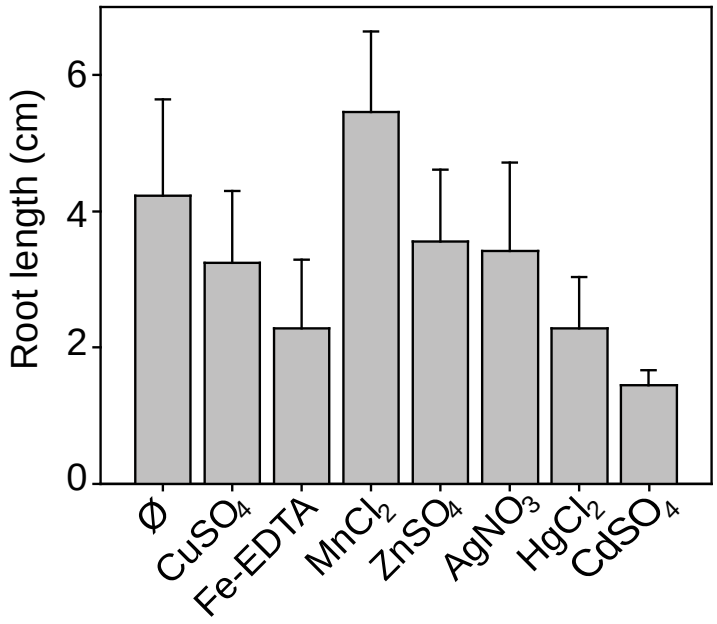

Figure A.2: Root-length of metal treated wild-type (Col-0) plants

Root length of 11 days old Col-0 seedlings grown vertically in the absence (Ø) or presence of different metals. Results represent the averages ± SD ($n \geq 13$ seedlings per treatment).

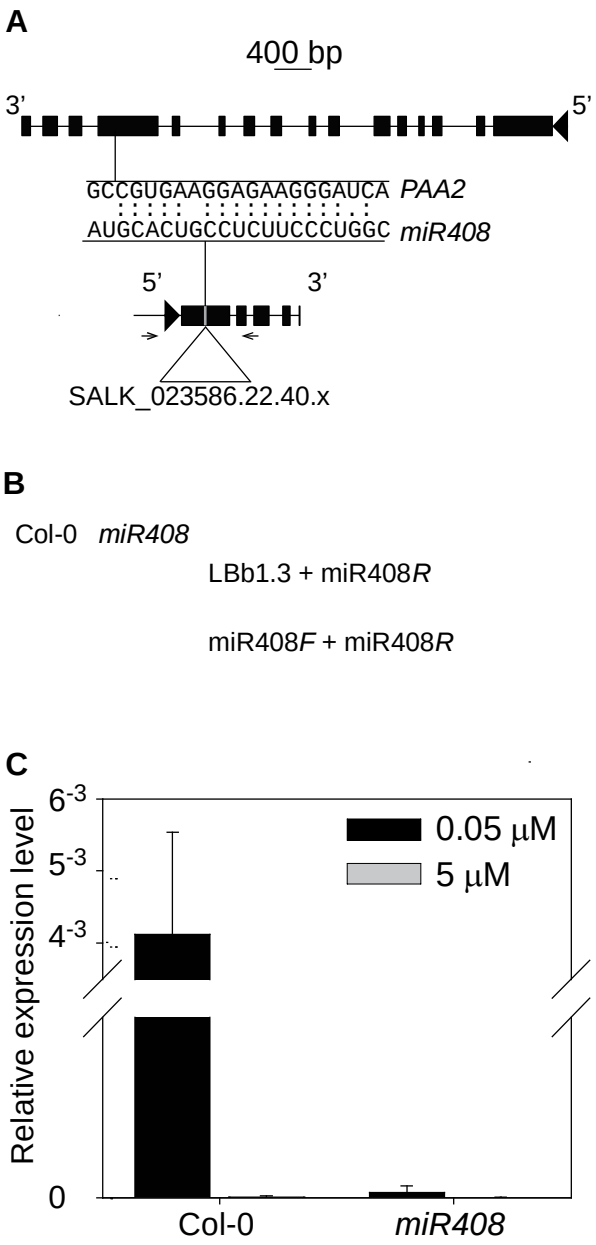

Figure A.3: *miR408* knock-out line characterization

(a) Map of the putative *miR408* targeting site within the *PAA2* coding sequence. The schematic drawing furthermore indicates the location of the T-DNA insertion within the *miR408* coding sequence. The primer binding sites for verification of the T-DNA insert are indicated. Exons are represented by black boxes and introns by black lines. (b) The presence of the T-DNA insert was analyzed by PCR using the primers listed in Table A.1. (c) Expression levels of mature *miR408* measured via stem-loop qRT-PCR in the wild-type (Col-0) and *miR408* knock out background. Plants were grown in the presence of 0.05 μM $CuSO_4$ (left, black bars) or 5 μM $CuSO_4$ (right, grey bars). Results represent the average of three biological replicates including two technical replicates each.

