Rapid report

The Clp protease system is required for copper ion-dependent turnover of the PAA2/HMA8 copper transporter in chloroplasts

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Summary

• The distribution of essential metal ions over subcellular compartments for use as cofactors requires control of membrane transporters. PAA2/HMA8 is a copper-transporting P1B-type ATPase in the thylakoid membrane, required for the maturation of plastocyanin. When copper is highly available to the plant this transporter is degraded, which implies the action of a protease. • In order to identify the proteolytic machinery responsible for PAA2/HMA8 turnover in Arabidopsis, mutant lines defective in five different chloroplast protease systems were analyzed. • Plants defective in the chloroplast caseinolytic protease (Clp) system were specifically impaired in PAA2/HMA8 protein turnover on media containing elevated copper concentrations. However, the abundance of a core Clp component was not directly affected by copper. Furthermore, the expression and activity of both cytosolic and chloroplast-localized superoxide dismutases (SODs), which are known to be dependent on copper, were not altered in the clp mutants, indicating that the loss of PAA2/HMA8 turnover in these lines was not caused by a lack of stromal copper. • The results suggest that copper excess in the stroma triggers selection of the thylakoid-localized PAA2 transporter for degradation by the Clp protease, but not several other chloroplast proteases, and support a novel role for this proteolytic system in cellular copper homeostasis.

Introduction

The correct allocation of essential trace elements over subcellular compartments that are separated by membranes presents an important challenge for all living organisms. Copper (Cu) is utilized as an enzyme cofactor by the vast majority of aerobic organisms (Ridge et al., 2008). An abundant cuproprotein in green cells of flowering plants and many algae is plastocyanin (PC), which is localized in the thylakoid lumen of chloroplasts, where it transports electrons between the cytochrome b6f complex and photosystem I (Weigel et al., 2003). The Arabidopsis thaliana genome encodes for two PC isoforms, PC1 and PC2, which have apparent redundant function in photosynthesis (Weigel et al., 2003; Pesaresi et al., 2009). Studies in Arabidopsis and poplar have indicated that Cu is preferentially allocated to PC when Cu supply is low, which was hypothesized to allow the plant to remain photosynthetically active (Abdel-Ghany & Pilon, 2008; Ravet et al., 2011). Prioritization is achieved through the expression of the so-called Cu-microRNAs (Burkhead et al., 2009), which direct the concerted down-regulation of transcripts that encode for seemingly nonessential cuproproteins. Among the Cu-microRNA targets are cytosolic and chloroplastic isoforms of copper/zinc superoxide dismutase (CSD), CSD1 and CSD2, respectively (Sunkar et al., 2006; Yamasaki et al., 2007). The expression of Cu-miRNAs is up-regulated when Cu availability is low and depends on the Cu-responsive transcription factor SPL7 (SQUAMOSA promoter binding protein-like7). At the same time, SPL7 promotes the expression of cell surface localized transporters and reductases, which function to increase cellular Cu intake (Yamasaki et al., 2009; Bernal et al., 2012).

PC is transported as an apoprotein into the thylakoid lumen by the Sec-pathway, which translocates proteins in an unfolded state (Cline et al., 1993). Thus, Cu has to be transported into the chloroplast stroma and further into the thylakoid lumen for PC...
maturation to occur. In Arabidopsis, the two P$_{1B}$-type ATPases PAA1/HEMA6 and PAA2/HEMA8 (for P-type ATPase of Arabidopsis 1 and 2 or Heavy Metal ATPase 6 and 8; hereafter referred to as PAA1 and PAA2) fulfill this Cu-transport function (Shikanai et al., 2003; Abdel-Ghany et al., 2005; Catty et al., 2011). PAA1 and PAA2 are localized to the inner chloroplast envelope and the thylakoid membrane respectively (Abdel-Ghany et al., 2005). PAA2 is involved in the homeostatic regulation of Cu allocation in the chloroplast as its abundance is c. 65% lower in elevated Cu compared to mild deficiency (Tapken et al., 2012). The down-regulation of PAA2 by Cu was demonstrated to be mediated by protein turnover and independent of SPL7 (Tapken et al., 2012). A number of protease systems have been identified in the plastids, many of which are likely derived from the prokaryotic ancestor of the chloroplast (for a comprehensive review see: Kato & Sakamoto, 2010). Mutations in protease subunits often lead to severe defects in chloroplast development and pleiotropic phenotypes, indicating that plastid proteases are crucial for the homeostatic control of organelar proteins (Olinares et al., 2011a).

