Regulation of Cu delivery to chloroplast proteins

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Abbreviations: CCS, copper chaperone for superoxide dismutase; Clp, casinolytic protease; COPT, copper transporter; CSD, Cu/Zn superoxide dismutase dismutase; Cu, copper; PAA1/2, P-type ATPase of Arabidopsis 1/2; PC, plastocyanin; COPT, copper transporter; CSD, Cu/Zn superoxide dismutase; PCH1, Plastid Copper Chaperone 1; PPO, Polyphenol Oxidase; SPL7, SQUAMOSA promoter binding protein-like7.

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Abstract

Plastocyanin is a copper (Cu)-requiring protein that functions in photosynthetic electron transport in the thylakoid lumen of plants. To allow plastocyanin maturation, Cu must first be transported into the chloroplast stroma by means of the PAA1/HMA6 transporter and then into the thylakoid lumen by the PAA2/HMA8 transporter. Recent evidence indicated that the chloroplast regulates Cu transport into the thylakoids via Clp protease-mediated turnover of PAA2/HMA8. Here we present further genetic evidence that this regulatory mechanism favors Cu delivery to the thylakoid lumen of plants. To allow plastocyanin maturation, Cu must first be transported into the chloroplast stroma by means of the PAA1/HMA6 transporter and then into the thylakoid lumen by the PAA2/HMA8 transporter. Recent evidence indicated that the chloroplast regulates Cu transport into the thylakoids via Clp protease-mediated turnover of PAA2/HMA8. Here we present further genetic evidence that this regulatory mechanism favors Cu delivery to the thylakoid lumen of plants. A key transcription factor mediating Cu homeostasis in plants is SQUAMOSA promoter binding protein-like7 (SPL7). SPL7 transcriptionally regulates Cu homeostasis when the nutrient becomes limiting by up-regulating expression of Cu importers at the cell membrane, and down-regulating expression of seemingly non-essential cuproproteins. It was proposed that this latter mechanism favors Cu delivery to the chloroplast. We propose a 2-tiered system which functions to control plant leaf Cu homeostasis: SPL7 dependent transcriptional regulation of cuproproteins, and PAA2/HMA8 turnover by the Clp system, which is independent on SPL7.

Introduction

Copper (Cu) is an essential micronutrient for the majority of aerobic organisms. In the cellular environment Cu exists in two oxidation states, Cu²⁺ and Cu³⁺, and is therefore an ideal cofactor for proteins performing redox chemistry. Cuproproteins in higher plants are important for essential housekeeping functions such as photosynthesis (plastocyanin) and respiration (cytochrome-c oxidase).³,5 Plastocyanin (PC) is one of the most abundant cuproproteins and is located in the thylakoid lumen of the chloroplast where it functions as an electron carrier between the cytochrome bd complex and photosystem II. PC is essential for photosynthesis of higher plants.⁴ Other abundant cuproproteins are the copper/zinc superoxide dismutases located in the cytosol (CSD1), and the chloroplast stroma (CSD2).⁵ Organisms that depend on Cu as a micronutrient have to balance its concentration within the cell to be able to sustain essential housekeeping processes, while avoiding its toxic side-effects.³ Such a homeostatic system relies on the coordination of sensing, transport, and compartmentalization of Cu within plant cells and organs.

The Cellular Cu Economy Model as Mediated by SPL7

SQUAMOSA promoter binding protein-like7 (SPL7) is a Cu-responsive transcription factor which appears to sense cytosolic Cu availability.⁶,⁷ In Cu-deficient conditions SPL7 activates the expression of the Cu importers COPT1, COPT2 and COPT6, as well as the root surface Cu reductase FRO4/FRO5, in order to increase Cu uptake from the soil and the apoplastic.⁶-⁹ SPL7 furthermore activates the expression of the Cu-miRNAs, which then down-regulate transcripts that encode for a diverse set of Cu proteins (Fig. 1).¹⁰,¹¹ The concerted down-regulation of seemingly non-essential Cu-binding proteins was proposed to aid in the prioritized delivery of the remaining Cu to PC and cytochrome-c oxidase, to maintain photosynthetic and respiratory...
functions. There is experimental support for miRNA-mediated Cu economy, both in Arabidopsis and poplar. Light should also affect Cu homeostasis because of the important role of Cu in photosynthesis. Indeed, phenotypes of the spl7 mutant are exacerbated by both low Cu and high light. The transcription factor Elongated Hypocotyl5 (HY5) mediates light responses and was found to physically interact with SPL7. Interestingly, many of the targets of SPL7, including COPTs and Cu-miRNAs, are co-regulated by HY5, which indicates that signals for light-regulated expression and Cu availability are integrated.

