Chapter 10

Trace Metal Utilization in Chloroplasts

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Summary

Redox reactions, which are central to metabolism, depend on redox active functional groups on enzymes such as cysteinyl thiols, organic cofactors like pyridine or flavin nucleotides, or metal cofactors such as Fe, Cu, Mn, or Mo. Accordingly, certain metals are nutritionally essential for life. The green organs of plants display the most descriptive symptoms of metal-deficiency because of the importance of the chloroplast in various metal-dependent redox pathways. The impact of trace element deficiency on chloroplast function at the molecular level has been studied most extensively in microorganisms such as the alga *Chlamydomonas*, for whom the growth media are more readily manipulated. Studies of trace metal distribution and its regulation in cyanobacteria are also relevant to our understanding of chloroplast processes. Fe is the most limiting metal nutrient for all forms of life. In chloroplasts, Fe is found as a redox-active cofactor in FeS centers, heme, mononuclear and di-iron enzymes, and also in ferritin, which functions as an iron "store" and iron "buffer" to maintain intracellular iron homeostasis. The plastid is the key organelle for heme biosynthesis, but FeS cluster synthesis occurs in both plastids and mitochondria. The machinery for cluster synthesis is derived from bacteria, with the process in mitochondria derived from the Isc system and that in plastids containing components of both the Suf system and the Isc system. In iron-deficient chloroplasts, the abundance of iron-containing proteins and specific chlorophyll-proteins is reduced by hierarchical post-transcriptional regulatory mechanisms that may receive signals from iron-dependent enzymes in the tetrapyrrole biosynthetic pathway. The abundant copper enzymes in chloroplasts include plastocyanin and, in some plants, polyphenol oxidase in the thylakoid lumen, and CuZn-superoxide dismutase in the stroma of plants but not green algae. Distributive copper transporters and chaperones are responsible for delivery of the metal to specific sub-organellar compartments. Again, a hierarchical pattern of copper allocation is noted, with plastocyanin receiving copper with higher priority in *Arabidopsis* where plastocyanin is essential, but not in *Chlamydomonas* where a heme protein can substitute for plastocyanin function. A master regulator of copper nutrition called Crr1 regulates degradation of apoplastocyanin in *Chlamydomonas*. The mechanisms of manganese delivery and distribution have not been studied in eukaryotic photosynthetic organisms but, by analogy to metal uptake pathways required for loading Mn-enzymes in bacteria and mitochondria, could involve MntA and MntH/Nramp-like transporters. Mn-deficiency impacts the water oxidation machinery in the chloroplast and also mitochondrial superoxide dismutase.

I. Introduction

A. The Transition Metals Function as Redox Catalysts

Redox reactions are central to metabolism. Biosynthetic reactions involve the reduction of inorganic compounds—CO_2, nitrate, N_2 and sulfate—to more reduced compounds such as carbohydrates, fatty acids and functional groups such as amines, alcohols and thiols. Energy producing reactions involve the oxidation of reduced organic compounds at the expense of a terminal electron acceptor, which is oxygen in most aerobic organisms. The relevant pathways are replete with enzymes that carry redox active cofactors, commonly pyridine and flavin nucleotides or various metal centers. Several transition metals are useful biological catalysts because ionic species of different oxidation states form stable chelates with functional groups found in proteins (Merchant and Dreyfuss, 1998). The use of particular metals in biology does not reflect their abundance in the earth's crust. Those elements that are bioavailable at neutral pH, either because of the high solubility of the aqua complexes of the low oxidations states (e.g. Cu^{2+} and Fe^{2+}) or of the corresponding hydrated oxyanions for the higher oxidation states (e.g. molybdate and vanadate), are used preferentially (Kaim and Schwederski, 1994; Raven *et al.*, 1999).

The use of these metals for catalysis in particular pathways makes organisms that use those pathways dependent on the availability of such metals, leading to the concept of essential or beneficial nutrients (Frieden, 1985; Marschner, 1995). Accordingly, organisms have evolved mechanisms for assimilating the essential metals from the environment, often accumulating them to high levels against a concentration gradient. Because the metal ions are reactive, the assimilation pathways are regulated by supply and demand, and when supply does not meet demand, adaptive mechanisms for

Abbreviations: ATPase – adenosine 5′-triphosphatase; EDTA – ethylenediaminetetraacetic acid; PSI – Photosystem I ; PSII – Photosystem II ; SOD – superoxide dismutase.

conserving, re-distributing and prioritizing the metal nutrient come into play. Based on its amount in an organism, the metal is classified as a micronutrient (e.g. Fe), a trace nutrient (e.g. Cu) or an ultra-trace nutrient (e.g. Se, Mo or Mn). The amount required is organism-specific. Plants carry out different metabolism than do animals, and therefore their micronutrient requirements are distinct.

B. Trace Metal Deficiency Impacts the Chloroplast

The impact of metal nutrition on the chloroplast has long been recognized because the symptoms of metal nutrient deficiency, which invariably include some form of "chlorosis" or chlorophyll-deficiency, are easily visualized in the green organs. There is a substantial amount of descriptive older literature on Fe-, Cu- and Mn-deficient plants, which established the importance of these metals in various metabolic pathways (Marschner, 1995). More recent research has focused on understanding the cell biology of metal homeostasis, and for this purpose, microorganisms such as algae and cyanobacteria have been useful because of the facility with which the growth media can be metal-depleted or fortified, coupled with the possibility of monitoring a large homogeneous population of cells.

C. Metalloprotein Assembly—Thermodynamics vs. Kinetics

It is useful to emphasize that the fundamental biochemical principles that are taught in the context of the so-called "central metabolic pathways" apply also to the metabolism of the metal nutrients. For instance, a requirement for catalysis of metalloprotein assembly was not appreciated historically because metal-binding to apoproteins was known to be a thermodynamically favorable reaction, based on the fact that metalloproteins are usually more stable than their corresponding apoproteins. Also, in the case of FeS centers, the uncatalyzed reaction occurred readily and produced the correct cluster as long as the appropriate reducing conditions were provided (Malkin and Rabinowitz, 1966). Nevertheless, metalloprotein assembly has been documented for many proteins to be selective *in vivo* relative to the uncatalyzed *in vitro* metal reconstitution reactions. Not surprisingly, the rate of assembly *in vivo* is much faster than for the corresponding uncatalyzed *in vitro* reaction. The use of assembly factors *in vivo*, aside from accelerating a specific reaction, also provides a means for regulation of metal cofactor utilization. This is relevant in a cell where a limiting micronutrient like iron may be required both for respiratory chain function as well as for photosynthesis. In this situation, iron delivery pathways to individual organelles allow assembly pathways to respond to metabolic demand. In a multicellular organism, there may also be differentiation of function and hence expression of particular metal-utilizing metabolic pathways in specialized organs or at particular developmental states, which suggests operation of inter-cellular signals for metal nutrient homeostasis. This area of metabolism is presently under-studied, especially in the context of chloroplast function in plants. The interested reader is referred to the work of Raven for an excellent treatment of variation in metal requirement in photosynthetic organisms in response to metabolic demand (Raven, 1988, 1990; Raven *et al.*, 1999).

Another point to consider is the use of equilibrium constants in describing intracellular metal distribution. This treatment is valid only in a system that is at equilibrium, which is distinctly not the case in a living cell. The assimilation and distribution of metal cofactors in most organisms requires an input of energy to maintain the system at a steady state away from equilibrium. This is grasped easily for metal transport (e.g. by metal-transporting P-type ATPases) but it applies also to other steps in metalloprotein assembly, including preparation of the apoprotein substrates (e.g. maintenance of ligand oxidation state) and formation of clusters (e.g. the Mn_4Ca complex involved in oxygen evolution). Theoretical calculations of concentrations of "free" metals or particular metal-ligand complexes based on equilibrium constants must be interpreted with caution because they may not reflect the true dynamic in a cell, which is in a non-equilibrium situation.