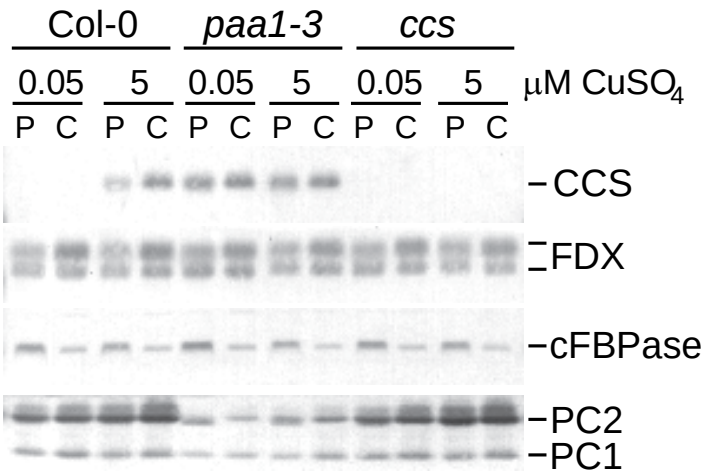

Figure A.4: Intactness of isolated protoplasts and chloroplasts

Immunoblot analysis of isolated protoplasts (P) and chloroplasts (C) from Col-0, *paa1-3* and *ccs* plants, grown in the presence of the indicated $CuSO_4$ concentrations. Protein, equivalent to 1.7 µg chlorophyll was loaded into each lane in order to be able to visualize small contaminations in each fraction. cFBPase serves as a cytosolic marker and ferredoxin (FDX) as stromal marker. Plastocyanin 2 (PC2) amounts increase with increasing Cu amounts, indicating that the Cu status of the cell was not drastically altered during the isolation process. CCS is dually located in the cytosol and stroma with a predominant presence in the chloroplastic compartment. Results are representative of two biological replicates.

APPENDIX II

Method development and preliminary observations of a Cu-dependent Clp mutant phenotype

In order to be able to detect PAA1 and PAA2 proteins in leaves, I had to develop a method to extract these transmembrane proteins in a reproducible manner. Furthermore, I aimed to measure the Cu content in isolated chloroplasts from plants grown in the presence of different Cu concentrations. Therefore I improved a small scale isolation protocol in which chloroplasts are isolated from protoplasts. This also enabled me to detect the native PAA1 and PAA2 in the chloroplasts for the first time. Here I am describing the methods in detail.

Results and Discussion

A2.1 Affinity purification of PAA1 and PAA2 antibodies

PAA1 and PAA2 antibodies had been raised in rabbit against the N-terminal domains of the respective proteins as described (Gogolin dissertation). These antigens included the amino acid residues 125-452 of PAA1 and 66-174 of PAA2. Purified and tag-cleaved proteins were available in 25 mM NaPi, pH 7.0 containing 500 mM NaCl. Affinity purification of the antibodies was performed using manufacturer's instructions (AminoLink® Coupling resin, Pierce). Before antigen coupling to the resin, 550 µl of 1M NaPi, pH 7.2 was added to 1-2 ml of purified protein. On average 3 mg of purified (1 - 2 ml) recombinant protein was bound to 3 ml resin slurry (final volume was 1.5 ml) in the presence of 50 mM cyanoborohydride in a 5 ml column. The composition of these and other solutions used are listed in Table A.3. Proteins were coupled for 30 min at room temperature under continuous slow end-over-end rocking in the fume hood, followed by further agitation over night at 4 ºC. The next day, the buffer was drained and the resin washed with 4 ml Coupling buffer. The remaining reactive sites in the resin were blocked by washing with 4 ml Quenching buffer. The resin was incubated in Quenching buffer and 50 mM cyanoborohydride for 30 min at room temperature and agitation in the fume hood. The resin was extensively washed with 5-times the resin bed volume with Wash solution prior to adding the antibody.

The resin was equilibrated using 6 ml Binding/Wash buffer. 2 ml of the final bleed of antibody production (of PAA1 and PAA2 antibody) was then diluted 1:1 in TBS and slowly pipetted on top of the resin bed. The flow-through was captured and again added to the resin. This was repeated five times to increase binding of the antibody to the antigen. After the fifth time, the flow of the column was stopped and the proteins incubated for another 30 min at 4 ºC before antibody elution. The resin was washed with 12 ml Binding/Wash buffer. The antibodies were eluted by adding 8 ml of Elution buffer to the resin. Sixteen fractions of each 500 µl were collected and the pH adjusted with 54 µl of Neutralization buffer. The ratio had been tested before through titration, to reach a pH of about 7.2 in the fraction. Protein abundance in the fractions was monitored using Bradford reagent (Bradford, 1976). Fractions 3-8 (total of 3 ml) usually contained the most protein and were pooled, the rest was discarded. The purified antibodies were subsequently concentrated via ultrafiltration about 8-fold by centrigugation at 4000 x *g* in the swing-out rotor S4180 for 5 min (Amicon Ultra Centrifugal Filters, Millipore, Carrigtohill Co., Cork, Ireland; Allegra 21R centrifuge, Beckman Coulter Inc., Brea, CA). Final protein concentrations in the approx. 200 µl antibody fraction were on average 3.03 µg/µl for PAA1 and 0.91 µg/ µl for PAA2. To the final purified antibody solution sodium azide was added to give 0.02% (w/v) for preservation. About 50 µl of the antibody was stored at 4 ºC for immediate use and the remaining antibody was aliquoted into 20 µl portions and stored in the -80 ºC freezer. For immunoblot analysis, PAA1 and PAA2 affinity purified antibodies were used in a 1:1500 dilution. The detection limit of the antibodies was tested on a dilution series on recombinantly expressed protein (Figure A.5).

