30

Comparative Analysis of Copper and Iron Metabolism in Photosynthetic Eukaryotes vs Yeast and Mammals

Sharon La Fontaine, Jeanette Quinn, and Sabeeha Merchant

1. INTRODUCTION

Copper and iron are essential micronutrients for all organisms because of their function as cofactors in enzymes that catalyze redox reactions in fundamental metabolic processes. Prominent examples of such enzymes include cytochrome oxidase in respiration, plastocyanin in photosynthesis, superoxide dismutase in oxidative stress, and ceruloplasmin in iron metabolism. Copper and iron carry out very similar functions in biology because both exhibit stable, redox-interchangeable ionic states with the potential to generate less stable electron-deficient intermediates during multielectron redox reactions involving oxygen chemistry. The major difference between copper and iron in biological systems derives from their individual ligand preferences and coordination geometries. The bioavailability of copper and iron is low so that organisms are faced with the challenge of acquiring sufficient copper and iron for cellular requirements while avoiding the buildup of levels that could lead to cellular toxicity. Over the last decade, it has become apparent that organisms have developed a suite of strategies to combat such challenges, so that an intricate balance between uptake, utilization, storage and detoxification, and efflux pathways for copper and iron exists.

A paradigm for copper and iron metabolism in eukaryotic cells has been developed through studies with the baker's yeast *Saccharomyces cerevisiae* (reviewed in refs. *1* and *2*). Genetic analysis of iron transport in *S. cerevisiae* has revealed a dependence of iron metabolism on adequate copper nutrition and this molecular connection has provided valuable insight into the basis for the well-established link between copper and iron nutrition in mammals. With the objective of comparing copper and iron metabolism in photosynthetic eukaryotes with that in yeast and mammals, this chapter summarizes the present state of knowledge of the relevant pathways in yeast and mammals, especially the basis for the copper–iron link, followed by a discussion of copper and iron metabolism in the model photosynthetic organisms *Arabidopsis thaliana* and *Chlamydomonas reinhardtii* and concludes with evidence for a copper–iron link in Chlamydomonas. The relevant pathways in other model organisms such as *Drosophila* and *Caenorhabditis elegans* will not be covered in this chapter, as our objective is to provide a background for comparison to plants rather than to embark on a comprehensive review of these processes in all model organisms.

From: Handbook of Copper Pharmacology and Toxicology
Edited by: E. J. Massaro © Humana Press Inc., Totowa, NJ

2. YEAST COPPER–IRON LINK

2.1. Copper

2.1.1. Uptake

In *S. cerevisiae*, high-affinity copper uptake is mediated by the *CTR1* and *CTR3* gene products (Ctr1p and Ctr3p, respectively) *(2–5)* and is associated with copper reductase activity of Fre1p and Fre2p to reduce Cu^{2+} to Cu^{1+} prior to uptake *(6,7)*. *CTR1*, *CTR3*, and *FRE1* are transcriptionally activated in response to copper deficiency by the copper-dependent transcriptional activator Mac1p *(2,8–10)*. Copper-dependent endocytosis of Ctr1p at low copper concentrations and degradation of Ctr1p in copper-replete cells provides an additional posttranslational level of regulation *(11)*. Three putative low-affinity copper-transport systems also have been described: Fet4p *(12)*, Smf1/Smf2 *(13,14)*, and Ctr2 *(15)*.

2.1.2. Distribution, Storage, and Regulation

Inside the cell, copper is bound by cytoplasmic copper chaperones and delivered to specific destinations within the cell. These chaperones include Cox17p, Lys7p, and Atx1p, for copper delivery to cytochrome oxidase in the mitochondria, cytosolic and mitochondrial superoxide dismutase, and the copper transporter Ccc2p in the secretory pathway, respectively *(2,16–20)*. Ccc2p is required for loading copper onto Fet3p, a multicopper oxidase, required for high-affinity iron transport across the plasma membrane *(21)*. The chloride-ion channel Gef1p, located in the same compartment, is also required for copper loading onto Fet3p *(22,23)*. Sco1p and Sco2p were identified as integral membrane proteins within the inner mitochondrial membrane and may be involved in the transfer of copper from Cox17p to cytochrome oxidase *(5,24,25)*.

With increased intracellular copper levels, copper-storage proteins such as the metallothioneins encoded by *CUP1* and *CRS5* are induced and sequester the copper *(8)*. Storage of copper in the vacuole may be an additional mechanism of preventing copper toxicity *(5)*.

Mac1p and Ace1p are the two copper-responsive transcriptional activators in *S. cerevisiae (2,9,10)*. Under copper-deficient conditions, Mac1p activates the expression of six genes, *CTR1*, *CTR3*, *FRE1*, *FRE7*, *YFR055w*, and *YJL217w*. With elevated copper levels, Ace1p activates the expression of copper-detoxification proteins, Cup1p and Crs5p.