D. Fe, Cu and Mn

The metals that are well-studied in the context of chloroplast biogenesis and function are Fe, Cu and Mn because of their abundance in the photosynthetic apparatus (Raven *et al.*, 1999). Also, the corresponding metalloproteins are readily monitored in the holoform by spectroscopic methods, and changes in metalloprotein expression are therefore easy to visualize. Other important metals such as Zn are spectroscopically silent, and hence, much less is known about the biogenesis of Zn-enzymes in the chloroplast despite their prevalence and abundance. The discussion in this chapter is restricted to the impact of Fe, Cu and Mn nutrition

on chloroplast function, especially the photosynthetic apparatus.

II. Fe

A variety of iron enzymes occur in chloroplasts: heme proteins like cytochromes and P450s, soluble and membrane-bound two- and four-iron-sulfur proteins, di-iron enzymes and mononuclear-iron enzymes, and iron bound to ferritin (Jäger-Vottero *et al.*, 1997; Kerfeld and Krogmann, 1998; Merchant and Dreyfuss, 1998; Briat *et al.*, 1999; Froehlich *et al.*, 2001; Berthold and Stenmark, 2003; Gray *et al.*, 2004; Tian and DellaPenna, 2004). These enzymes function in electron transfer reactions of the photosynthetic apparatus and in various redox reactions in pathways that produce secondary metabolites and natural products. Because iron is an actively acquired nutrient that limits most life forms, organisms generally do not excrete iron when it is in intracellular excess beyond what is needed for maintenance of the iron enzymes, but rather store it. The reactivity of iron in an aerobic environment requires that it be stored in a protected form, as in the protein ferritin. When iron is required for de novo synthesis of iron-containing proteins, it can be mobilized from the stored form, and when it is released as iron-containing enzymes are degraded, the store can be re-built. Ferritin is therefore a key component of iron homeostasis.

A. Ferritin

The protein ferritin accounts for stored iron in a plant and is the source of iron for chloroplast development. The reader is referred to substantial reviews by Briat and Theil and their co-workers for details of ferritin chemistry and biology (Briat and Lobréaux, 1997; Briat *et al.*, 1999; Curie and Briat, 2003; Theil, 2003, 2004). The protein consists of 24 subunits that bind up to 4.5×10^3 atoms of iron as ferric-oxy-hydroxide within an internal core. This mineral core is built by movement of iron into the core and oxidation of ferrous to ferric ions by the ferroxidase activity of the ferritin subunits (Lawson *et al.*, 1989). Plant ferritins are distinguished from animal ferritins by being localized within an organelle, the plastid, and by their subunit composition. While animal ferritins consist of two types of related chains, H and L, plant ferritins have a single type of chain. The plant ferritin subunit, encoded by a multigene family, is more closely related to the H-chain but also has features of the L chains that facilitate iron nucleation and yield a stable core (Van Wuytswinkel *et al.*, 1995; Wardrop *et al.*, 1999). The mineral core in plant ferritin is also unique in containing a high proportion of phosphate like bacterioferritins (Waldo *et al.*, 1995). The proteins encoded by the *FER* genes in plants and algae include an N-terminal plastid-targeting sequence in the precursor, and an N-terminal region in the mature protein that is a determinant of stability and protease susceptibility *in vitro* (Ragland *et al.*, 1990; Wardrop *et al.*, 1999; La Fontaine *et al.*, 2002). Interestingly, plastid ferritin is quite distinct from bacterioferritin found in cyanobacteria, which indicates that chloroplast ferritin is a function acquired from the host rather than retained from the endosymbiont (Laulhere *et al.*, 1992).

Ferritin expression is determined by multiple signals because of the nutritional importance of iron, the variation in iron demand at different stages of growth, and the potential for toxicity of iron in an aerobic environment. Ferritin abundance is regulated by changes in RNA abundance through transcriptional regulation and polypeptide abundance through post-transcriptional mechanisms in response to multiple signals, including iron supply, developmental stage, and wounding (Lescure *et al.*, 1991; Lobréaux and Briat, 1991; Ragland and Theil, 1993; Fobis-Loisy *et al.*, 1996; Tarantino *et al.*, 2003). Therefore, it is important to monitor protein abundance for a picture of ferritin action *in vivo*, while RNA abundance only presents a picture of the potential or capacity for ferritin action. Recent studies in animals and plants suggest that ferritin can also be found in mitochondria under certain conditions (Levi *et al.*, 2001; Zancani *et al.*, 2004), which adds another layer of complexity in understanding the biology of ferritin. Another relevant aspect of ferritin function is the amount of iron in the mineral core, which can change during growth and development (e.g. van der Mark *et al.*, 1981), but this has not been studied systematically.

The four ferritin-encoding genes in *Arabidopsis*, *FER1*, *FER2*, *FER3* and *FER4*, show unique patterns of expression in response to iron nutrition, environmental stress and developmental stage, which reinforces the importance of plastid Fe homeostasis for plant growth and physiology (Petit *et al.*, 2001; Tarantino *et al.*, 2003). Ferritin accumulates at high levels in non-green plastids and decreases in abundance as they green, suggesting that ferritin is the source of iron found in heme- and other iron-containing proteins in the photosynthetic apparatus, although the direct movement of labeled Fe from ferritin to an Fe-containing enzyme has not been monitored. As the leaf gets older, the ferritin

content decreases until the organ is at the stage of senescence, when the ferritin content increases again, presumably to accommodate iron that is released from enzymes as the proteins of the chloroplast are degraded. The re-appearance of ferritin is attributed to increased gene expression and de novo synthesis (Tarantino *et al.*, 2003). Ferritin accumulation is also increased under conditions of oxidative stress, which presumably exacerbate the toxicity of iron (Briat *et al.*, 1999; Petit *et al.*, 2001).

Although ferritin accumulation involves post-transcriptional regulatory mechanisms, when a soybean *FER* cDNA was over-expressed in tobacco plants via a 35S promoter-driven construct, ferritin did over-accumulate in the mature plants (Van Wuytswinkel *et al.*, 1998). The plants accumulated more iron but displayed symptoms of iron-deficiency (i.e. inter-veinal chlorosis) even when the ferritin was targeted to the plastid. These studies emphasize the role of plastid ferritin in iron homeostasis and the function of ferritin as an iron "buffer" that keeps iron available in the cell but in a non-toxic form. This work also raised the question of how iron might be mobilized from ferritin. The more abundant ferritin in the over-expressing plants catalyzes and accommodates the over-chelation, but the fact that it is not available for normal chloroplast development indicates that the iron mobilization system can not circumvent the over-chelation. Because oxidation is involved in the deposition of iron in the ferritin core, it is generally assumed that reduction is required for iron mobilization and there are some experiments that support this notion (e.g. Bienfait and van den Briel, 1980). In that *in vitro* study, a connection between copper and ascorbate- and oxygen-dependent iron mobilization from ferritin was noted, suggesting perhaps a role for an enzyme like ascorbate oxidase. Nevertheless, an *in vivo* connection has not yet been established. The models considered for iron release from ferritin in animal cells favor either lysosomal degradation of the protein and/or unfolding of the iron cores, but mobilization of iron from the mineral would still depend on reduction (reviewed by Theil, 2004). Besides serving as a buffer for iron, ferritin is also an important store of Fe for the next generation and does accumulate in seeds (Masuda *et al.*, 2001).