However, despite their pivotal physiological functions, very few specific substrates are known for the various protease systems in the chloroplast. Exceptions are the processing peptidases which remove targeting signals from precursors during protein translocation (Teixeira & Glaser, 2013). The turnover of PAA2 constitutes a unique example of regulated protein turnover at the thylakoid membrane, because it occurs in response to an environmental stimulus and impacts Cu homeostasis. We therefore aimed to identify the organellar protease(s) that mediates this process.

Materials and Methods

Plant materials and growth conditions

Arabidopsis Col-0 was used as a wild-type control and all mutant lines have been described previously: clpc1-1 (SALK_014058, Sjögren et al., 2004), clpc2-2 (hsp93-III-1, SAIL_622_B05, Kovacheva et al., 2007), clpd (SAIL_77_G05, reviewed in Olinares et al., 2011a), clpr2-1 (SALK_046378, Rudella et al., 2006; Kim et al., 2009), clps1 (SAIL_326B_G12, Nishimura et al., 2013), sppA-1 (GABI-Kat 142B03, Wetzsel et al., 2009), var2-5f5/5f2H2-5, 7 (Sakamoto et al., 2004), prep1xprep2 (Nilsson Cederholm et al., 2009), deg2, deg5, deg7, deg8 (Sun et al., 2010), pc2 (Weigel et al., 2003), paa1-3 (Shikanai et al., 2003) and paa2-1 (Abdel-Ghany et al., 2005). The clpr2-1/R2:His line used for complementation has been described (Rudella et al., 2006). For in vitro growth, Arabidopsis seeds were surface sterilized according to Tapken et al. (2012). Plants were grown on half-strength Murashige and Skoog (MS) medium (Murashige & Skoog, 1962; Caisson Laboratories, North Logan, UT, USA) solidified with 0.6% Phytagel™ (Sigma-Aldrich) and supplemented with 1% sucrose, at pH 5.8. Plants were grown in a temperature-controlled growth chamber at a photon density of 120 μmol m$^{-2}$ s$^{-1}$ in a 12h:12h light:dark cycle at 23°C. Copper (Cu) was added as copper sulfate (CuSO$_4$) and concentrations in the media are indicated for each experiment. Cycloheximide treatments were performed in liquid culture as described (Tapken et al., 2012).

Protein extraction, immunoblot analysis, enzyme activity assay, elemental analysis and statistics

Proteins for denaturing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis were extracted as described (Tapken et al., 2012). After fractionation by SDS-PAGE, proteins were electroblotted onto a nitrocellulose membrane (Trans-Blot® Pure Nitrocellulose, 0.2 μm; Bio-Rad, Hercules, CA, USA). The antisera for cFBPase and CSD2 were obtained from Agrisera (Vännäs, Sweden). Affinity purified antibodies for PAA1, PAA2 (Tapken et al., 2012) and ClpR2 (Asakura et al., 2009) have been described. For the enzymatic activity of SODs, total leaf protein was extracted under nondenaturing conditions and in-gel activity staining was performed as described (Beauchamp & Fridovich, 1971; Abdel-Ghany et al., 2005). Elemental analysis of leaf samples was conducted after digestion with concentrated nitric acid of washed and dried samples, essentially as described (Ravet et al., 2011). All assays have been performed at a minimum for three biological replicates. Representative results for immunoblots are shown. The software ImageJ (NIH, Bethesda, MD, USA) was used on digitized immunoblots to quantify protein abundance (Tapken et al., 2012). Statistical analysis was performed by Student’s t-test using the software JMP (version 9.0.2; SAS Institute, Cary, NC, USA). Significant differences are reported where appropriate and represent values of P < 0.05.