2.2. Iron

2.2.1. Uptake

Iron uptake into *S. cerevisiae* involves several different assimilatory pathways depending on the chemical source of iron and its concentration. In general, all of the pathways require reduction of Fe^{3+} to Fe^{2+} by one or more products of the *FRE1–FRE7* genes *(14,26)*. Under iron-limiting conditions, high-affinity uptake is mediated by the inducible Fet3p/Ftr1p complex in the plasma membrane *(1,14,27)*. Fet3p is a multicopper oxidase *(28)* whose active sites are related to multicopper oxidases such as laccase, ascorbate oxidase, and ceruloplasmin. Fet3p functions to oxidize Fe^{2+} to Fe^{3+} at the cell surface; Fe^{3+} is then delivered from Fet3p to the associated Fe^{3+} permease, Ftr1p, for transport into the cell *(14,27)*. Highly related pathways operate in other fungi. In *Schizosaccharomyces pombe*, Frp1 is related to the Fre reductases, and Fio1/Fip1 forms an iron-uptake complex related to Fet3p/Ftr1p *(29)*. The fungal pathogen *Candida albicans* also uses a similar pathway, involving a surface-associated reductase, Cfl1p *(30)*, two Ftr1p-related permeases, CaFtr1 and CaFtr2 *(31)*, and a Fet3p-like component *(30)*. Expression of the permeases and their associated oxidases is regulated by iron nutritional status *(27,29)*, whereas some reductases are regulated by iron status and others by both copper and iron—the latter category functioning in both copper and iron assimilation *(10,14)*. Under iron-replete conditions, low-affinity systems, such as Fet4p,

with broad transition metal specificity *(12,32)*, or Smf1/Smf2, which were originally identified as manganese transporters *(13)*, operate. When iron is available in complex with siderophores, it can enter cells either through the Fet3p/Ftr1p complex after reduction by the Fre proteins and subsequent release of the iron from the siderophore, or if the Fet3p/Ftr1p is inoperable, by the ARN family of siderophore transporters through an endocytic pathway *(33–38)*.

2.2.2. *Distribution, Storage, and Regulation*

Whereas the Fet3p component of iron assimilation is conserved from yeast to man, the mechanism of iron storage appears diverse. *S. cerevisiae* lacks genetic information for ferritin *(8)*; rather, iron (with other ions) is stored in the vacuole *(39,40)*. A homolog of the oxidase–permease complex Fet3p/Ftr1p, designated Fet5p/Fth1, was localized to the vacuole and proposed to function under iron starvation conditions to mobilize stored iron from the vacuole *(41)*.

Another key organelle in iron metabolism is the mitochondrion. Not only is it an important target site for iron utilization, but it is also a major player in the maintenance of cellular iron homeostasis. Yfh1p regulates mitochondrial iron accumulation, possibly at the level of efflux *(42,43)*, Ccc1p limits mitochondrial iron uptake *(44)*, Nfs1p is involved in maintaining or synthesizing cytosolic Fe–S proteins *(45,46)*, Atm1p is a transporter of the inner mitochondrial membrane *(47)*, and Ssc2p is a protein of the Hsp70 class of chaperones with a role in Yfh1p maturation *(48)*. Although there are as yet no defined chaperones for the delivery of iron to specific intracellular targets, the IscA family of proteins are candidates for iron delivery to sites of iron–sulfur cluster assembly *(49)*.

Iron uptake in *S. cerevisiae* is regulated in response to cellular iron levels via the iron-responsive transcriptional activator Aft1p. Under iron-limiting conditions, Aft1p-mediated activation leads to induction of *FRE1, FRE2, FTR1, FET3, FET5*, and *FTH1*, as well as the induction of expression of the genes involved in copper transport, *CCC2* and *ATX1 (8)*. A further level of regulation of iron uptake exists that is mediated through Tpk2, a catalytic subunit of the yeast A kinases (PKA) *(50)*. According to the proposed model, during fermentative growth on glucose Tpk2, activated by cAMP, represses genes involved in iron uptake, but as the glucose is depleted, Tpk2 activity is inhibited, thus relieving the repression of the iron-transport genes. This derepression allows iron transport into the cell for incorporation into respiratory enzymes and permits growth on nonfermentable carbon sources.

2.3. *Copper–Iron Link*

In *S. cerevisiae*, the copper–iron link is evident at both the level of protein biosynthesis and function and at the level of gene regulation. Copper is required for the assembly and function of the high-affinity Fet3p/Ftr1p iron-uptake complex, and thus for high-affinity iron uptake. Therefore, *S. cerevisiae* cells that are copper deficient are also iron deficient. The iron-uptake genes are regulated as a function of iron availability, so that the expression of these genes is activated in cells that are starved for iron. Because the Fre1 reductase functions in copper and iron uptake, its expression is induced by both copper and iron deficiency, whereas the copper-transport genes (*CCC2* and *ATX1*), whose activity is critical for the production of functional iron-uptake proteins, are induced by iron deficiency, but not by copper deficiency *(51–54)*. In addition, in *S. pombe*, a copper-responsive transcription factor, Cuf1, activates *CTR4* gene expression for copper uptake under copper-starvation conditions while mediating repression of the iron-uptake genes under these same conditions to prevent futile expression of these genes when there is insufficient copper cofactor available to produce functional iron-uptake proteins *(55)*. Therefore, an intricate system of interplay among transport, storage, chaperone and regulatory proteins exists to maintain copper and iron homeostasis in *S. cerevisiae* and *S. pombe*.

3. MAMMALIAN COPPER–IRON LINK

Although evidence for a physiological link between copper and iron metabolism in mammals was elucidated approximately 75 yr ago *(56,57)*, the molecular basis for such a link is only now beginning

to unfold. The discovery of ceruloplasmin *(58)*, the ceruloplasmin homologs Fet3p in yeast *(28)* and Hephaestin in mice *(60)* and a role for all of them in iron metabolism have been pivotal in furthering our understanding of the copper–iron connection in mammals *(1,59)*.