B. Heme, FeS and Fe Cofactor Synthesis

During development of the chloroplast in germinating seedlings, the disappearance of ferritin is correlated with the appearance of iron-containing catalysts such as cytochromes that contain heme (or Fe-protoporphyrin IX) and iron-sulfur proteins (of either the Fe_2S_2 variety as in ferredoxin or the Fe_4S_4 variety as in PSI). It is possible that iron distribution to various enzymes is regulated based on physiological demand for, or importance of, particular metabolic pathways (see below). For an understanding of the principles underlying the regulation, it is first useful to have a description of the iron-utilizing pathways.

The biosynthesis of heme and FeS centers represent major iron utilizing pathways in the cell, and the organelles (mitochondria and plastids) contain an abundance of these redox cofactors. In *Saccharomyces cerevisiae*, inhibition of mitochondrial heme synthesis blocks transcriptional activation of the iron uptake genes, and disruption of iron-sulfur metabolism results in mitochondrial iron accumulation and hence altered cellular iron homeostasis, substantiating the importance of cofactor biosynthesis in iron homeostasis (e.g. Knight *et al.*, 1998; Li *et al.*, 1999; Lange *et al.*, 2000; Crisp *et al.*, 2003).

1. Heme

In fungi and animal cells, the heme biosynthetic pathway is distributed between the cytosol and the mitochondrion. In plants, the entire pathway, from δ-aminolevulinate to Fe-protoporphyrin IX, is localized in plastids, but the last two enzymes—protoporphyrinogen oxidase and ferrochelatase—occur also in the mitochondrion (Chow *et al.*, 1997; Lermontova *et al.*, 1997; Beale, 1999; Watanabe *et al.*, 2001). In *Arabidopsis*, two ferrochelatase-encoding genes, *FC-I* and *FC-II*, are expressed under different conditions (Singh *et al.*, 2002). Interestingly, *FC-I*, whose product is probably localized to both plastids and mitochondria, showed increased expression in leaves upon wounding or upon treatment with salicylic acid, which suggested that an increased potential for heme synthesis, presumably to provide cofactors for induced P450s (see below), is part of the defense response. It is not presently known whether heme found outside the plastid, in peroxisomes (peroxidases), cytosol (hemoglobin), endoplasmic reticulum (P450s and non-heme oxygenases), or the cell wall (peroxidases), is derived from the plastid pool or the mitochondrial pool.

Iron deficiency impacts heme levels and reduces the content of cytochromes in the photosynthetic apparatus (Duggan and Gassman, 1974; Moseley *et al.*, 2002a). Because the tetrapyrrole pathway is feedback regulated by heme, synthesis of δ-aminolevulinate is promoted

under these conditions (Cornah *et al.*, 2003; Franklin *et al.*, 2003).

For many organisms, heme is a source of iron, especially under conditions of nutritional deficiency. Heme or Fe-protoporphyrin IX is oxidized by a mixed function-type oxidase reaction, which requires molecular oxygen and a reductant, to Fe-biliverdin (usually the α isoform) and CO. This reaction, catalyzed by a heme oxygenase, is driven by removal of product through the action of biliverdin IXα reductase (Franklin *et al.*, 2003). The step also releases iron bound to the tetrapyrrole. In animals, fungi and bacteria, a heme oxygenase is a key target of iron deficiency because it releases iron from heme for use in other iron-containing enzymes (e.g. Poss and Tonegawa, 1997; Protchenko and Philpott, 2003; Frankenberg-Dinkel, 2004; Skaar *et al.*, 2004).

The role of ferrochelatase as an iron-utilizing enzyme and heme oxygenase as an iron-releasing enzyme in plastid iron homeostasis is intriguing but not yet analyzed thoroughly in plants. The major role of the plastid heme oxygenases lies in the production of bilins for light harvesting and signaling (Willows *et al.*, 2000; Terry *et al.*, 2002). In a red alga, the gene *pbsA* in the plastid genome, encoding a heme oxygenase, is transcriptionally activated by iron-deficiency (Richaud and Zabulon, 1997). In *Chlamydomonas* neither of the two typical heme oxygenase-encoding genes appears to be transcriptionally regulated by iron nutrition (S. Merchant, unpublished results), and the question of regulation by iron has not yet been addressed for the various heme oxygenases of plants. Nor has the issue of whether plastid heme levels may signal organelle or cellular iron status, as evidently is the case for mitochondrial heme in yeast, been addressed.

2. FeS

a. Discovery and Function of Prototypical Bacterial Nif, Isc and Other Components

The biosynthesis of FeS centers requires mobilization of sulfur from cysteine, mobilization of Fe, assembly of the cluster and transfer of the cluster to apoenzymes (Merchant and Dreyfuss, 1998; Lill and Kispal, 2000; Frazzon and Dean, 2003). Although not all of the molecular events are understood, the necessary components have been defined in many organisms through classical and reverse genetic approaches. The first components were identified in the context of nitrogenase function (called NifS and NifU) and, subsequently, related molecules encoded by *isc* genes in *Escherichia coli* were shown to be responsible for the assembly of clusters in various iron-sulfur proteins (Zheng *et al.*, 1993; Takahashi and Nakamura, 1999). Sulfur is mobilized from cysteine by NifS/IscS in a pyridoxal phosphate-dependent reaction catalyzed by a cysteine desulfurase, which yields alanine and sulfane sulfur. This activity is required also for the synthesis of other sulfur containing compounds in bacteria such as thiamine and thionucleosides. NifU, a three-domain protein, is responsible for building the FeS cluster (Yuvaniyama *et al.*, 2000). An N-terminal iron-binding domain related to IscU is the assembly scaffold and binds a "transient" cluster through three cysteine residues (Agar *et al.*, 2000). IscU interacts with the chaperone system, Hsc66 and Hsc20, encoded in the *isc* operon (Table 1) (Hoff *et al.*, 2000). The middle domain of NifU holds a permanent FeS cluster, presumably with redox function during cluster assembly. The C-terminal domain, called the NFU domain or CnfU, is involved in transferring the FeS cluster to apoproteins. The domain contains a CxxC motif of unknown function (Frazzon *et al.*, 2002). IscA is another cluster assembly component providing a scaffold for transient formation and binding of an FeS cluster, but how it relates to IscU function is not understood (Ollagnier-de-Choudens *et al.*, 2001; Cupp-Vickery *et al.*, 2004). A ferredoxin (itself containing an FeS cluster) is associated with the *isc* operon and is required for cluster biogenesis, presumably for reducing ferric to ferrous iron. This ferredoxin interacts physically with IscA (Ollagnier-de-Choudens *et al.*, 2001).

Interestingly, under anaerobic but not aerobic conditions, a simple system consisting solely of NifS- and NifU-homologues is sufficient for FeS cluster assembly in *E. coli* lacking both the *isc* and *suf* (see below) operons (Ali *et al.*, 2004). This suggests that the additional components of the Isc system evolved to accommodate an aerobic environment.

In a genetic screen for defects in FeS cluster metabolism in *Salmonella enterica*, two new assembly factors were identified, called ApbC and ApbE (Skovran and Downs, 2003). Phenotypic analysis suggests that these proteins are required for the maintenance (repair) or assembly of oxygen-labile FeS clusters in the enzymes ThiH (required for thiamine biosynthesis), succinate dehydrogenase (containing multiple FeS clusters) and aconitase. The specific role of the proteins is not known, but ApbC does

Table 1. Biochemical functions required for cluster biosynthesis in bacteria and organelles. A list of components and types of activities required for FeS cluster assembly in various organisms. See text for details.