A2.2 PAA1 and PAA2 protein extraction from leaves and Immunoblot analysis

The general protein extraction buffer that is used for the isolation of soluble proteins, was not able to release PAA1 and PAA2 from the membranes. The general extraction buffer contains 0.2% Triton-X 100 as detergent. Triton-X 100 is non-ionic with a relatively small head group and it is likely not strong enough to dissolve all transmembrane proteins and the phospholipids and glycolipids of the chloroplast

membranes. Therefore, I decided to isolate the proteins by using a sodium dodecyl sulfate (SDS) loading buffer, which contains 10 % SDS. SDS is an ionic surfactant with an anionic sulfate group. However, SDS interferes with Bradford reagent and the extraction buffer also contained bromophenol blue. Thus, the use of Bradford reagent for protein quantification in the sample was not possible. In order to be able to load comparable samples, we normalized to the amount of tissue. Equal loading was further verified by probing for the non-Cu regulated cytosolic fructose-1,6-bisphosphatase (cFBPase).

Rosette leaves of *in vitro*-grown 18 day old plants were gently pulled from the agar, cut right above the root base and immediately frozen in liquid nitrogen. Samples were stored at -80 ºC until use. For protein extraction, mortar and pestle were pre-cooled with liquid nitrogen and the samples ground to a very fine, white powder. The weight of the powder was determined and the tissue homogenized in 3-times the volume of extraction buffer per weight of sample, using a micro pestle. Best results were obtained with 50 - 80 mg of tissue per extraction. Samples were immediately heated to 95 ºC for 5 min before removing the insoluble fractions through centrifugation at 14,000 rpm in a tabletop centrifuge at 4 ºC for 15 min. The samples were then either directly used for analysis on a 10 cm x 10 cm long 10 % SDS-PAGE or stored at -20 ºC. For long-term storage, aliquots of about 50 µl of the supernatant were frozen in separate microcentrifuge tubes to prevent multiple freezing-thawing events. A maximum of two heating/thawing cycles is advised. After freezing, the samples were again heated to 95 ºC for 5 min, followed by a quick vortex and 10 min centrifugation at 14,000 rpm at 4 ºC. For the detection of PAA1, a minimum of 10 µl and for PAA2 13 µl extract were loaded into each well. For immunoblot analysis, proteins were transferred by electroblotting to a nitrocellulose membrane at 15 V over night (Trans-Blot®Pure Nitrocellulose, 0.2 µm, Bio-Rad, Hercules, CA). Then the membrane was incubated for 1 hour in 3 % milk and TBS-T under slow agitation or at 4 ºC over night (3 % fat-free milk powder (w/v) in 1x TBS-T). Before the membrane was incubated in the primary antibody, it was cut at about the 64 kDa to avoid cross reaction to RubisCO. This results in more intense PAA1 and PAA2 bands and the antibody solution can be reused more often. Primary antibodies (PAA1 and PAA2) were used in a 1:1500 dilution in 1 % milk in TBS-T with 0.02 % azide and incubated for 2 hours at room temperature and slow agitation. Following

was a washing step with TBS-T for at least 1 hour in which the buffer was changed twice. The secondary antibody was diluted a 1:10,000 in TBS-T and incubated for 1 hour at room temperature and slow agitation (anti rabbit IgG, produced in goat, Sigma-Aldrich, MO). Before visualization of the proteins, the membrane was rinsed one last time for 1 hour in TBS-T with two buffer changes, followed by 15 min in TBS and 5 min in AP buffer. Protein bands were visualized through the activity of alkaline phosphatase, conjugated to the secondary antibody, in the presence of BCIP and NBT (Sigma-Aldrich, MO).

A2.3 Chloroplast isolation from Protoplasts

Arabidopsis was grown *in vitro* in Phytatrays™ (Sigma-Aldrich, MO) for 4-5 weeks on half-strength MS, solidified with 0.6% Phytagel™ (Murashige and Skoog, 1962); Caisson Laboratories, North Logan, UT; containing 0.05µM $CuSO_4$, Sigma-Aldrich, MO) and supplemented with 1% sucrose and more $CuSO_4$ as necessary. 6-8 plants were sowed per Phytatray™. Individual leaves were removed from the plant and then cut into 1 mm thin strips, using a razor blade. Blades were exchanged after about 6 leaves. The middle rib was avoided. Both sides of the leaves were dipped into the enzyme solution (see below). 10 ml enzyme solution was used in a 100x15 mm round petri dish. In total 10 - 12 plants were used for one chloroplast isolation experiment. If more leaves are to be digested, the volume of the enzyme solution has to be adjusted accordingly. Only fully submerged leaves are being digested. A vacuum was applied for about 10 min to enhance access of the enzymes to the leaf tissue and the leaves were subsequently digested over night in the dark. The following day, protoplasts were separated from the leaf by gentle swirling of the petri dish for about 1 min. Then, W5 solution was added in a 1:1 ratio of enzyme solution. Protoplasts were filtered through a pre-wetted (W5) 215 µm nylon mesh into a 50 ml conical tube on ice. For protoplast transfer, a 25 ml wide-opening pipet was used. All further procedures were performed in the dark and on ice. Protoplasts were checked for integrity using a microscope with 20x magnification. The protoplasts were then pelleted using the swing-out rotor S4180 (Allegra 21R centrifuge, Beckman Coulter Inc., Brea, CA) at 200*x g* for 2 min at 4 ºC. Accelaration and decellaration were set at 6 and 5