3.1. Copper

3.1.1. Absorption

The first step in the proposed scheme for copper transport through the body is the absorption of dietary copper through the intestinal mucosa *(61,62)*, although the precise mechanism of copper absorption is not known. A candidate protein for copper uptake into intestinal cells is hCtr1, a homolog of the yeast high-affinity copper transporter Ctr1p *(63)*. Recently, the mouse homolog was shown to be essential *(64)*. Inside the cell, copper is likely to be bound by the human counterparts of the yeast intracellular copper chaperones, Atox1 (Hah1), hCox17, and hCCS, for copper delivery to the secretory pathway, mitochondria, and Cu/Zn-superoxide dismutase (SOD), respectively *(5,17,18,65–67)*. In intestinal enterocytes and the majority of nonhepatic cells, Atox1 delivers copper to the Ccc2p homolog, the Menkes protein (MNK) located at the trans-Golgi network (TGN) *(66)*. MNK, in turn, transports copper into the TGN lumen for incorporation into copper-dependent enzymes that are transported by the secretory pathway, such as lysyl oxidase *(68)*. An additional role of MNK is in the efflux of excess copper from the cell, which is achieved through a mechanism whereby copper concentrations above a certain threshold level stimulate a steady-state shift in the distribution of MNK from the TGN to the plasma membrane *(69,70)*. Under normal steady-state conditions, MNK continually recycles between the TGN and plasma membrane *(71,72)*. Therefore, MNK located at the basolateral surface of intestinal epithelial cells may also serve to transport copper across the basolateral membrane into the bloodstream.

3.1.2. Distribution

Albumin, the major copper carrier in the circulation, transports copper via the portal blood to the liver *(73)*. Here, the MNK counterpart and homolog of Ccc2p, the Wilson protein (WND), is also located at the TGN and relocalizes in elevated copper to an unidentified vesicular compartment, possibly en route to the biliary canalicular membrane *(74–76)*. In the liver, WND functions to excrete the excess copper into the bile *(77)* and to transport much of the copper that enters hepatic cells into the TGN lumen for incorporation into ceruloplasmin *(78)*. The ceruloplasmin holoenzyme, which contains six copper atoms *(79)*, is secreted from the liver and carries > 95% of total serum copper for delivery to tissues *(80)*. In addition, ceruloplasmin, via its ferroxidase activity, mediates iron release from the liver, also for delivery to tissues *(81)*.

3.1.3. Regulation

In mammals at a whole-body level, copper absorption from the small intestine is, to some extent, regulated, with increased uptake when the animal is copper deficient and decreased uptake when the diet is adequate in copper *(82)*. Details of the molecular mechanisms regulating absorption remain to be elucidated. The control of copper excretion, which is also an important part of the protection against excess copper exposure, is regulated by the liver, with excess copper being disposed of through the bile *(83)*. Within the cell, excess copper is detoxified by at least two mechanisms; sequestration by metal-binding proteins such as metallothioneins (MT) and enhanced export by the copper ATPases, MNK, and WND *(84)*. Although a definitive biological role for MTs remains elusive despite years of research, MTs are known to be small cysteine-rich proteins that bind heavy metals, including copper, zinc, and cadmium, through cysteine thiolates *(85,86)*. There are 17 genes encoding four isoforms of mammalian MTs (MT-1 to MT-4), each of which have subtypes. Induction of MT synthesis occurs at the level of transcription by heavy metals themselves (Zn^{2+}, Cu^{2+}, and Cd^{2+}) through metal-regulatory elements (MREs) tandemly arranged within the 5′ flanking regions of MT genes *(87,88)*. In contrast, the regulation of copper export by MNK and WND occurs at a post-translational and enzymatic level that couples copper efflux to the intracellular copper concentration *(69,74,75,77)*.

3.2. *Iron*

3.2.1. *Absorption*

The current model of iron uptake and utilization begins with the absorption of iron from nutritional sources across the intestinal epithelium. Iron, taken into the body as Fe^{3+}, is reduced to Fe^{2+} by Dcytb and perhaps other as yet unidentified ferrireductases and transported across the apical surface of enterocytes lining the gut by the divalent metal-ion transporter DMT1 (DCT1, Nramp2) *(89–92)*. The HFE protein has a role in regulating intestinal iron absorption *(91,93,94)*. HFE associates at the cell surface with β_2-microglobulin and the transferrin receptor, but the significance of these associations remains to be fully elucidated *(91)*. However, the discovery of a reciprocal regulation of HFE and DMT1 *(95)* led to the proposal that HFE regulates trafficking of DMT1 to the plasma membrane and therefore iron uptake, depending on cellular iron concentrations *(96)*. Inside the cell, Fe^{2+} is incorporated into iron-containing proteins, bound to ferritin for storage and detoxification, or it is directed to the mitochondria for heme synthesis and assembly of nonheme iron proteins (97). The Frataxin protein regulates mitochondrial iron accumulation although its precise function remains to be defined *(1)*. Although chaperones for delivering iron to intracellular targets are believed to exist, these putative chaperones have not been identified *(98)*.