## Chloroplast Cu Delivery: Cu Chaperones and Cu-Transporting P-type ATPases

PC acquires its cofactor in the thylakoid lumen. Both cytosolic CSD1 and stromal CSD2 receive Cu from the soluble Cu chaperone for SOD (CCS). The cytosolic and chloroplastic isoforms of CCS are encoded by the same gene, but the use of alternative transcriptional start sites results in dual localization. Cu distribution between the cytosol, stroma and thylakoid lumen is mediated by 2 P-type ATPase transporters called PAA1/HMA6 and PAA2/HMA8 (from here on called PAA1 and PAA2). PAA1 is localized in the envelope, and PAA2 in the thylakoid membranes. Most known Cu-transporting P-type ATPases accept Cu from a Cu-chaperone which delivers the substrate ion directly to a platform formed by a region of the transporter that includes transmembrane regions 6–8. The recently discovered protein called Plastid Copper Chaperone 1 (PCH1), which is conserved in green eukaryotes, delivers Cu to PAA1. PCH1 presumably delivers Cu to PAA1 in the inter membrane space of the plastid envelope, but direct evidence for this localization of PCH1 is still lacking. In many species, such as Arabidopsis, PCH1 is encoded by an alternatively spliced mRNA, which is derived from the PAA1 gene sequence. In other plants, PCH1 is encoded by a separate gene, which probably evolved following gene duplication and sequence divergence of an ancestral PAA1 sequence. By quantitative RT-PCR, we measured the relative abundance of the PAA1-derived mRNA splice forms in Arabidopsis seedlings grown on agar media and found that the smaller mRNA isoform, which encodes PCH1, is about 5-times higher expressed when compared to the full PAA1 mRNA (not shown). This relatively high PCH1 expression might facilitate the delivery of Cu to the surface of the inner envelope. In vitro, CCS can bind to PAA2 and even deliver Cu for transport. However, loss of CCS has no effect on the activity or abundance of PC in planta.

## Regulation of Chloroplast Cu Delivery

The question arises if SPL7 can also influence chloroplastic Cu, or if the chloroplast maintains some autonomy to regulate its own Cu requirements. Modulation of the abundance of PAA1 or PAA2 can be a mechanism to control chloroplast Cu delivery. In Arabidopsis, we observed that PAA2 protein abundance is highest in Cu deficient conditions and decreases in the presence of Cu in the growth medium in both Columbia and Landsberg accessions. Regulation of PAA2 appears to be conserved throughout the Arabidopsis genus since we observed Cu-dependent modulation of PAA2 levels in all the natural accessions we tested (Fig. 2A). Interestingly, PAA2 of Arabidopsis and other species has a putative Cu-miRNA targeting site for miR408, which would suggest that in those species PAA2 mRNA could be subject to downregulation when Cu levels drop. However, we previously confirmed that PAA2 is not a target of miR408 and that the PAA2 transcript is not affected by Cu in Arabidopsis. Instead it was shown that PAA2 protein levels are controlled by protein degradation independently of SPL7 activity. In contrast, protein abundance of PAA1 is unaffected by the Cu status of the plants.

What could control PAA2 turnover in response to Cu? We previously observed that PAA2 protein accumulation is significantly increased in paa1 mutants in which the Cu content of the chloroplast is decreased to about half of the wild-type levels. Therefore, it can be hypothesized that low Cu amounts in the chloroplast stroma signal decreased turnover of PAA2. The PAA2 transporter also is in contact with the thylakoid lumen where PC is localized. Two PC isoforms, PC1 and...
PC2, are encoded in the Arabidopsis genome; the PC2 protein becomes increasingly abundant when Cu is added to the growth medium, while PC1 abundance is not affected.18,19 By contrast to what is observed in paa1 mutants, PAA2 transporter abundance is always low in a pc2 mutant, regardless of the provided Cu concentration in the growth medium.16 It therefore can be reasoned that levels of PC, the ultimate Cu acceptor in the lumen, may directly and positively control PAA2 stability. In a novel approach we now generated a paa1–3 pc2 double mutant in order to identify the ultimate determinant for PAA2 stability; chloroplastic Cu levels, or abundance of PC2 (Fig. 2B). Strikingly, in the paa1–3 pc2 double mutant PAA2 protein abundance is still affected by Cu, and mimics the wild-type. Thus, we conclude that regulation of PAA2 degradation does not depend on direct interaction with PC. Instead, it seems likely that elevated Cu levels within the stroma of chloroplasts trigger PAA2 degradation. The paa1–3 and pc2 mutations are both likely to affect Cu levels in the stroma (decrease and increase, respectively). In the double mutant the additive effect apparently results in the restoration of Cu levels similar to those observed in the wild-type. This hypothesis is further supported by the analysis of Cu-dependent SOD activities (Fig. 2B), which give an indirect approximation of stromal and cytosolic Cu levels for these mutants in the absence of direct measurements, which would be very challenging. The relatively higher stromal CSD2 activity in pc2, even in relatively low Cu conditions, indicates that more Cu is available in the stroma in the pc2 mutant compared to the wild-type. Since stromal Cu and CSD2 abundance are well correlated, it might be argued that CSD2 or CCS are somehow involved in PAA2 turnover regulation. However, in a previously characterized CSD2 overexpression line, PAA2 protein abundance resembles that of the wild-type in all Cu conditions that we tested (Fig. 2C). Therefore, CSD2 does not appear to affect PAA2 stability. Interestingly, CCS which was shown to bind to PAA2 in vitro might affect PAA2 protein abundance since a ccs mutant was shown to have slightly