Function	Gene
Sulfur mobilization from cysteine	bacterial IscS, NifS
	mitochondrial Nfs1
	bacterial and plastid SufS[3]+SufE
ATP dependent sulfur transfer?	bacterial and plastid SufC+SufB+SufD
ATP-dependent Repair / synthesis of (oxygen labile Fe_4S_4) clusters	bacterial ApbC / Mrp
	cytosolic Cfd1p / Nbp35p
	plastid Hcf101
Cluster assembly and provision of scaffold for assembly	bacterial NifU-N terminus, IscU
	mitochondrial Isu1, Isu2
Cluster assembly	bacterial IscA
	mitochondrial Isa1, Isa2
	plastid ISAI[3]
	bacterial and plastid SufA[4]
Cluster transfer	bacterial NifU-C terminus with conserved CxxC motif
	mitochondrial Nfu1
	plastid NFUs
Reductant	bacterial NifU-permanent cluster in central portion
	bacterial Fdx
	mitochondrial Yah1
Chaperones	bacterial ATP-dependent Hsp70, HscA, Hsc66 +
	bacterial J-type co-chaperone, HscB, Hsc20
	mitochondrial Ssq1 + Jac1
Iron metabolism	mitochondrial frataxin

[3] Also called CsdB in *E. coli* or NifS-Type II.
[4] SufA is related to IscA and the nomenclature used depends on genic context (i.e. whether the gene occurs in a *suf* vs. *isc* operon).

catalyze ATP hydrolysis and the *apb* mutants (unlike *isc* mutants) can be suppressed by exogenous iron. These features are reminiscent of the essential P-loop ATPase Cfd1p in *S. cerevisiae*, which is involved in repair or synthesis of the aconitase cluster in the cytosol, and indeed, Cfd1p and ApbC share sequence similarity (Roy *et al.*, 2003). Cfd1p contains a conserved CxxCxxC motif, of which the first two cysteine residues are functionally important and conserved also in ApbC.

b. Eukaryotic Homologs of Nif and Isc Components Function in Mitochondria

The discovery of homologs in eukaryotes of the proteins encoded in the bacterial *nif*/*isc* operon led to the description of a related machinery in mitochondria, consisting of Nfs1 (related to NifS), Nfu1 (related to the C-terminal domain of NifU), Isu1/2 (related to the N-terminal domain of NifU), Isa1/2 (related to IscA), Yah1 (a mitochondrial ferredoxin) and the molecular chaperone system, Ssq1 plus Jac1 (related to Hsc66 and Hsc20) (Table 1) (Garland *et al.*, 1999; Kispal *et al.*, 1999; Li *et al.*, 1999; Schilke *et al.*, 1999; Jensen and Culotta, 2000; Lange *et al.*, 2000; Mühlenhoff *et al.*, 2002). In *S. cerevisiae*, and probably animals as well, this machinery appears to be the source of clusters for all FeS proteins in the cell (Lill and Kispal, 2000), although additional components, like Cfd1p, Nar1p and Nbp35p, are required for the synthesis of extra-mitochondrial clusters (Roy *et al.*, 2003; Balk *et al.*, 2004).

Nevertheless, several lines of evidence suggested that plastids make their own FeS clusters. First, isolated plastids could incorporate sulfur from cysteine into acid-labile clusters in ferredoxin in a reaction requiring ATP and NADPH (Takahashi *et al.*, 1986; Takahashi *et al.*, 1991a; Takahashi *et al.*, 1991b). Second, newly imported apo-ferredoxin could be converted into the holoform *in vitro* in isolated plastids in the absence of cytosol (Li *et al.*, 1990; Pilon *et al.*, 1992). And third, most of the iron in the plant cell is plastid-localized (see above). Therefore, it was concluded that plant cells must have at least two FeS assembly machineries, one in the mitochondrion and one in the plastid.

c. Plastid Components That are Nif/Isc-Related

Analysis of the *Arabidopsis* genome revealed two *nifS*-related genes. One, At*NFS1*, is suggested to

encode a mitochondrially-targeted protein, and another, At*NFS2*, a plastid-localized one with cysteine desulfurase activity (Kushnir *et al.*, 2001; Léon *et al.*, 2002). Subsequently, five NFU proteins, containing the conserved C-terminal CxxC motif, encoded by At*NFU1* through *NFU5* were described as well as a plastid-localized IscA homolog, AtISA1 (Léon *et al.*, 2003; Yabe *et al.*, 2004). The NFU proteins were distinguished into two sub-types, the NFU1-3 type[1] being plant-specific and proposed to be plastid-localized based on immunodetection, GFP fusions and *in vitro* import studies, and NFU4 and NFU5 being mitochondrial. A T-DNA insert in the *NFU* gene on chromosome V, *CNFU2* or *NFU2* (encoding a plastid-type protein), resulted in decreased abundance of photosystem I, ferredoxin, sulfite reductase, and reduced stromal iron-sulfur cluster assembly activity (Touraine *et al.*, 2004; Yabe *et al.*, 2004). On the other hand, Fe_3S_4 glutamate synthase activity was not reduced, nor was the abundance of the Fe_2S_2 Rieske protein or subunits of the cytochrome b_6f complex reduced, suggesting distinct pathways for plastid iron-sulfur cluster assembly (Touraine *et al.*, 2004).

d. The More Recently Discovered Suf System Functions in FeS Cluster Assembly in Bacteria and Plastids

The Suf pathway was revealed through analysis of suppressors of *E. coli* strains in which the ISC machinery was deleted (Takahashi and Tokumoto, 2002). In the suppressed strain, the Suf pathway, which is important in bacteria under conditions of oxidative stress and iron limitation (Nachin *et al.*, 2003; Outten *et al.*, 2004; Wang *et al.*, 2004), is mis-expressed, allowing the SUF system to cover the loss of the ISC system. Homologs of the SUF components (called SufABCDES) are found in many bacteria, archaea and plastid-containing eukaryotes (Ellis *et al.*, 2001), and the operation of the Suf pathway in *Arabidopsis* plastids was demonstrated recently (Xu and Møller, 2004). As mentioned above, anaerobic conditions facilitate cluster assembly. The corollary is that conditions that favour oxidation reactions make cluster maintenance and assembly more difficult. The use of the Suf system in cyanobacteria and chloroplasts perhaps represents an adaptation to greater oxidative stress in this compartment relative to the mitochondrion.

SufC, a cytoplasmic ABC-type ATPase in bacteria, is a key component of the SUF system because of its high degree of conservation and the severity of phenotype associated with loss of function (Nachin *et al.*, 2003). SufC associates with SufB and SufD to form a complex that is required for "repair" of labile FeS clusters that are damaged during oxidative stress, which may mean energy-dependent insertion of Fe^{2+} into Fe_3S_4 centers.

SufS is related to NifS and in bacteria constitutes one subunit of a cysteine desulfurase. SufS also exhibits selenocysteine lyase activity, which is required for the synthesis of selenoproteins. The second subunit, SufE, enhances the cysteine desulfurase over the selenocysteine lyase activity (Loiseau *et al.*, 2003). The plastid SufS, called CpNifS or AtNFS2, also shows both cysteine desulfurase and selenocysteine lyase activity (Pilon-Smits *et al.*, 2002). Interestingly, the recombinant protein shows only a fraction (1 to 2%) of the activity of the endogenous protein in stromal extracts in the assembly of iron sulfur clusters of ferredoxin (Ye *et al.*, 2004). One possibility is that only a fraction of the recombinant protein is active. Another possibility, raised by the function of SufE in bacteria, is that the *in vivo* reaction involves other factors, such as a plastid SufE-homolog (Xu and Møller, 2004). The latter model is supported by the observation of a high molecular weight CpNifS-containing complex (Ye *et al.*, 2004). SufS and NifS are related, but the key difference may be the dual role of SufS in both Se and S metabolism, dependent on interaction with SufE. SufA is related to IscA and by analogy probably has a role in cluster assembly.

e. Directly Discovered Plastid Components

In a screen for mutants of *Arabidopsis* defective in the assembly of PSI, Meurer and co-workers identified HCF101 as a candidate FeS cluster assembly factor in the plastid stroma (Lezhneva *et al.*, 2004; Stöckel and Oelmüller, 2004). They proposed a role for HCF101 in the assembly of Fe_4S_4 clusters as opposed to Fe_2S_2 clusters based on a drastic decrease in the abundance of PSI reaction center polypeptides PsaA and PsaB, attributable to degradation of the apoproteins as a result of a post-translational block in assembly. A less dramatic increase in the peripheral subunits argued for an effect of HCF101 on cofactor biogenesis and, more specifically Fe_4S_4 centers, owing to a 50% decreased abundance of ferredoxin thioredoxin reductase but not of ferredoxin or the Rieske FeS protein.