3.2.2. *Distribution*

Iron also is transported across the basolateral surface of enterocytes into the blood for transport throughout the body. This export of iron from the enterocyte is mediated by a basolateral transporter Ferroportin1 *(99)*/IREG1 *(100,101)*, although the species of iron transported has not been established. Iron is carried throughout the body, fulfilling the physiological iron requirements of cells and tissues, bound to the plasma protein transferrin. The loading of iron onto transferrin requires oxidation from Fe^{2+} to Fe^{3+}, which may be mediated by either or both of the multicopper ferroxidases, Hephaestin and ceruloplasmin. Hephaestin may act together with Ferroportin1 at the basolateral surface of enterocytes to oxidize Fe^{2+} to Fe^{3+} prior to export into the plasma *(102)*. Alternatively or in addition, plasma ceruloplasmin oxidizes Fe^{2+} to Fe^{3+} subsequent to export into blood plasma for loading onto transferrin. An additional important role of ceruloplasmin is the mobilization of iron from tissues such as the liver where ceruloplasmin is synthesized *(59)*. In cells other than those of the intestine, iron is taken up bound to transferrin. The transferrin receptor at the cell surface binds diferric transferrin and is internalized and taken up into endosomes *(103)*. The Fe^{3+} is released from transferrin into the acidified endosome and reduced to Fe^{2+} *(97)*, followed by transport into the cytoplasm by DMT1 *(89,104)*.

3.2.3. *Regulation*

The regulation of intestinal iron uptake is mediated through iron-dependent transcriptional and translational control of DCT1 *(105)*. The iron concentration within the cell is controlled by reciprocal regulation of ferritin and the transferrin receptor at the posttranscriptional level. This regulation is mediated by iron-regulatory proteins (IRPs) 1 and 2 that bind iron-responsive elements (IREs) located in the 5' and 3' untranslated regions of ferritin and transferrin receptor mRNAs, respectively, to modulate the amount of each protein that is produced based on the cellular iron status *(90,91,97,106–108)*. IREs or IRE-like elements have been identified in the UTRs of other proteins, including an IRE-like element within the 5' UTR of the IREG1 mRNA and the 3' UTR of DMT1 mRNA *(90,91,106)*, but regulation of DMT1 and IREG1 by mechanisms involving this element have not been characterized.

3.3. *Copper–Iron Link*

Although the existence of a copper–iron link was documented in nutritional studies many decades ago, the discovery of the molecular basis for this link is relatively recent. In particular, the multicopper ferroxidases form the basis of the copper–iron connection. Ceruloplasmin is central to interorgan copper and iron metabolism. The copper atoms carried by ceruloplasmin not only serve to satisfy the

copper requirements of body tissues but are also critical for its catalytic function as a ferroxidase for iron mobilization and release from the liver for transport around the body. The assumption is that copper is also critical for the ferroxidase function of Hephaestin, mediating iron absorption from the small intestine into the bloodstream. Hence, iron metabolism is critically dependent on copper both at the level of absorption from dietary sources and transport around the body.

Clearly, there are parallels between the yeast and human copper–iron transport systems that extend to the fact that the dependence of iron metabolism on copper has been maintained over a vast evolutionary distance. Many of the metabolic catalysts are conserved, such as Atx1p/Atox1, Ccc2p/Menkes–Wilson ATPases, Fet3p/ceruloplasmin–Hephaestin, Yfh1p/Frataxin, Ctr1p/hCtr1, Cox17p/hCox17, Lys7/CCS, and the Sco proteins; yet others are distinct between yeast and man, such as Ftr1p vs IREG1 or DMT1, and Dcytb vs the Fre reductases.

4. COPPER–IRON LINK IN PHOTOSYNTHETIC ORGANISMS

Besides having the same copper and iron requirements as human and yeast cells, photosynthetic organisms have additional requirements for these metals in the plastid, where they are utilized as the redox active cofactors in many metabolic processes, including, in particular, the proteins involved in photosynthetic electron transfer in the chloroplast. Iron-containing cytochromes, Fe–S centers (>20 atoms per PSII/PSI unit), and copper-requiring plastocyanin are abundant proteins in photosynthetic organisms. Metal metabolism in this context presumably requires a completely separate set of factors in addition to those required for cellular and mitochondrial iron and copper metabolism. A further challenge for photosynthetic organisms is that biologically useful levels of soil copper and iron are low. The variability in the mineral composition of soils means that for plants to survive and grow they must adapt to the variation in supply of copper and iron. As with yeast and humans, the essential elements of copper and iron metabolism include transport, storage and detoxification, and utilization. The transport process can be further subdivided into reduction, uptake, and intraorganellar and intracellular transport. The storage and detoxification of copper and iron may involve not only sequestration by cellular proteins but also compartmentalization into vacuoles and plastids, which, in turn, requires proteins for transport into these compartments. Most of our knowledge about copper and iron metabolism in photosynthetic organisms comes from studies on the vascular plant *Arabidopsis thaliana*, the unicellular green alga *Chlamydomonas reinhardtii*, and the cyanobacterium *Synechocystis* 6803, which serves as a model for chloroplast biology. The biology of metal metabolism in these organisms is ripe for study because the mechanisms of copper and iron homeostasis in photosynthetic organisms are less well characterized than in humans and yeast.