respectively for this and all following centrifugation steps. Importantly, mannitol concentrations higher than 400 mM inhibit protoplast peletting and therefore recovery. Therefore, the clear supernatant was removed by pipetting and the pelleted protoplasts washed with 10 ml Half and half solution (equal volumes of W5 salts and mannitol wash). Protoplasts were resuspended by gentle end-over-end rocking. After resuspension they were again peletted at 200 x *g* for 2 min at 4 ºC in the same rotor. The supernatant was removed and protoplasts taken up in 1 ml Half and half solution. The amount of protoplasts was determined using a hemocytometer and their concentration adjusted to 10^6 protoplasts/ ml with Half and half solution. On average the amount of isolated protoplasts per 10 ml enzyme solution was 3.11 ±1.5 mio protoplasts/ml. After pelleting, using the same settings as described above, protoplasts were taken up in 800 µl chloroplast isolation buffer (CIB) containing BSA. With a wide-mouth pipettor they were then transferred into a 10 ml syringe with a filter adapter. The adapter contains an 18 µm mesh through which the protoplasts were forcefully pressed (Component Supply Co., Fort Meade, FL). Lysed cells were collected in a pre-cooled 2 ml centrifuge tube with a round bottom (Life Science Products, Inc., Denver, CO). Lysed protoplasts were then centrifuged at 1125 x *g* for 2 min at 4 ºC. The supernatant was removed and the pellet resuspended in 400 µl CIB containing BSA. Chloroplasts were then loaded on top of a 40 % Percoll gradient (Amersham Biosciences AB, Uppsala, Sweden) and centrifuge again for 5 min at 1625 x *g*. Intact chloroplasts were recovered from the bottom of the tube by resuspension in 1 ml CIB without BSA. Chlorophyll content was determined according to Bruinsma (Bruinsma, 1961). Yields averaged 138 µg/µl ± 66 chlorophyll. All samples were adjusted to 1 µg/µl chlorophyll by a last centrifugation step at 1625 x *g* for 5 min in the swing-out rotor. The supernatant was discarded and the chloroplasts resuspended in the corresponding volume of CIB without BSA. Samples were stored at -80ºC.

A2.4 Cu detection in Protoplasts and Chloroplasts

Protoplasts and chloroplasts were isolated as described (2.3). Before protoplasts and chloroplasts were dried prior to mineralization, the microcentrifuge were washed over night in 10% HCl, then rinsed 3 times in double distilled water and dried. Protoplasts and chloroplasts equivalent to 20 - 50 µg chlorophyll were then dried over night at 70 ºC in a pre-washed centrifuge tube. After the samples were completely dry, 40 µl nitric acid was added and the samples vortexed rigorously for one minute in the fume hood. Samples were then spun down briefly to collect the entire liquid at the bottom of the tube. The digestion took place in a sand bath at 95 ºC over night. To relief pressure from inside the tube, a small whole was punched into the centrifuge cap and the caps were closed. The next day, the samples were diluted 1 in 10 with double distilled water to a final volume of 400 µl. For ion chromatography analysis, the sample was further diluted 10-fold. Samples were run at a flow rate of 1 ml/min. The analysis was performed on a Dionex ion chromatography system (Sunnyvale, CA) as described (Shikanai et al., 2003).

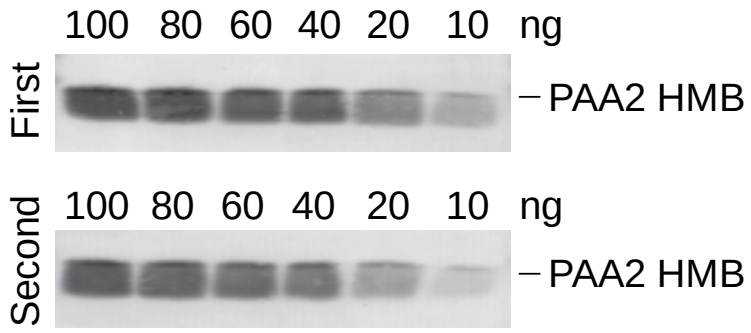

Figure A.5: Test of antibody detection level

Shown is the detection level of the PAA2 antibody after two independent antibody purifications. A dilution series of the recombinantly expressed antigen (PAA2 (N-terminus)) was probed with the purified antibodies. The antibodies were able to detect as little as 10 ng of recombinant protein.

Table A.3: Composition of solutions used

Affinity purification of antibodies	
Coupling buffer	0.1 M sodium phosphate, 0.15M NaCl, pH 7.2
Cyanoborohydride solution	5 M $NaCNBH_3$ in 1M NaOH
Quenching buffer	1 M Tris/HCl, pH 7.4
Wash solution	1 M NaCl
1x TBS	50 mM Tris/HCl, pH 7.5, 150 mM NaCl
Elution buffer	0.2 M glycine/HCl, 2.5
Neutralization buffer	1 M Tris/Hcl, pH 9
Protein extraction from leaves and Immunoblot	
Extraction buffer (2x SDS loading buffer)	250 mM Tris/HCl pH 6, 20% glycerol, 4% sodium dodecyl sulfate (SDS), 0.1% (w/v) bromophenol blue, 80 mM dithiothreitol (DTT)
1x TBS-T	50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 0.1 % Tween-20
AP-buffer	100 mM NaCl, 100 mM Tris pH 9.5, 5 mM $MgCl_2$
BCIP (5-bromo-4-chloro-3'-indolyphosphate)	20 mg/ml in 100 % DMF
NBT (nitro-blue tetrazolium)	70 mg/ ml in 70 % DMF
Protoplast and chloroplast isolation	
Pre-enzyme solution	20 mM MES, pH 5.7, 400 mM Mannitol, 20 mM KCl
Final enzyme solution	To Pre-enzyme solution add: 1.5 % (w/v) Cellulase, 0.4 % (w/v) Macerozyme, 10 mM $CaCl_2$, 0.1 % (w/v) BSA
Note: Heat to 55 ºC for 10 min before adding $CaCl_2$ and BSA	
W5	MES/KOH pH 5.7, 154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl
Half and Half	11 mM MES/KOH pH 5.7, 77 mM NaCl, 63 mM $CaCl_2$, 2 mM KCl, 200 mM Mannitol
1x CIB +BSA	HEPEs/KOH pH 8.0, 200 mM Mannitol, 5 mM EGTA, 5 mM EDTA, 10 mM $NaHCO_3$, 0.1 % BSA (w/v)