[1] The nomenclature of Leon et al. (2003) is used here owing to precedence. The gene names for the mitochondrial forms are at*NFU1* and at*NFU2* and for the plastid forms at*CNFU1* through at*CNFU3* in the work of Yabe et al. (2004).

The sequence relationship between HCF101 and ApbC (Lezhneva *et al.*, 2004), and also between HCF101 and Cfd1p, solidifies its role in FeS biogenesis in the plastid, as does its iron-dependent expression (Stöckel and Oelmüller, 2004), but what specifically that role might be is unclear. HCF101 also contains a conserved CxxC motif (Lezhneva *et al.*, 2004). As appropriate for a gene encoding a PSI assembly factor, *HCF101* is expressed in green organs (Stöckel and Oelmüller, 2004). The *Arabidopsis* genome encodes three different HCF101-like proteins—HCF101, HCF101-L1 and HCF101-L2, but L1 and L2 have not been characterized functionally as yet.

HCF101 is highly conserved with homologs in all kingdoms of life. These have been classified into four groups (Lezhneva *et al.*, 2004). HCF101 itself belongs to Class 1. The Class 2 form (L1 in *Arabidopsis*) is proposed to function in mitochondria based on the presence of an apparent N-terminal pre-sequence. The Class 3 form (L2 in *Arabidopsis*) functions in the cytosol and nucleus based on the location of the yeast representative, Nbp35p (Hausmann *et al.*, 2005), and the Class 4 form is in the cytosol. It is possible also that one of the *Arabidopsis* homologs is a redundant factor in the plastid (accounting perhaps for the weak impact of loss of HCF101 on ferredoxin thioredoxin reductase).

It is likely that a continued classical genetic approach to the study of plastid FeS cluster biogenesis could reveal new components besides those related to the products of the well-studied *isc* operon and the more recently discovered *suf* operon. A recent publication suggests that *Arabidopsis* APO1 may be involved in Fe_4S_4 cluster biosynthesis (Amann *et al.*, 2004), but the pleiotropic impact of loss of APO1 on membrane structure and the absence of homologs in *Chlamydomonas* and cyanobacteria suggest that APO1 may be more generally involved in a development-specific aspect of thylakoid biogenesis.

3. Hydrogenase Fe Cluster

Besides heme and FeS centers, there are other iron-containing cofactors in enzymes, including mono- and di-iron enzymes as well as uncharacterized iron sites (Fox, 1998; Plank *et al.*, 2001; Berthold and Stenmark, 2003; Hausinger, 2004). One of these is the bi-nuclear Fe center of hydrogenase in *Chlamydomonas* (Happe *et al.*, 1994). Genetic analysis of hydrogenase-minus mutants revealed two new enzymes, HydE and HydG belonging to the "Radical SAM" family (jSofia *et al.*, 2001), which are required for production of active hydrogenase (Posewitz *et al.*, 2004). The restricted occurrence of HydE and HydG homologs in Fe-hydrogenase containing prokaryotes is consistent with the proposed function in hydrogenase assembly. By analogy to the biosynthesis of the nitrogenase cluster, the authors of this work suggest that HydE and HydG may be involved in mobilization of iron for assembly of the so-called H-cluster of the [Fe] hydrogenase, which also requires CN, CO and the di(thio-methyl)amine ligand, but they do not rule out a function in in situ generation of the iron-coordinating ligands. Heterologous expression of hydrogenase in *E. coli* requires only the HydE and HydG factors in addition to the gene for the apoprotein, indicating that these are probably the most critical assembly factors.

C. Transport of Iron into Chloroplasts

If ferritin is assembled with iron in the plastid, then there must be a mechanism for iron transport into the chloroplast. Also, under conditions of iron deficiency, it may be necessary to re-allocate iron from one compartment to another (such as the mitochondrion where iron is required for the function of respiratory enzymes), and intracellular transporters are expected to be central to organelle metal homeostasis. However, such molecules have not yet been discovered, although the Nramp transporters are candidates. The Nramp proteins, originally identified in mammals as iron transporters that affect resistance to microbial infection, are considered to be broad specificity, divalent cation transporters (Gunshin *et al.*, 1997). They are encoded in plant and other eukaryotic genomes as multi-gene families with members displaying distinct patterns of expression in response to divalent cation nutrition, suggesting that they may have metal-specific roles *in vivo*. While they are generally considered to be assimilatory transporters, they are found also in intracellular membranes in *S. cerevisiae* (reviewed by Van Ho *et al.*, 2002), and it is possible that they function to deliver metal ions into and out of organelles in plants. NRAMP3 in *Arabidopsis* localizes, e.g., to the vacuolar membrane (Thomine *et al.*, 2003).

In *S. cerevisiae*, members of the carrier family—Mrs3p and Mrs4p—appear to be involved in iron transport across the mitochondrial inner membrane (Foury and Roganti, 2002; Mühlenhoff *et al.*, 2003; Kunji, 2004; Lesuisse *et al.*, 2004). Although the *Arabidopis* genome encodes 58 members of the mitochondrial carrier family, the probability that Mrs3p and Mrs4p homologues would function in chloroplasts is low because the plastid inner envelope transporters tend to be

distinct from the mitochondrial carriers (Flügge *et al.*, 2003; Picault *et al.*, 2004).

D. Fe-Deficiency Impacts the Photosynthetic Apparatus

The abundance of the photosynthetic apparatus in chloroplasts and the numerous iron-containing redox-active proteins therein, plus the loss of chlorophyll proteins as a marker for iron-deficiency, meant that studies of the impact of iron-deficiency on plastid biochemistry have focused on photosynthesis (e.g. Terry, 1983; Terry and Abadía, 1986). Nevertheless, the discovery of di-iron enzymes in desaturation and other fatty acid modification reactions, and in carotenoid synthesis (Cunningham and Gantt, 1998; Shanklin and Cahoon, 1998), and the recent molecular identification of cytochrome P450 enzymes functioning in the biosynthesis of carotenoids, oxylipins and other isoprenoid-derived compounds, indicates the importance of iron in many other physiological processes including the production of defense metabolites (Froehlich *et al.*, 2001; Helliwell *et al.*, 2001; Tian and DellaPenna, 2004).