4.1. Arabidopsis

Arabidopsis thaliana is a small weed in the mustard family, formally known as the family Brassicaceae. It has become an invaluable tool for studying plant physiology, biochemistry, development, and molecular genetics, and joins organisms such as *Escherichia coli*, yeast, *Drosophila*, *Caenorhabditis elegans*, and mice as vehicles for providing insight into fundamental biological processes.

4.1.1. Copper: Transport and Distribution

Copper assimilation and its regulation in plants is largely uncharacterized. Two approaches are commonly used: the identification of genes or expressed sequence tags (ESTs) encoding metal transporters based on sequence relationships, and functional complementation of well-characterized *S. cerevisiae* mutants.

4.1.1.1. COPT1

One of the first Arabidopsis genes encoding a copper transporter to be identified by the latter approach was *COPT1*, which encodes a highly hydrophilic protein that was capable of rescuing a *S. cerevisiae ctr1* strain defective in high-affinity copper uptake *(15)*. In copper-replete plants, *COPT1*

Table 1
Copper Transporters, Chaperones, and Homeostasis Factors in *Arabidopsis thaliana*

Gene	*Homolog*	*Putative function*	*Experimental evidence*	*Reference/ accession*
COPT1	Functional homolog of *S. cerevisiae CTR1*	Copper transporter; distributive	Rescues *S. cerevisiae ctr1* mutant	*15*
COPT1 homologs	*COPT1*	*Copper transporter*	Based on sequence similarity	CAB51175 chromosome 2, section 151
RAN1	Wilson/Menkes proteins, *S. cerevisiae CCC2*	ATP-dependent intracellular copper transporter	Rescues *S. cerevisiae ccc2* mutant	*110*
RAN1 homolog	*RAN1*	ATP-dependent copper transporter	Based on sequence similarity	AAF19707
PAA1	Wilson/Menkes proteins, *Synechococcus PacS*	ATP-dependent copper transporter in plastid	Based on sequence similarity	*111,112*
COX17 homologs	Yeast, mammalian *COX17*	Copper chaperone *(cytochrome* oxidase assembly factor)	Based on sequence similarity	*113* BAB02169 chromosome 1 and BAC F8L10
CCS1 homologs	Yeast *LYS7*, mammalian *CCS1*	Cu/Zn-SOD copper chaperone	Rescues *lys7* mutant; binds metal	*114* AF061517 AF179371 BE038022
CCH	Yeast *ATX1*, mammalian *ATOX1*	Copper chaperone	Rescues *S. cerevisiae atx1* mutant	*115*
CutA homolog	*E. coli CutA*	Copper homeostasis protein; plastid localization	Binds copper; is imported into plastids	AF327524 *112,116*
SCO1 homolog	Yeast, mammalian *SCO1*	Copper transport	Based on sequence similarity	AAF07830
COX11 homolog	Yeast, mammalian *COX11*	Cytochrome oxidase Cu_B center assembly factor; possible copper chaperone	Based on sequence similarity	AAG00893

is expressed in flowers, stems, and leaves, but not in roots *(109)*, suggesting that the gene product might function in distribution rather than in assimilation. The expression of *COPT1* in copper-deficient plants has not yet been analyzed. Two sequences, highly related to *COPT1*, are evident in the complete genome of Arabidopsis; the genes seemed to be expressed, but their function in metal metabolism has not been determined (Table 1). Perhaps one of these may represent an assimilatory pathway component. Two less closely related sequences that are not known to be expressed also were identified.

4.1.1.2. RAN1 AND THE CPX FAMILY OF METAL TRANSPORTERS

The family of ethylene receptors comprises an important function for copper in plants. These molecules use copper as a cofactor at the binding site for this gaseous plant signaling molecule *(117)*. Genetic analysis of the ethylene signaling pathway in Arabidopsis led to the identification of the *RAN1* locus, which encodes a copper transporter related in sequence and in function to *CCC2* of *S.*

cerevisiae (118,119). In plants, RAN1 is proposed to function by analogy to Ccc2p to load copper proteins such as the ethylene receptors. Phenotypic analysis of a strong *ran1–3* allele suggests that RAN1 may function also to load copper proteins in addition to the ethylene receptors.

A homolog of RAN1 has been annotated in the Arabidopsis genome as a copper-transporting ATPase, but its functional relationship to RAN1 is not known (Table 1). The occurrence of highly related sequences in the EST database for other plant species suggests that the RAN1 homolog is expressed. Williams and co-workers *(120)* have noted that the Arabidopsis genome encodes additional CPx-type metal-transporting ATPases, including PAA1 identified previously as a putative plastid-localized copper transporter *(111)*, HMA1 and HMA4, and more recent database analyses reveal yet more sequences in this family. A reverse genetic approach to their study should add much to our knowledge of plant metal-transport mechanisms.

In the meantime, cyanobacteria, which are useful models for chloroplast biology, have been used to deduce a role for CPx-type transporters in metal delivery to the plastid *(121–125)*. Two such proteins, PacS located in the thylakoid membrane *(121)* and CtaA *(122)*, were found to be required for copper delivery to plastocyanin in the thylakoid lumen, and by analogy, these authors proposed that similar proteins might function in the plastid thylakoid membrane and envelope, respectively, for delivery to plastid copper enzymes *(125)*. PAA1 is one good candidate for a plastid-localized copper transporter *(111)*. Synechocystis mutants disrupted for PacS and CtaA function displayed an increased requirement for iron *(125)*, suggesting a connection between copper and iron metabolism in this organism and, perhaps by extension, in plastids as well (*see* Section 4.2.).