Results

We tested 11 Arabidopsis mutant lines with defects in five different chloroplast protease systems for their PAA2 abundance: stromal and thylakoid lumen Deg (deg2,5,7,8), stromal caseinolytic protease (Clp) (clpc1-1, clpc2-2, clpd, clpr2-1), thylakoid SppA (sppA-1), stromal Prep (prep1xprep2) and thylakoid Fsh (var2-5). The presence of sucrose in the in vitro media had the advantage that the most severe phenotypes of these protease mutants were suppressed. In addition, Cu levels were easily controlled. PAA2 protein abundance responded to Cu availability and was comparable to the wild-type in all lines except for clpc1-1 and clpr2-1 (Fig. 1a). When compared to the Col-0 wild-type, PAA2 protein abundance was increased over four-fold in the presence of elevated Cu in clpc1-1 and clpr2-1 and Cu addition no longer significantly affected PAA2 (Fig. 1b). ClpC1 is a AAA+/HSP93 chaperone and ClpR2 is a subunit of the Clp core complex of the stromal localized Clp system (Olinares et al., 2011a). We tested if the regulatory mechanism can be restored in a complemented line of the clpr2-1 allele (Fig. 1c). Indeed, PAA2 abundance was again reduced and the effect of Cu was restored, indicating that ClpR2 is required for PAA2 regulation by Cu (Fig. 1c).

PAA2 is most similar in sequence to PAA1, which is located in the inner envelope and thus at least partially exposed to the stroma. Therefore we tested if PAA1 is also a Clp substrate (Fig. 2a). In clpc1-1 and clpr2-1, PAA1 protein abundance was comparable to the wild-type (Fig. 2a). Copper (Cu) addition had no effect on PAA1 protein abundance in either wild-type or the two mutants. These observations indicate that Cu-mediated regulation by Clp is specific to PAA2. Protein accumulation is the result of the balance.
of synthesis and degradation. Therefore, increased PAA2 abundance in Clp mutants might be explained either by decreased PAA2 turnover or by a more indirect effect on translation of either PAA2 itself or of a stabilizing factor. To analyze if the Clp system is directly required for PAA2 turnover, we interrupted protein synthesis in 10 d-old seedlings through a 24 h treatment with 100 \( \mu M \) cycloheximide (CHX) in the presence or absence of additional Cu (Tapken et al., 2012). The clpr2-1 allele could not produce enough biomass in this condition and therefore we limited the analysis to a comparison of clpc1-1 to the wild-type. PAA2 protein accumulated to high levels and was not significantly reduced by CHX treatment in clpc1-1, independent of the presence of Cu. By contrast, CHX strongly affected PAA2 abundance in the wild-type, which is especially evident when 5 \( \mu M \) total CuSO\(_4\) was present (Fig. 2b). Quantification of three biological repeats showed that the PAA2 protein levels were 5.4-times higher in the clpc1-1 mutant when compared to the wild-type in the presence of Cu and CHX (\( P < 0.05 \)). Together the results presented in Figs 1 and 2 strongly suggest that the effect of the clpc1-1 and clpr2-1 mutations cannot be explained by a lack of Cu in the chloroplasts.