General principles concerning the impact of iron-deficiency on photosynthesis in plants have not yet emerged because it is difficult to directly compare individual studies with different plant material at different stages of growth and under various conditions of other nutrients. Therefore, our understanding of iron-deficiency adaptation of the photosynthetic apparatus comes largely from studies of microorganisms like cyanobacteria and green algae where iron nutrition can be readily and uniformly controlled (Moseley *et al.*, 2002a; Michel and Pistorius, 2004). Some well-documented changes in response to iron-deficiency in cyanobacteria include the replacement of iron-containing ferredoxin by iron-free flavodoxin, a decrease in the ratio of PSI to PSII from about 4:1 to 1:1, and the de novo synthesis of a new antenna for photosystem I consisting of the IsiA polypeptide or CP43' (Laudenbach *et al.*, 1988; Laudenbach and Straus, 1988; La Roche *et al.*, 1996; Bibby *et al.*, 2001; Boekema *et al.*, 2001). PSI is also a prime target in iron-deficient plants, presumably because of its high iron content (three Fe_4S_4 clusters), but the other two adaptations are not known to occur in chloroplasts (Nishio *et al.*, 1985). The *Chlamydomonas* genome appears to encode several different chloroplast-targeted ferredoxins. Two of these genes show a reciprocal pattern of expression dependent on iron nutrition, but the physiological function of this pattern is not known (N. Fischer and J.-D. Rochaix, personal communication; A. Terauchi and S. Merchant, unpublished results). Because ferredoxin is the source of electrons for many biosynthetic pathways in the plastid, it is possible that synthesis of alternate forms of ferredoxin may determine allocation of reducing power under iron-deficient conditions.

In a recent study with *Chlamydomonas*, a distinction was made between iron deficiency vs. iron-limitation (La Fontaine *et al.*, 2002; Moseley *et al.*, 2002a). *Iron-deficient* cells are defined as those that are not chlorotic but where the assimilator iron uptake genes, *FOX1*, *FTR1* and *FEA1*, are fully induced, whereas *iron-limited* cells are defined as symptomatic (i.e. chlorotic) and the rate of cell division is reduced. In *Chlamydomonas*, a progressive modification of the photosynthetic apparatus was observed. In marginally iron-deficient cells, the LHCI antenna was found to be physically and functionally uncoupled from PSI and this was correlated with an altered association of the PSI-K polypeptide (Fig. 1), which functions to facilitate the transfer of excitation energy from the peripheral

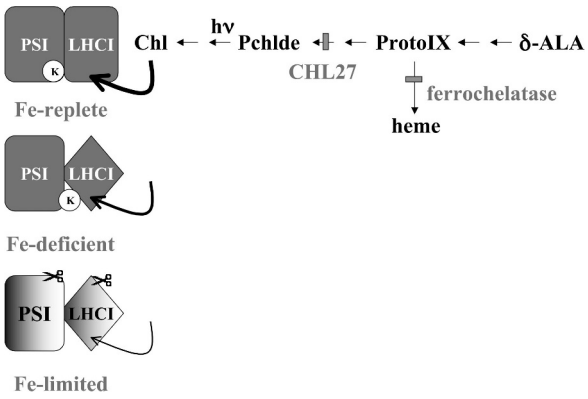

Fig. 1. The PSI-LHCI interaction is dependent on Fe nutrition. In Fe-replete cells, LHCI is physically and functionally associated with PSI in order to optimize energy transfer. As the cells anticipate iron-deficiency (i.e. in the situation where iron uptake is enhanced), the photosynthetic apparatus is modified to decrease excitation energy input into the PSI reaction center. This occurs via a loosening of the interaction between LHCI and PSI through a change in the association of PSI-K with PSI, and also by a change in the polypeptide composition of the LHCI complex (indicated by the change in shape of LHCI from rectangular to rhomboidal). The rationale for this modification is to avoid oxidative stress resulting from loss of iron from labile Fe_4S_4 clusters in PSI. When iron is limiting cell division, despite the expression of high affinity transporters, the abundances of PSI and the LHCI antenna are down-regulated by induced proteolysis to conserve iron (indicated with the scissors symbols). The plastid iron status may be sensed by flux through the tetrapyrrole pathway. For regulating chlorophyll protein abundance, the level of chlorophyll synthesized is relevant, and this is sensitive to iron nutrition because the aerobic oxidative cyclase (CHL27) is an iron-containing enzyme. For regulating heme proteins, the level of heme may be relevant, and this is clearly dependent on iron availability at the step catalyzed by ferrochelatase.

antenna to the reaction center (Jensen *et al.*, 2000). This response was suggested to function as a protective mechanism to avoid photo-oxidative damage resulting from damaged or improperly assembly FeS clusters in PSI. And indeed, iron-deficiency or loss of LHCI antenna can "rescue" the light sensitivity of a *Chlamydomonas* strain lacking PsaF (Hippler *et al.*, 2000; Moseley *et al.*, 2002a).

As iron-deficient *Chlamydomonas* cells progress to iron limitation, specific subunits of the LHCI antenna proteins are degraded, and the abundance of both photosystems and the cytochrome complex is reduced to about 5% of the level maintained in iron-replete cells coincident with a decreased rate of cell division. The abundance of the ATP synthase is unchanged by iron deficiency. Interestingly, the chlorophyll content on a per cell basis is maintained at about 50% of the level found in iron-replete cells, with most of the chlorophyll associated with LHCII complexes that are not associated with a reaction center. This pigment may serve as a reservoir of chlorophyll for de novo synthesis of photosystems during the re-greening process initiated by iron nutrition. Although de novo chlorophyll synthesis does occur even in iron limited cells, this pigment must be selectively allocated to the LHCII antenna.

E. Fe-Sensing

The impact of Fe-deficiency on the biosynthesis of chlorophyll (or chlorophyll proteins) was noted decades ago and is a classic symptom of Fe-deficiency (Bogorad *et al.*, 1958; Machold, 1971; Spiller and Terry, 1980; Terry, 1980). The aerobic oxidative cyclase or CHL27, now known to be a di-iron enzyme, was suggested to be a key target of iron-deficiency (Spiller *et al.*, 1982; Tottey *et al.*, 2003). The loss of chlorophyll proteins in iron-deficiency was therefore attributed to reduced flux through the chlorophyll biosynthetic pathway (Spiller and Terry, 1980). However, as mentioned above, iron-deficiency chlorosis represents a specific re-programming of chlorophyll protein synthesis rather than a general decrease in all chlorophyll proteins. Because a *Chlamydomonas* mutant with reduced cyclase activity recapitulated parts of the program initiated by marginally iron-deficient cells, it was suggested that the cyclase might represent an iron sensor in the plastid (Moseley *et al.*, 2002a). According to this model, the occupancy of the active site iron in CHL27 would be proportional to iron availability in the plastid. As the cell, and hence the organelle, becomes iron-deficient, the rate of chlorophyll synthesis is decreased, leading to restriction of chlorophyll for the de novo synthesis of chlorophyll proteins. If there is a hierarchical allocation of chlorophyll to particular apoproteins (and there is considerable evidence for this), then the program of chlorophyll-protein complex accumulation can be linked to plastid iron status. In the case of PSI, it appears that the stability of PsaK is very sensitive to iron-nutrition status as well as chlorophyll biosynthesis. The model therefore suggests that occupancy of the pigment sites in PsaK determines the association of this polypeptide with PSI, and hence the functional association of the LHCI antenna with PSI (Fig. 1). Nevertheless, a causal connection between chlorophyll binding to PsaK and its assembly with PSI has not yet been established. An attractive aspect of this model is that it distinguishes between iron sensing in the plastid vs. iron sensing in the nucleus to control the expression of iron assimilatory genes. On the other hand, the mechanism is clearly relevant only in the context of the photosynthetic apparatus. Whether other plastid types have mechanisms for signaling and responding to iron nutrition is not known. One can envision a similar mechanism for regulation of heme protein accumulation in all plastid types via the action of ferrochelatase, but so far there are no studies that test this idea.