4.1.1.3. Copper Chaperones

A copper metallochaperone, designated Atx1, was recently identified in *Synechocystis* PCC6803 and shown to interact with the CtaA copper importer to acquire and then deliver copper to the PacS copper ATPase (*125a*). An Arabidopsis homolog of a copper chaperone (mammalian *ATOX1* and Saccharomyces *ATX1*) was identified as a gene that was upregulated during senescence (Table 1). This gene, called *CCH*, can functionally complement an *atx1* mutant yeast strain for both antioxidant and copper-chaperone activity *(115)*, and the encoded protein may also function in intercellular copper transport in plants *(126)*. Because *CCH* is induced during senescence, a role in sequestering copper following degradation of copper-containing proteins has also been proposed. *RAN1* is upregulated during leaf senescence as well, suggesting that CCH and RAN1 may function together to recycle copper during senescence *(127)*. Genes encoding functional metallothioneins had been identified previously in *A. thaliana (128)*. One of these, *MT1*, is induced during leaf senescence and also in leaves with higher than normal copper concentrations, whereas another, *MT2*, is expressed in leaves prior to senescence. It is suspected that these metallothionein-like proteins may function in copper homeostasis to prevent copper toxicity from copper released during degradation of senescing leaf proteins *(127)*.

Homologs of two other copper chaperones, Cox17p and Lys7p/CCS1, also are found in the Arabidopsis genome (Table 1). CCS1, which binds metal *(114)*, is proposed to be targeted to the plastid as is appropriate because Cu,Zn-SOD is present in this organelle in plants. These homologs can complement the appropriate Saccharomyces mutant, suggesting that the pathways for copper delivery are conserved between plants, mammals, and fungi, but it should be noted that their function and subcellular localization in plants has not yet been studied. It is certainly possible that the plant homologs have analogous rather than homologous functions. Genome analysis has revealed Sco1 and Cox11 homologs as well (Table 1), but these have not been subject to experimental analysis at any level.

A number of other sequences and putative open reading frames with the chaperone-like metal-binding site MxCxGC (or simply a CxxC metal-binding site) can be found in the Arabidopsis genome. One family of these proteins has been characterized and consists of C-terminal prenylated proteins that bind transition metals *(129)*. Because these proteins might be membrane associable, one hypoth-

esis is that they may function in copper delivery to extracellular plasma membrane or secreted copper proteins. Another possibility is that they might be involved in some other aspect of metal homeostasis, such as metal sequestration for resistance.

4.1.2. Iron: Acquisition and Storage

4.1.2.1. Copper-Idependent Acquisition

All plants must extract iron from insoluble Fe^{3+} complexes in the soil and convert it to the more soluble Fe^{2+} form for transport into the cell. Two types of iron acquisition mechanisms have been described for plants: Strategy I and Strategy II. Neither pathway seems to require a copper-containing ferroxidase as noted for animals, fungi (*see* Sections 2.2. and 3.2.) and algae (*see* Section 4.2.2.). Nevertheless, the mechanisms of iron movement within the plant, from the roots to the shoots, is not well characterized at the molecular level, and there is some indication that copper deficiency can cause a localized iron deficiency. Therefore, a connection with copper may yet be discovered.

In Strategy I plants (dicotyledons and nongraminaceous monocotyledons), Fe^{3+} is released from chelates by reduction and Fe^{2+} is taken up by a Fe^{2+}-specific transporter. A putative ferric chelate reductase-encoding gene, *FRO2*, was identified on the basis of its relationship to yeast (*FRE*) and mammalian (gp91phox) homologs *(130)*. *FRO2* is upregulated in iron deficiency and complements *frd1* mutants. Roots of the *frd1* mutants display impaired ferric chelate reductase activity and iron uptake *(131,132)*; hence *FRO2* is a key component of iron assimilation. Several homologs of *FRO2* are found in the Arabidopsis genome (Table 2); their function is not known yet but it is assumed that they participate in iron assimilation or distribution within the plant. A cytochrome-b_5 reductase, encoded by *NFR*, with Fe^{3+}-chelate reductase activity also was cloned from maize and a homolog was identified in Arabidopsis *(138)*. Because the protein is localized to the tonoplast, its role in assimilation is questioned, but a role in intracellular iron or copper homeostasis is still open *(140)*. Nevertheless, genetic evidence for NFR function in metal metabolism is not yet available. In addition to iron reduction, iron solubility in the rhizosphere is increased through activation of a specific H^+-ATPase. The gene *AHA2* encodes one member of a large family of H^+-ATPases in Arabidopsis; it is upregulated under iron deficiency and therefore assigned function in iron assimilation *(109)*.