A2.5 Genotyping of PAA1 mutants

The PAA1 and PAA2 mutant lines have been generated using EMS mutagenesis (Shikanai et al., 2003; Abdel-Ghany et al., 2005). Genotyping was therefore conducted in a two-step process which involved the amplification of a DNA fragment containing the mutation and the subsequent digestion of this PCR fragment with restriction enzymes. The mutations either caused or removed a restriction site in this fragment. Here I describe a faster and reliable method which only involves one PCR for genotyping of these and other mutants. It is based on the methods described for single-base mutants called Amplification-Refractory Mutation System PCR (ARMS) (Haque et al., 1998; Little, 2001).

The theory This protocol is for genotyping SNPs or mutants with point mutation by using only PCR. It is based on the need for the polymerase to have a base-paired 3'-end in order to start amplification from the primer. The protocol needs 3 primers: 1 universal reverse primer, 1 wild-type specific primer and one mutant specific primer. The amount and strength of mismatches as well as the annealing temperature will have to be tested for each primer pair individually. The PCR will be done in two separate reactions containing the specific primer pairs. If a PCR product can be detected in both reactions, then the plant is heterozygous for the wild-type and the mutant gene. If a product exclusively occurs in one of the samples, then the plant is homozygous for that gene.

Primer design The reverse primer does not have to be designed in a modified way. It should however match the melting temperature of the two forward primers. The design of the forward primers is different. Both the wild-type and mutant-specific forward primer will have a mismatch in the second but last base from its 3'-end. Usually the nucleotide base present in the genomic sequence is chosen for that (see Figure A.6, the nucleobase G in this example). This makes it more likely that a mismatch or weak binding of the last base will not lead to amplification through PCR. The last base will then be designed to specifically recognize the wild-type or the mutant sequence respectively. Elongation will only occur if the last base of the primer binds complementary to the DNA strand. The mismatch-strength of the last base should be designed as strong as possible to avoid false positive PCR products. The mismatch strength

increases as follows: no mismatch (AT, CG), weak (CA, GT), medium (AA, GG), strong (CC), very strong (GA, CT, TT) (Bui and Liu, 2009). The primer sequences used in the genotyping of PAA1 mutants is shown in Table A.4.

The PCR cycle parameters were as follows: Initial heating at 96 ºC for 5 min, followed by 35 cycles of: 96 ºC for 30 sec, 57 ºC for 40 sec and 72 ºC for 2 min and 30 sec. The amount of DNA template is 5 µl of a 90 µl DNA extraction in water (Shorty prep, see Chapter 4). The total PCR reaction volume is 20 µl.

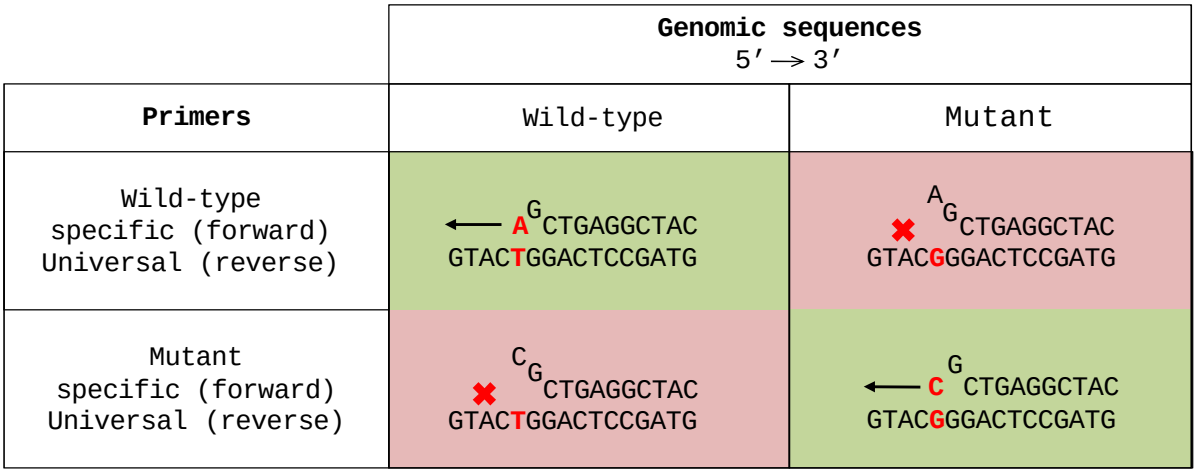

Figure A.6: Schematic representation of the ARMS-PCR

This matrix was modified after Little, 2001. It illustrates the four combinations of primer and genotypes present when using ARMS for genotyping purposes. The single-stranded genomic sequence is always shown on the bottom, while the upper sequence represents the primers. The penultimate base is designed to form mis-matches. The ultimate 3'-primer is specific to the wild-type and mutant sequence, this base is depicted in red. If this base is able to bind complementary to the genomic DNA, elongation will occur (black arrow, green background). If the second but last as well as the 3'-end base cannot complement, no amplification of the product is expected (red x, red background).

Table A.4: Primers used for genotyping PAA1 mutants

Shown are the primers used for genotyping the Arabidopsis PAA1 mutants listed below using ARMS-PCR. *paa1-3* can be genotyped without ARMS-specific primers, because more than one base is different between the wild-type and the mutant. However, the same PCR reactions as described for ARMS are required.