Figure 2. Regulation of PAA2 protein abundance by Cu is evolutionary conserved in Arabidopsis and is controlled by Cu levels in the stroma. (A) Cu-dependent regulation of PAA2 protein abundance in Arabidopsis accessions. L Cu: 0.05 μM CuSO4 and H Cu: 7.5 μM CuSO4. (B) (top panel) The homozygous paa1–3 and pc2 single mutants were crossed and the F2 generation screened for homozygous double mutant offspring.13,18 L Cu: 0.05 μM CuSO4 and H Cu: 7.5 μM CuSO4. *denotes unrelated band. CSD2 protein serves as an indicator for cellular Cu abundance. Fructose-1,6-bisphosphatase (cFBPase) served as loading control.16 (bottom panel) SOD activity profiles as a proxy for subcellular Cu availability in the same plant samples. Total soluble proteins (30 μg) were fractioned on a non-denaturing 15% acryl amide gel and stained for total SOD activity.12 (C) Comparison of PAA2 abundance in the Col0 wild-type and a CSD2-OX (overexpressor) line by immunoblot analysis. L Cu: 0.05 μM CuSO4 and H Cu: 5.0 μM CuSO4. The CSD2 OX line was previously characterized (CSD2ox-1).24 For all immunoblots and SOD activity gels, plants were grown for 18 d in vitro on agar-solidified half-strength Murashige and Skoog medium.16,23 Total protein was extracted from rosette leaves separated by SDS-PAGE, and probed with the indicated antibodies. Methods for plant growth, protein extraction and antibodies have been described.16 Equal loading was ensured through probing of the non-Cu-responsive cytosolic cFBPase.
increased PAA2 protein albeit that Cu still affected PAA2 turnover in this line.\textsuperscript{15,16} Thus even if loss of CCS does not affect PC it is still possible that CCS affects the regulation of PAA2 stability.\textsuperscript{12,16}

Recently, the stromal Clp protease was identified as responsible for Cu-dependent PAA2 turnover.\textsuperscript{20} Clp proteases consist of a proteolytic barrel-shaped core with a relatively narrow entry pore in which the proteolytic active sites are shielded from the surrounding stroma.\textsuperscript{21} Substrates are thought to be threaded into the core by associated Clp chaperones.\textsuperscript{21} Mutants defective in the Clp core and chaperone subunits, strongly over-accumulated PAA2 even when grown in slight Cu excess.\textsuperscript{20} Cu itself did not affect the abundance of the Clp core and it is unlikely that Cu would affect its protease activity in general.\textsuperscript{20} Then how could PAA2 turnover by Clp be linked mechanistically to Cu? As a Cu-transporting P-type ATPase, PAA2 will have to bind its substrate. Both elevated stromal Cu and lack of PC as an acceptor in the \textit{pc2} line will result in a relatively higher proportion of PAA2 interacting with Cu, which we propose then destabilizes the protein by making it a more suitable Clp substrate. The double mutant analysis depicted in Fig. 2 suggests that Cu bound by PAA2 on the stromal site may be critical for the control of its protein turnover (Fig. 3). We propose that an increase of Cu in the stroma and/or increase of Cu bound to PAA2 increases the susceptibility for selection as a substrate by the Clp chaperones leading to its turnover.

**Integrating the 2 Cu Economy Models**

Previous work suggests that in \textit{paa2} mutants more Cu is retained in the cytosol compared to the wild-type, because it was observed that SPL7-mediated down-regulation of cuproproteins is diminished even in Cu-deficient conditions.\textsuperscript{14} Thus the absence of PAA2 appears to affect not only chloroplastic, but also cellular Cu distribution. With the regulation of PAA2 protein turnover by the Clp protease we can now propose an expanded model of Cu homeostasis in Arabidopsis (Fig. 3):
In Cu deficient conditions, SPL7-mediated up-regulation of Cu acquisition and Cu economy are active, while PAA1 transports available Cu into the stroma and the increased abundance of PAA2 further facilitates the prioritization of Cu delivery to PC.10,11 With an increase of the chloroplastic Cu pool, PAA2 protein is turned over in an SPL7-independent manner. This in turn may lead to a feedback regulation, in which Cu is now retained in a central regulator for copper homeostasis in Arabidopsis. Plant Cell 2009; 21:347-61; PMID:19122104; http://dx.doi.org/10.1105/tpc.109.076031.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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