Two types of iron transporters are known in plants: IRT1 and the NRAMP family (Table 2). IRT1 was identified independently by functional complementation of *S. cerevisiae fet3/fet4* or *smf1* mutants *(133–135)*, whereas the NRAMP family from plants was identified on the basis of their homology to fungal and mammalian NRAMPs and tested for function by complementation of *fet3/fet4* or *smf1 S. cerevisiae* mutants *(136,137)*. In Arabidopsis, *IRT1* is expressed in roots and is upregulated under iron-deficient growth conditions, implying a role in Fe uptake *(133)*. IRT1 is one member of a large family of divalent cation transporters called the ZIP family that is distributed widely in nature *(135)*. The NRAMP family of transporters *(120)*, including NRAMP 1, 3, and 4, is also implicated in iron assimilation by virtue of their increased expression in iron-deficient plants and their ability to rescue *fet3/fet4* mutants *(136,137)*. Both the IRT1 and NRAMP type of transporters seem to have broad metal specificity for divalent, cations but their physiological specificity may well be more narrow. Because both types of transporters take up Fe^{2+}, a role for a ferroxidase (as in mammals, fungi, and algae) is not evident.

The Strategy II iron acquisition mechanism is found in grasses (graminaceous monocotyledons). The roots of these plants secrete phytosiderophores which chelate Fe^{3+}, and a siderophore transporter, YS1, takes up the resulting complex *(139,141)*. YS1, which was cloned from an *Ac*-tagged *ys1* allele, belongs to the major facilitator superfamily. The mutants display interveinal chlorosis that can be rescued by direct application of iron to the leaves or via the xylem after the roots are excised *(142)*. Like the enzymes involved in phytosiderophore biosynthesis, iron–phytosiderophore uptake is induced under iron-deficient conditions through increased expression of the *YS1* gene. Arabidopsis contains 8 *YS1*-like genes, designated *YSL1* through *YSL8*, but whether they all function in siderophore metabolism remains to be determined *(139)*.

Table 2
Iron Assimilation Components in *Arabidopsis thaliana*

Gene	*Homolog*	*Function*	*Experimental evidence*	*Reference*
IRT1	ZIP family	Iron transporter	Rescues *S. cerevisiae ftr3/4*double mutant; expressed in roots; induced in Fe deficiency	*133–135*
Nramp1, Nramp3, Nramp4	Nramp family	Iron transporters	Rescues *S. cerevisiae ftr3/4* double mutant; increase in mRNA accumulation in Fe deficiency	*136,137*
FRO2	*S. cerevisiae FRE1, S. pombe FRP1,* and mammalian gp91phox	Iron-chelate reductase	Increased accumulation of *FRO2* mRNA in Fe deficiency; complements *frd1* mutant	*130*
FRO2 homologs	*FRO2*	Iron-chelate reductases	Based on similarity to *FRO2*	*130*
NFR homolog	Maize *NFR*	Iron reductase	Based on sequence similarity	*138*
YSL1-8	Maize *YS1*	Siderophore uptake	Based on sequence similarity	*139*

4.1.2.2. Iron Storage

Iron is stored within plants in ferritin, which is localized in the plastids in the stroma *(143)*. Ferritin is found primarily in roots and leaves of young plants with much lower levels in mature plants *(144)*. The native molecule consists of 24 subunits of a single type of chain that is related to animal ferritin chains with an additional plant-specific N-terminal sequence in the mature protein known as the extension peptide. The plant ferritin chain has the iron oxidation site found in animal ferritin H chains and also the carboxylates of the L chain that are responsible for efficient mineralization of the core.

Ferritin has a storage function; it accumulates in seeds during maturation and is mobilized by degradation of the protein during germination *(141)*. The abundance of ferritin in the stroma of the plastid decreases as chloroplasts develop, and this corresponds to the movement of iron from the stroma to the thylakoid membrane where iron-containing electron-transfer proteins reside. Ferritin is found only at low levels in mature plants. When ferritin is incorrectly expressed in mature plants, it results in iron-deficiency chlorosis, confirming that ferritin sequesters iron and supporting the model that ferritin serves as an iron reservoir for iron supply to the photosynthetic apparatus in developing leaves *(145)*. Ferritins probably function also for transient iron storage (e.g., as is necessary during senescence or other situations where iron proteins are degraded) *(144)*. This might explain the anomalous finding that mRNAs encoding ferritin are upregulated during Fe deficiency; in Fe-deficiency-induced chlorosis, Fe might be released as PSI is degraded (*see* Section 4.2.2.2.). Thus, plant ferritin accumulation is regulated at the level of transcription in response to iron levels among other stimuli *(146)*. In soybean, transcriptional regulation is mediated by an iron-regulatory element (FRE) and iron-sensing trans-acting factor *(147)*, whereas in *A. thaliana* and maize, an iron-dependent regulatory sequence (IDRS) was recently identified within the promoter region of the respective ferritin genes (*AtFer1* and *ZmFer1*) and shown to be involved in iron-responsive transcriptional control *(148)*.

4.1.3. Copper–Iron Link

Evidence in support of a definitive link between copper and iron metabolism in vascular plants remains to be firmly established. It is well accepted that ferric reductase is specifically induced in response to iron deficiency. However, in pea plants, copper deficiency also stimulates elevated root ferric reductase activity, as well as rhizosphere acidification, another response that is induced by iron deficiency *(149,150)*. Cohen et al. *(149)* suggested that the copper–iron-deficiency-induced activity derives from a single ferric reductase enzyme and, furthermore, that the copper deficiency causes a localized iron deficiency in the plant, which consequently leads to induction of the ferric reductase activity, a scenario that parallels the human and yeast copper–iron link.