Mutation	Specificity	Sequence (5′→3′)	Tm (ºC)	Product size (bp)	Sense
paa1-1	Wild-type	TAAAAAATATTTTGGGCTAGAG**c**C	59	335	Forward
	Mutant	TAAAAAATATTTTGGGCTAGAG**c**T	57		Forward
	Universal	GCAGAGAGTGCCATCACTCC	59		Reverse
paa1-2	Wild-type	CTCTGGCATCATCTAATGTT**a**G	57	442	Forward
	Mutant	GCTCTGGCATCATCTAATGTT**a**A	59		Forward
paa1-3	Wild-type	AGATATCATTATTCTCGATGTTGG	59	352	Forward
	Mutant	GTCAGATATCATTATTCTCGTAGG	61		Forward
	Universal	CTAGCTGAAGCAACTTGAGGC	59		Reverse
paa1-4	Wild-type	TGATGGGCAATCGGTTAAC**g**C	59	346	Forward
	Mutant	TTGATGGGCAATCGGTTAAC**g**T	59		Forward
paa1-5	Wild-type	ATGTTAATAGCTTTTGTATTGCT**a**G	59	366	Forward
	Mutant	TATGTTAATAGCTTTTGTATTGCT**a**A	59		Forward
	Universal	ATAAGGAAAACATAACCCAAGCG	59		Reverse
paa1-6	Wild-type	GCTGCTGCTCTGGCATCA**c**C	61	429	Forward
	Mutant	GCTGCTGCTCTGGCATCA**c**T	59		Forward
paa1-2,1-4, 1-6	Universal	TCCATCGCATCAAGCAACTGC	59		Reverse

A2.6 Physiological response of *clpc1-1* and *clpr2-1* to high Cu treatment

Both Clp mutants accumulate significantly higher amounts of PAA2 protein than the wild-type (Chapter 3). PAA2 functions in Cu import into the thylakoids and thus I tested if the increase in PAA2 protein abundance would lead to Cu sensitivity in *clpc1-1* and *clpr2-1*. Parameters for Cu toxicity were: PSII efficiency (Fv/Fm), root-length and biomass. Col-0, *clpc1-1* and *clpr2-1* were grown in biological triplicates as described before (Chapter 2 and 3) in the presence of the indicated Cu concentrations for 18 days (Fv/Fm, biomass). For root-length measurements, plants were grown on vertical plates for 10 days.

Overall the Cu treatments did not significantly affect the root-length of the wild-type (Figure A.7, *A* and *B*). Root length development was variable especially in the *clpr2-1* line. Overall, *clpr2-1* seems to be impaired in root growth independently of the Cu-treatment and therefore no clear trend evolved concerning a Cu-mediated phenotype. The increase in Cu concentration however, had a small but detectable impact on the root growth of *clpc1-1*. Follow-up experiments will have to include higher Cu concentrations (e.g. 40 µM $CuSO_4$), in which also the wild-type clearly develops a phenotype.

Photosynthetic efficiency is expected to be negatively affected in deficiency and very high Cu concentrations. The response of the wild-type and *clpc1-1* is comparable and slightly decreases in Cu-deficient conditions (Figure A.7, *B*). *clpr2-1* has been reported to be involved in chloroplast development and thus a photosynthetic phenotype was expected (Rudella et al., 2006). Indeed, photosynthetic efficiency is strongly impaired in this line. Interestingly, its Fv/Fm is highest in low Cu concentrations and decreases drastically at levels that are not are considered toxic (Burkhead et al., 2009).

Higher Cu concentrations seem to have an effect on the biomass of *clpc1-1* and *clpr2-1*, while the wild-type was virtually unaffected. Figure A.7 *C* shows the biomass of the three genotypes with respect to the biomass they develop when grown in the presence of 5 µM $CuSO_4$. The wild-type only lost about 2% of its biomass on 30 µM Cu compared to 5 µM Cu, while *clpc1-1* lost 14 % and *clpr2-1* 56 % (see also Figure A.8).