III. Cu

Three abundant plastid proteins that contain copper are CuZnSOD in the plastid stroma and plastocyanin and polyphenol oxidase in the thylakoid lumen (Jackson *et al.*, 1978; Kieselbach *et al.*, 1998). These proteins are not present in all chloroplasts; the chlorophyte algae (such as *Chlamydomonas*) contain only plastocyanin, *Arabidopsis* has CuZnSOD and plastocyanin, and tomato and spinach have all three proteins. The relative proportion of each protein in a given plant cell depends of course on the organ, the developmental state and environmental conditions (e.g. Sabeeha Merchant, Last and Gray, 1989; Perl-Treves and Galun, 1991; Thygesen *et al.*, 1995; Thipyapong *et al.*, 1997). The use and distribution of copper within plastid compartments and the impact of deficiency on plastid function is therefore likely to vary. Cyanobacteria, like the green algae, do not have CuZnSOD or polyphenol oxidase, but on the other hand, they do have a respiratory copper-containing oxidase.

A. Cu-Protein Assembly

1. Transporters

Once copper is taken up into the plant cell, presumably by a member of the COPT1 family of transporters

(Sancenón *et al.*, 2003; Sancenón *et al.*, 2004), it needs to be distributed to organelles for the biosynthesis of copper-enzymes like cytochrome oxidase, CuZnSOD, and plastocyanin, and to other compartments for the biosynthesis of various intra- or extra-cellular multi-copper oxidases such as ascorbate oxidase and laccase. Some members of the so-called CPx-type heavy-metal transporting ATPases are responsible for intracellular copper distribution (reviewed by Williams *et al.*, 2000). Three of these enzymes have been characterized in *Arabidopsis*. RAN1 is responsible for loading copper in the secretory pathway for the biosynthesis of the ethylene receptor, while PAA1 and PAA2 function to deliver copper to the chloroplast for CuZnSOD and plastocyanin (Hirayama *et al.*, 1999; Shikanai *et al.*, 2003; Abdel-Ghany *et al.*, 2005).

PAA1 and PAA2 carry N-terminal MxCxxC metal-binding domains, CPC ion-transduction motifs, classical P-type ATPase phosphorylation and phosphatase domains, and ATP-binding sites. PAA1 is proposed to localize to the envelope membranes and PAA2 to the thylakoid membrane based on *in vitro* import experiments and localization of GFP fusion proteins (Shikanai *et al.*, 2003; Abdel-Ghany *et al.*, 2005). This distribution is analogous to the localization of two copper transporting P-type ATPases in cyanobacteria, CtaA and PacS. The former is located in the cell membrane and functions in copper acquisition, whereas the latter is located in the thylakoid membrane and functions to deliver copper to the lumen (Kanamaru *et al.*, 1994; Phung *et al.*, 1994; Tottey *et al.*, 2001). The phenotypes of *paa1* and *paa2* mutants are entirely consistent with this model. Plants carrying mutations in *PAA1* show reduced abundance of both plastocyanin and CuZnSOD whereas *paa2* plants have less plastocyanin (Shikanai *et al.*, 2003; Abdel-Ghany *et al.*, 2005). Although copper transporting activity has not been shown for either PAA1 or PAA2, the impact of copper nutrition on the phenotype is consistent with their function in copper transport. Specifically, the phenotype of *paa2* alleles was exacerbated in medium containing low copper and suppressed in medium containing extra copper. Also, while both *paa1* and *paa2* mutants had normal leaf copper content, when metal content was analyzed after sub-cellular fractionation, *paa1* chloroplasts were found to have less copper than those from wild-type, and *paa2* showed less copper in the thylakoid fractions but essentially normal copper content in intact chloroplasts. PAA1 is expressed in all organ types, reflecting a need for copper in all plastid types, while PAA2 expression was detected only in green organs, consistent with a function in photosynthesis (Abdel-Ghany *et al.*, 2005).

Interestingly, the genome of *Cyanidioschizon merolae* lacks a PAA1 or PAA2 homolog, but this is not incompatible with the fact that the organism also lacks a gene for plastocyanin and must use only a *c*-type cytochrome for photosynthesis (see below) (Hanikenne *et al.*, 2005).

2. Chaperones

The concept of a copper chaperone for delivery of copper between proteins developed through genetic analysis of copper homeostasis in *S. cerevisiae* and subsequent analysis of the function of homologous proteins in other organisms (O'Halloran and Culotta, 2000). Yeast Atx1p (homologs known as HAH1, Atox1 and CCH) is a small protein containing a metal-binding site that interacts specifically with the metal-binding site on Ccc2p (a P-type ATPase). By analogy, a stromal chaperone for copper delivery from the envelope to the thylakoid, and perhaps another in the lumenal compartment for delivery from PAA2 to apoplastocyanin, is predicted. In cyanobacteria, an Atx1-related molecule, identified through a two-hybrid interaction with the metal-binding domains of PacS and CtaA, was shown to function as a copper chaperone for plastocyanin and cytochrome oxidase assembly (Tottey *et al.*, 2002). Proteins carrying candidate copper chaperone motifs can be identified in the *Arabidopsis* genome, but these have not yet been analyzed functionally. It is also possible that because of the small size of Atx1-like copper chaperones, some candidate molecules have not been predicted accurately or they have escaped detection because of the less significant BLAST (homology) scores.

A small copper-binding protein, related to a copper homeostasis factor in bacteria called CutA, was shown to be chloroplast-localized (Burkhead *et al.*, 2003). Its physiological function has not been deduced, but it may well function in copper trafficking and recycling (see below).

B. Cu Deficiency and Regulation by Cu

1. Chlamydomonas

Because plastocyanin is the most abundant copper protein in a photosynthetic cell, it is a prime target in the face of copper-deficiency. In cyanobacteria and green algae, there is a well-regulated "back up" system, in which a heme protein, called cytochrome c_6,

is induced in copper-deficiency to compensate for the loss of plastocyanin in the electron transfer chain (Wood, 1978; Sandmann *et al.*, 1983; Merchant, 1998). Accordingly, copper is not essential for photosynthesis in these organisms. The regulatory events have been studied most thoroughly in the *Chlamydomonas* model (Merchant, 1998). The *CYC6* gene for cytochrome c_6 is associated with copper-response elements that serve as binding sites for a transcriptional activator, Crr1, in copper-deficient cells (Quinn and Merchant, 1995; Quinn *et al.*, 2000; Eriksson *et al.*, 2004). In this situation, plastocyanin is rapidly degraded because of the reduced thermodynamic stability and increased protease-susceptibility of the apo-protein vs. the holoprotein, and the Crr1-dependent expression of a degrading activity (Merchant and Bogorad, 1986; Li and Merchant, 1995; Eriksson *et al.*, 2004). In *crr1* mutants, apo- and some holo-plastocyanin accumulate even in copper-deficient cells, owing presumably to the lack of the protease. The *crr1* mutation, therefore, by affecting the "salvage" of copper from plastocyanin, has an impact on respiratory growth in addition to its impact on photosynthesis owing to loss of *CYC6* expression (S. Tottey, S. Nakamoto, J. Kropat and S. Merchant, unpublished results).

Besides regulating plastocyanin and cytochrome c_6 abundance, Crr1 also controls the expression of *CPX1* and *CHL27A*/*CHL27B*, encoding oxygen-dependent enzymes (coproporphyrinogen oxidase and aerobic oxidative cyclase) in the tetrapyrrole biosynthetic pathway (Eriksson *et al.*, 2004). *CPX1* encodes a plastid-targeted isoform that is about 10- to 20-fold up-regulated in copper-deficiency, while the expression of *CPX2*, encoding possibly a mitochondrial isoform, is unaffected by copper (Hill and Merchant, 1995; J. Kropat and S. Merchant, unpublished results). Copper nutrition and Crr1 reciprocally regulate the expression of *CHL27A* and *CHL27B* (Moseley *et al.*, 2000; Moseley *et al.*, 2002b). Both isozymes are plastid-localized but they may be differently distributed between the envelope and thylakoid membranes within the plastid (M. Allen and S. Merchant, unpublished results). The rationale for *CPX1* and *CHL27A*/*CHL27B* regulation by copper is not known, but it does point to a previously unrecognized connection between copper and the tetrapyrrole pathway.