**Discussion**

The turnover of PAA2 can be observed for plants grown in vitro in the presence of sucrose. These growth conditions further allowed us to reveal that the Cu-induced turnover of PAA2 is dependent on the Clp system. ClpC1 and ClpR2 are both nuclear encoded proteins and subunits of Clp, which functions within the chloroplast stroma. The Clp system is of prokaryotic origin and composed of a Cu levels were lower in clpc1-1 and clpr2-1. We therefore compared tissue Cu levels for wild-type plants and the two clp mutants grown at low or high Cu concentrations. As expected, Cu feeding increases its content in the leaves, but there is no difference between the wild-type and mutant lines (Fig. 3a). Similarly, no significant differences were observed between the lines for the leaf content of other elements tested (Supporting Information Fig. S2). The abundance and activity of chloroplastic CSD2 is strongly affected by Cu availability in the chloroplast (Shikanai et al., 2003; Abdel-Ghany et al., 2005) and can therefore be used as a reliable indicator for its Cu status. If there is less Cu in chloroplasts of clpc1-1 and clpr2-1, then we would expect reduced CSD2 abundance and activity. Clearly, CSD2 is not affected in clpc1-1 and clpr2-1. By contrast, its activity is even slightly elevated in the two clp mutants grown in low Cu conditions (Fig. 3b). Together the observations in Fig. 3 strongly suggest that the effect of the clpc1-1 and clpr2-1 mutations cannot be explained by a lack of Cu in the chloroplasts.
barrel-shaped proteolytic core with associated chaperones (Olinares et al., 2011a). The core is comprised of five different proteolytic ClpP, and four different nonproteolytic ClpR subunits, which together organize into two asymmetric heptameric rings. The resulting core structure harbors the proteolytically active sites occluded within its center (Olinares et al., 2011b). The opening of the Clp core is thought to be too narrow for large folded proteins to enter and thus substrates need to be initially recognized for degradation, unfolded and then actively threaded through the axial pores (Adam et al., 2006; Clarke, 2012). In plants this is thought to be dependent on the three putative ATP-dependent AAA+/HSP93-type Clp chaperones ClpC1, ClpC2 and perhaps ClpD (Sjögren et al., 2014). The strongest change in PAA2 abundance was observed in the clpr2-1 mutant allele. It carries a T-DNA insertion 7 base pairs upstream of the translational start codon, which results in a 50% reduction of ClpR2 mRNA levels (Rudella et al., 2004; Majeran et al., 2000). However, in plants which lack core Clp subunits, protein abundance of all photosynthetic thylakoid complexes are reduced by half, without a specific effect on the cytochrome b6f complex (Rudella et al., 2006; Zybailov et al., 2009; Kim et al., 2013; Nishimura et al., 2013). In Chlamydomonas reinhardtii, Clp has been implicated in the specific degradation of the thylakoid-located cytochrome b6f complex under nitrogen (N)-starvation or when biogenesis of the Rieske subunit is blocked (Majeran et al., 2000). However, in plants which lack core Clp subunits, protein abundance of all photosynthetic thylakoid complexes are reduced by half, without a specific effect on the cytochrome b6f complex (Rudella et al., 2006; Zybailov et al., 2009; Kim et al., 2013). A previously identified candidate for a ClpC1 target in plants is the thylakoid membrane-bound chlorophyllide a oxygenase, which converts chlorophyll a to chlorophyll b and accumulates in a clpC1 (Clp chaperone) mutant (Nakagawara et al., 2007). However, Clp chaperones may have additional biological roles as these proteins can also function as part of the chloroplast protein import machinery (see: Jarvis, 2008). Indeed chlorophyllide a oxygenase, was not reported to over-accumulate in clpr2-1 (Clp core) in which the ratio of chlorophyll a and b was also similar to the wild-type (Rudella et al., 2006). Protease mutants have furthermore the tendency to increase general chaperone
activity (CPN60/20, HSP70, HSP90, ClpB3) within the plastids to cope with increasing amounts of incomplete and mal-formed proteins (see Olinares et al., 2011a). In this context, a number of observations give confidence that PAA2 over-accumulation in clp mutants is a reflection of the direct involvement of the Clp system. Out of the 11 tested protease mutant lines only two, clp-c1-1 and clp-r2-1, showed a significant increase of PAA2 protein accumulation, which indicates that only the Clp system is specifically required for PAA2 turnover. Further, the Cu-dependent regulation is restored through complementation of clp-r2-1. Importantly, clp-c1-1 is deficient in a chaperone while clp-r2-1 is deficient in the Clp core, yet both affect PAA2. While most homoygous ClpP and ClpR mutants are seedling lethal or unable to set seed, both clp-c1-1 and clp-r2-1 are relatively mild alleles, capable of completing a full lifecycle and exhibiting a viridescent phenotype and reduced growth on soil or MS medium. Furthermore, Cu levels used in this study had no additional effect on phenotypes of any of the Clp mutants tested here (Supporting Information Fig. S3). This reduces the likelihood for indirect pleiotrophic phenotypes. In addition, the thylakoid photosynthetic machinery is, on a whole cell bases, decreased in abundance to c. 50% in various clp mutants including clp-r2-1; however the thylakoid protein PAA2 is four times more (not less) abundant in this mutant compared to the wild-type. Finally, PAA2 accumulation in the wild-type responds to a defined physiological trigger, low Cu availability, and PAA2 turnover is induced by elevated Cu levels that are well below toxicity. At the same time PAA2 transcript levels do not respond to Cu (Tapken et al., 2012). Indeed, PAA2 accumulation on low Cu in the clp mutants is not a result of transcript up-regulation or protein synthesis. It is rather the result of reduced turnover (Fig. 2b); at the same time loss of function of the two Clp subunits does not affect indicators of the chloroplasts Cu status (Fig. 3b).