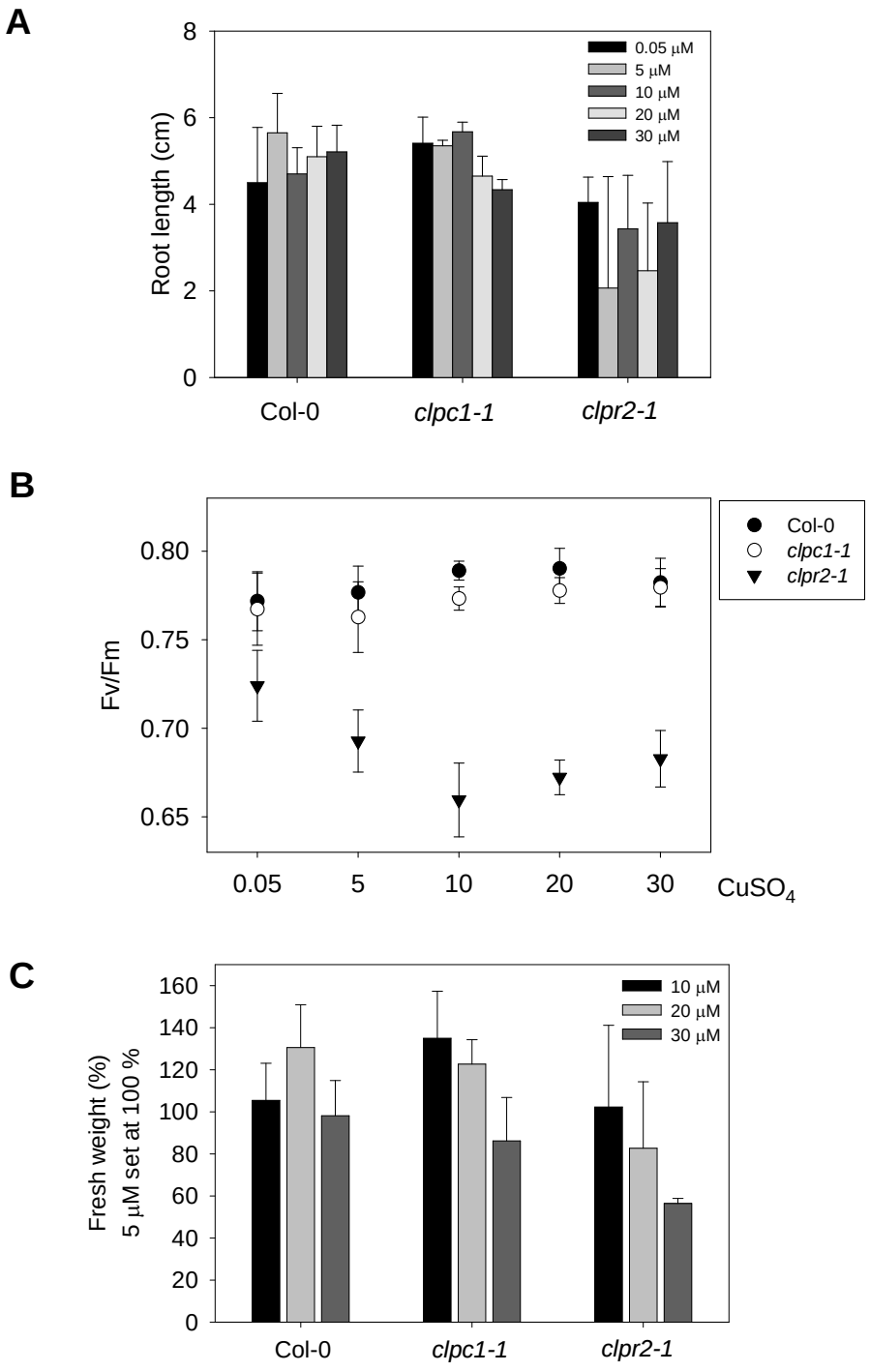

Figure A.7: Affect of high Cu treatment on the physiology of *clpc1-1* and *clpr2-1*

(A) Root-lengths of wild-type, *clpc1-1* and *clpr2-1* in the presence of increasing levels of Cu. Plants were grown for 10 days on vertical plates. For Fv/Fm (B) and biomass (C) measurements, plants were grown for 18 days in the presence of the indicated Cu conditions. (C) Presented is loss of biomass in percent, compared to the biomass at 5 µM $CuSO_4$, normalized for each individual genotype. The results are the mean ± S.D. for biological triplicates, (B) and (C) and 4 technical replicates for (A).