2. Arabidopsis

Copper-deficiency has not been studied systematically in *Arabidopsis* but the work on PAA1 and PAA2 function revealed that the standard medium for *Arabidopsis* growth in the laboratory is probably slightly copper-deficient (Abdel-Ghany *et al.*, 2005). The addition of copper to that medium stimulates the accumulation of both plastocyanin and CuZnSOD with a more noticeable effect for SOD. The authors concluded that under conditions of copper limitation there is preferential allocation of copper to plastocyanin (for which there is no substitute in *Arabidopsis*) vs. CuZnSOD (for which there is a substitute). In fact, the expression of FeSOD is increased to compensate for loss of CuZnSOD.

3. Redistribution of Copper

Several lines of evidence indicate that metals can be redistributed from the chloroplast to other organelles or even secreted from the cells. When copper-replete *Chlamydomonas* cells become deficient, copper is reallocated from plastocyanin in the chloroplast to cytochrome oxidase in the mitochondrion (S. Tottey, S. Nakamoto, J. Kropat and S. Merchant, unpublished results). In vascular plants, the copper content of senescent tissue decreases. This process is correlated with an increase in the content of a copper chaperone, CCH, in the vascular tissue (Mira *et al.*, 2001). These processes probably require the action of transporters, chaperones or copper-binding proteins to move copper from a stable intracellular site in a protein, but the relevant molecules have not yet been identified and the process has not been subject to genetic analysis.

IV. Mn

Mn is nutritionally essential for all living organisms (Frieden, 1985; Marschner, 1995). It functions as a redox catalyst because it can occur stably in a cell in many different oxidation states, and this is its role in Mn-containing SOD and in PSII. Mn^{2+} can also activate water to generate a strong nucleophile for hydrolytic reactions (as in the enzyme arginase) or it can stabilize a leaving group (as in the nucleotide products of a glycosyl transferase reaction), but its role in these types of reactions in the plastid are not specifically described in the literature.

A. Manganese Transport

The bulk of the manganese in a photosynthetic cell is found in PSII in the chloroplast lumen. The mechanism of assembly of this cluster is not well understood even though PSII biogenesis has been subject to considerable genetic dissection in both cyanobacteria

and *Chlamydomonas* (Pakrasi, 1995). Pakrasi and coworkers approached this problem in the *Synechocystis* model and discovered the MntABC system for manganese ion uptake into bacterial cells. In more recent work, they show that cyanobacteria contain two pools of manganese, a storage pool that is released upon treatment with EDTA but whose maintenance is energy-dependent, and a second pool in PSII that is derived from the storage pool (Keren *et al.*, 2002). By analogy, there must be mechanisms for Mn^{2+} transport into the chloroplast across the inner envelope membrane plus a mechanism for transport across the thylakoid membrane. The identity of the transporters in the chloroplast is unknown. The MntABC system appears to be strictly bacterial, indicating the operation of another system for chloroplasts.

The Nramp proteins (reviewed by Williams *et al.*, 2000; Forbes and Gros, 2001) are excellent candidates for a manganese delivery system to plastids. These molecules are proton-coupled divalent cation transporters that show broad substrate specificity in many *in vitro* experiments but it is likely that some members of the gene family are Mn^{2+}-specific *in vivo*. The bacterial homologs of the Nramps, called MntH, indeed appear to be Mn^{2+} selective (Kehres and Maguire, 2003) and all three Nramp homologs in *Chlamydomonas* show increased expression only in response to manganese- but not iron- or zinc-deficiency (S. Tottey, J. Kropat, E. del Rio and S. Merchant, unpublished results). Plant genomes contain multiple Nramp homologs with functionally distinct roles based on sub-cellular location, organ-specific pattern of expression, metal specificity, and pH sensitivity (Belouchi *et al.*, 1997; Curie *et al.*, 2000; Thomine *et al.*, 2000; Thomine *et al.*, 2003). While some members of the family are likely involved in iron homeostasis, others could function in manganese metabolism. But the role of plant *NRAMP* expression and function in manganese nutrition has received less attention.

A possibility for manganese acquisition by the plastid, hinted at by the intracellular organelle localization of Nramp homologs Smf1p and Smf2p in *S. cerevisiae* (reviewed by Van Ho *et al.*, 2002) is that one or more Nramps may be involved. In this context, it is worth noting that mitochondria also have a significant manganese requirement (e.g. for MnSOD) and the question of allocation of manganese to plastids vs. mitochondria in plants has not been addressed. In *S. cerevisiae*, a member of the carrier family has been proposed as a facilitator for mitochondrial manganese acquisition for MnSOD biogenesis (Luk *et al.*, 2003). It is not known whether Mtm1p is actually a Mn^{2+} transporter. The mitochondrial carriers are evolutionarily distinct from most of the known plastid inner envelope translocators, and so it does not necessarily follow that a homolog of *S. cerevisiae MTM1* would function in plastid Mn-protein assembly. The diversity of Mn^{2+} transporters known in nature—MntA, MntH and perhaps the Mtm1p carrier—leaves open the possibility that a completely novel molecule operates in the plastid.

A recent comparative analysis of algal genomes revealed members of the cation diffusion facilitator family (called MTP proteins in plants) that may be manganese transporters, and it is suggested that one or more of these molecules could be plastid-localized (Hanikenne *et al.*, 2005).

B. Manganese Deficiency

The importance of manganese in the photochemical reactions of photosynthesis was recognized half a century ago because of the impact of Mn deficiency on phototrophic growth and oxygen evolution in algae (Pirson, 1955). The symptoms of Mn-deficiency in plants are noted as leaf discoloration, which implies an impact at the level of the chloroplast, but the biochemical consequences of deficiency have not been investigated. Mn-deficient *Chlamydomonas* cells show loss of PSII and MnSOD activity and a sensitivity to peroxides but not paraquat or Rose Bengal (S. Tottey, M. Allen, J. del Campo, J. Kropat and S. Merchant, unpublished results). Whether the oxidative stress occurs at the level of plastid or mitochondrion redox metabolism is not known.

V. Questions for Future Investigation

The metabolism of the transition elements is intimately inter-related. For instance, in many organisms, including algae, fungi and mammals (although not plants), a copper-containing enzyme is required for high affinity iron uptake (Askwith and Kaplan, 1998; La Fontaine *et al.*, 2002). Therefore, copper-deficiency generates secondarily an iron-deficiency. A connection between copper and zinc metabolism is known in humans, where excess zinc in the diet blocks copper intake (Kumar *et al.*, 2003). Recently we noted a role for manganese in iron assimilation in *Chlamydomonas* (S. Tottey, M. Allen, J. del Campo, J. Kropat and S. Merchant, unpublished results). The use of microarray and proteomic approaches to study metal homeostasis at a whole genome level is ideal for the discovery of such inter-relationships.

In a recent microarray study on iron-deficient yeast, the concept of metabolic re-modeling was noted where certain iron-utilizing pathways are down-regulated in favor of parallel pathways that are less iron-dependent (Shakoury-Elizeh *et al.*, 2004). This phenomenon is well known in the context of the photosynthetic apparatus where flavodoxin can substitute for ferredoxin or cytochrome c_6 for plastocyanin, and for the SODs where Mn-SOD is up-regulated to compensate for the loss of CuZnSOD in the copper-deficient rat (Hutber *et al.*, 1977; Wood, 1978; Merchant and Bogorad, 1987; Bottin and Lagoutte, 1992; Lai *et al.*, 1994). It is possible that there are back-up systems for other metalloenzymes in nature and these may also be discovered through the whole genome analyses.

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