How chloroplast ClpC chaperones recognize their substrates is still a largely open question. In Escherichia coli (E. coli), ClpS is involved in substrate selection and delivery to the bacterial ClpAP; in particular ClpS recognizes proteins with an N-terminal degradation signal (N-degron), as well as aggregated proteins (Sauer & Baker, 2011). Through phylogenetic and in silico analyses, it was shown that angstosperma contains a homolog, named ClpS1 which interacts with ClpC1,2 chaperones (Nishimura et al., 2013). The Arabidopsis ClpS1 null mutant (clps1) lacks a visible growth phenotype, similar to the E. coli clpS null mutant. Affinity purification identified eight candidate ClpS1 substrates and interaction with five substrates strictly depended on two conserved ClpS1 residues involved in N-degron recognition (Nishimura et al., 2013). Using the clps1 null mutant, it was clear however that in vivo PAA2 degradation did not depend on ClpS1 (Supporting Information Fig. S4). The mechanism of Clp-mediated PAA2 turnover could involve either an effect of Cu on the protease itself or binding of Cu to the substrate, increasing its availability through an unknown mechanism. PAA2 turnover is reduced when Cu levels are lower. Given the known biochemical properties of Clp, a serine protease, it is difficult to envision a mechanism by which Cu could have an activating (in Cu excess) or inhibitory (on low Cu medium) effect. We also observed that Cu does not affect ClpR2 protein abundance (Supporting Information Fig. S1) and therefore the assembly of the Clp system is most likely not affected by Cu availability. We mined publicly available RNAseq data (Bernal et al., 2012) and found that neither ClpC1, ClpR2, nor any other chloroplast protease subunit, is regulated at the transcript level in response to Cu or via SPL7. This agrees with the notion that Clp is not regulated by Cu. We therefore consider it likely that a Cu-dependent conformational change of PAA2 is the trigger for substrate recognition by Clp. In the absence of sufficient amounts of apo-PC, when Cu cannot be donated efficiently to the thylakoid lumen or with relatively high Cu in the stoma, PAA2 would be more likely in a Cu-bound state. Conformational changes in P1B-type ATPases can be inferred based on 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). Indeed, the Cu-transporting P\(_{1B}\)-type ATPase CopA of Archaeoglobus fulgidus is postulated to follow the E1/E2 Albers-Post model for transport (Argiello et al., 2007). Other conformational changes that are not primarily associated with Cu transport can also be envisioned for Cu-transporting P-type ATPases (Rosenzweig & Argiello, 2012).

By analyzing fresh weight and the fluorescence parameter \(F_{v}/F_{m}\), which estimates photosystem II capacity and is a general indicator for stress within the chloroplast (Maxwell & Johnson, 2000), we did not observe significant Cu-induced phenotypes in clp-c1-1 even in the presence of up to 30 \(\mu\text{M}\) CuSO\(_{4}\) whereas only small effects of Cu were seen for clp-r2-1 (Supporting Information Fig. S5). Most likely any effects of Cu are masked by other phenotypes in these clp mutants. Nevertheless, we suggest that Clp-mediated turnover of PAA2 is physiologically relevant for Cu homeostasis. In a developing green leaf, the thylakoid lumen-localized PC is the primary recipient of Cu. However, free Cu is potentially cytotoxic in its ionic form and plants need an efficient system to avoid overloading of the thylakoids to prevent damage to the photosynthetic apparatus (Yruela et al., 1996; Kipper et al., 2003). Therefore, the chloroplast needs a reliable mechanism to decrease ion flow to this compartment when Cu levels increase. Conformational changes in PAA2 in a Cu-dependent manner could be a simple and direct gauge for the Cu status of the thylakoids. Clp-mediated PAA2 protein turnover will result in relatively increased Cu availability for the maturation of other Cu enzymes such as CSDs, which are transcriptionally up-regulated when Cu levels rise (Yamasaki et al., 2007). In this way, chloroplast Cu homeostasis is integrated with cellular and whole-plant regulation of Cu uptake and use as mediated by the SPL7 master regulator.

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**References**


**Supporting Information**

Additional supporting information may be found in the online version of this article.

**Fig. S1** Chloroplast copper (Cu) availability does not affect ClpR2 abundance.

**Fig. S2** Elemental analysis of iron (Fe), sulfur (S) and zinc (Zn) contents in wild-type and caseinolytic protease (Clp) mutants.

**Fig. S3** Phenotypes of wild-type and Clp mutants grown on 0.05 and 5 μM copper sulfate (CuSO₄).

**Fig. S4** ClpS1 is dispensable for PAA2 degradation in response to Cu.

**Fig. S5** Analysis of the effects of Cu on phenotypes of Clp mutants grown in Murashige and Skoog (MS) medium.

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