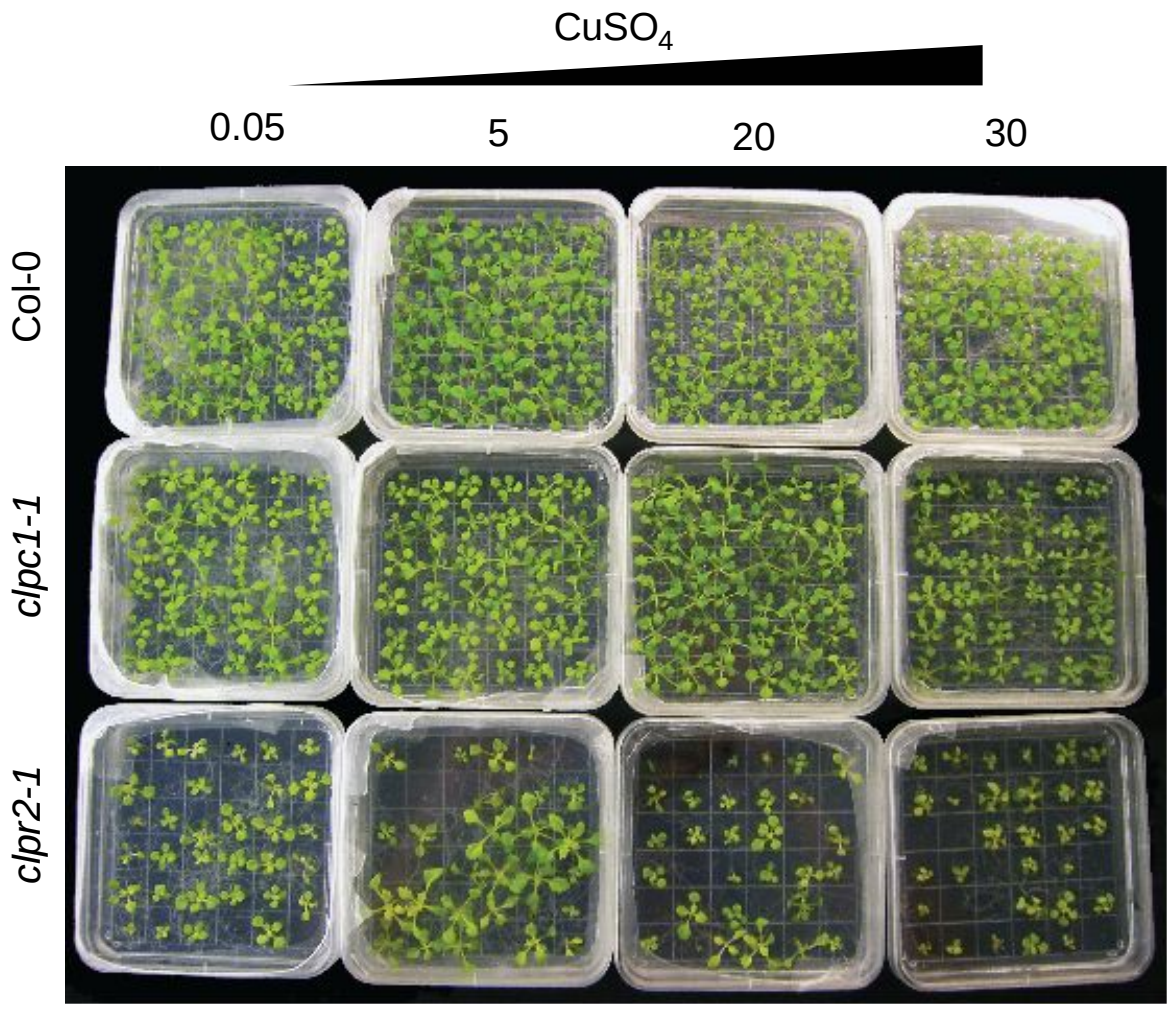

Figure A.8: Cu-dependent phenotype of *clpc1-1* and *clpr2-1*

Representative phenotype of 18 day-old Col-0, *clpc1-1* and *clpr2-1* seedlings when grown in the presence of the indicated Cu concentrations *in vitro*.

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APPENDIX III

Curriculum vitae

Wiebke Tapken, M.Sc.

Mailing Address
Biology Department
200 West Lake Street
Fort Collins, CO, 80523-1878, USA
Office Phone: 970 491 5307
wtapken@lamar.colostate.edu

Home Address
500 West Prospect Rd, Apt 20L
Fort Collins, CO, 80526, USA
Tel.: 970 231 9657

Education

PhD (Botany), GPA 4.0: Colorado State University (USA), Aug 2008 - planned Oct 2012

Dissertation: "Cu transport into the chloroplast and its implications for Cu homeostasis in *Arabidopsis thaliana*"

Supervisor: Dr. Marinus Pilon

M.Sc.: University of Technology Braunschweig (Germany), Oct 2003 - May 2008

Thesis: "Relevance of the molybdenum cofactor sulfurase ABA3 for selenium tolerance in *Arabidopsis thaliana*"

Supervisor: Dr. Ralf R. Mendel

Other relevant employment

Technical Assistant, Helmholtz Center for Infection Research, Braunschweig (Germany), Oct - Dec 2007

Publications (peer reviewed journals)

1. **Tapken, W.**, Pilon, M. (2012): *Regulation of Copper Homeostasis in Plants* (Metals in Cells), John Wiley& Sons, Inc., New York (*in review*)

2. **Tapken, W.**, Ravet K., Pilon, M. (2012): Plastocyanin controls the stabilization of the thylakoid Cu-transporting P-type ATPase PAA2/HMA8 in response to low copper in *Arabidopsis*. *J Biol Chem*, **287** (22): 18544 - 18550

3. Chiang, Y.-H., Zubo, Y.O., **Tapken, W.**, Kim, H.J., Lavanway, A.M., Howard, L., Pilon, M., Kieber, J.J. and Schaller, G.E. (2012): Functional Characterization of the GATA Transcription Factors GNC and CGA1 Reveals Their Key Role in Chloroplast Development, Growth, and Division in *Arabidopsis*. *Plant Physiol*, **160**: 332 - 348

4. Pilon, M., Ravet, K., **Tapken, W.** (2011): The biogenesis and physiological function of chloroplast superoxide dismutases. *Biochim Biophys Acta*, **1807**: 989-998

5. Pilon-Smits, E.A.H., Quinn C.F., **Tapken, W.**, Malagoli M., Schiavon, M. (2009): Physiological functions of beneficial elements. *Curr Opin Plant Biol*, **12**: 267-274

Teaching Experience

Colorado State University (USA)

BZ 441 - Plant Physiology (Undergraduate laboratory), every Spring 2009 - 2012

LIFE 102 - Introduction to Biology (Undergraduate laboratory), Fall 2008

University of Technology Braunschweig (Germany)

ZB 05 - Cell Biology of Plants (Undergraduate laboratory), Fall 2007

Mentoring

4 Undergraduates during the PhD (USA)

Shaad Bakeet, Emma Jobson, Steven Chow, Moritz Wilch

Award

German Academic Exchange Service (DAAD): Stipend for Scientific Education Abroad (2008)

Oral communications

Copper10 conference, Alghero (Italy), 2010

Regulation of the P_{1B}-type ATPase PAA2/HMA8 by its own substrate. Wiebke Tapken* and Marinus Pilon
*First author presented the talk

Plant Supergroup, Colorado State University, Fall 2010

Plant Biology Congress, Freiburg (Germany), 2012

Plastocyanin controls the stabilization of the thylakoid chloroplast Cu transporter PAA2/HMA8 in response to low Cu. Wiebke Tapken*, Karl Ravet and Marinus Pilon
*First author presented the talk

Poster Presentations

Gordon Research Conference, Cell Biology of Metals, RI (USA), 2009

Copper10, Alghero (Italy), 2010

Plant Biology Congress, Freiburg (Germany), 2012

Professional service

Ad-hoc reviewer for *Plant Physiology*, December, 2011

Languages

German (native), English (fluent)