In Arabidopsis, iron deficiency leads to an increase in both ferric reductase activity and copper-chelate reductase activity at the root surface *(131)*. Both activities are absent in *frd1-1* mutants *(131)* and are restored in transgenic plants expressing *FRO2 (130)*. Although these data suggested that the ferric reductase can also reduce Cu^{2+} to Cu^{1+}, the accumulation of copper (as well as manganese and zinc) was not inhibited in *frd1-1* mutants, leading to the conclusion that other factors must be involved in regulating divalent cation influx *(131)*.

Much is still to be learned about the complex mechanisms underlying copper and iron homeostasis in plants, from uptake by the roots to transport into, and storage and utilization by the cells of the various plant tissues. Although molecular approaches to identify and functionally characterize plant genes and proteins are proceeding at a rapid pace, significant insights into plant metal metabolism also can be gained from studying other model photosynthetic organisms such as the unicellular green alga, *Chlamydomonas reinhardtii*.

4.2. Chlamydomonas

The unicellular green alga *Chlamydomonas reinhardtii* is also a genetically tractable organism that can be grown on very highly defined media consisting of simple salts, which facilitates studies involving trace micronutrients such as copper. Therefore, it has served as a model for the analysis of copper metabolism in the context of the photosynthetic apparatus. The organism grows heterotrophically as well as photoautotrophically, so that mutants affected in metal supply to the photosynthetic apparatus can be maintained and analyzed *(151)*. Under photoautotrophic conditions, the chloroplast is a sink organelle for metal micronutrients like copper and iron, whereas under heterotrophic conditions, copper and iron are distributed to both the mitochondrion as well as the plastid. The mechanisms for metal homeostasis involving the plastid are only beginning to be studied. The recent generation of an EST database for *C. reinhardtii* has provided an opportunity for comparative analysis of metal metabolism in this organism relative to plants and animals.

4.2.1. Copper: Adaptation to Deficiency

4.2.1.1. Plastocyanin vs Cytochrome-c_6

Chlamydomonas reinhardtii has provided a fascinating system for the study of biological compensation for copper deficiency since Wood (1978) *(152)* recognized that the organism remains photosynthetically competent in copper-deficient medium by replacing an abundant copper protein, plastocyanin, with an iron-containing cytochrome, cytochrome-c_6. Our laboratory has since discovered that this occurs through regulated proteolysis of apoplastocyanin (so that the protein is degraded in –Cu cells but accumulates as the holoprotein in +Cu cells), and transcriptional activation of the *Cyc6* gene (encoding cytochrome-c_6) *(153,154)*. Interestingly, the degree of transcriptional activation is proportional to the perceived copper deficiency *(155)*. In a fully copper-supplemented medium (> 400 n*M* for a late log-phase culture), the photosynthetic apparatus maintains several million molecules of plastocyanin per cell. When the medium copper concentration falls below the amount necessary to provide copper at this stoichiometry, the *Cyc6* gene is activated to the extent necessary to compensate for the loss of plastocyanin. Genetic analysis of plastocyanin mutants (*pcy* strains) demonstrated that the activation of the *Cyc6* gene responded to perception of copper levels rather than to the perception of holoplastocyanin deficiency because strains lacking plastocyanin (e.g., frame shift

or stop codon in the structural gene for preapoplastocyanin) still display copper-responsive *Cyc6* expression *(156,157)*. Therefore, a copper sensor must exist in this organism.

4.2.1.2. Copper Assimilation

In addition to the regulation of alternate (Cu vs Fe) carriers for the photosynthetic apparatus, there are other adaptations to copper deficiency. As is the case generally for nutrient deficiencies, an assimilatory pathway is activated in copper deficiency *(158)*. This pathway involves a reductase component and a transporter. Because the pathway is activated coordinately (with respect to medium copper concentration) with *Cyc6* expression, it is likely that the assimilatory components are downstream targets of the same signal transduction pathway. When medium copper content is measured in Chlamydomonas cultures grown in copper-deficient versus copper-sufficient or copper-excess conditions, one notes that there is no residual copper in the medium as long as this nutrient is below that required to fill the active site of all copper enzymes. Once the copper content of the medium exceeds that required to saturate the copper enzymes, net copper uptake ceases and the excess copper remains in the medium. The mechanism underlying this tight homeostatic control is not known. Is there a balance between an influx versus efflux transporter? Or is the uptake transporter regulated at multiple points (e.g., posttranslationally and also transcriptionally) to achieve this level of control? In this context it should be noted that activation of assimilatory pathways puts the organism at risk for toxicity when the nutrient is resupplied. Hence, it is reasonable to imagine multiple levels of control operating to balance intracellular copper levels in the face of deficiency on the one hand and overload on the other.

Chlamydomonas does not seem to have genetic information for metallothioneins and probably uses only phytochelatins for detoxification of copper, cadmium, mercury, and other heavy metals *(159–161)*, although it is possible that the metallothioneins of Chlamydomonas have not yet been discovered. Perhaps genomics will provide the answer to this question.

4.2.1.3 Other Targets

Three other plastid-localized enzymes are regulated by copper availability in Chlamydomonas: coproporphyrinogen oxidase (Cpx1) encoded by *Cpx1 (161)*, and two putative diiron enzymes encoded by *Crd1* and *Cth1 (162)*. The function of the latter is not known, but one possibility is that they are required for iron metabolism in the plastid. Because Chlamydomonas uses a multicopper oxidase for iron assimilation (*see* Section 4.2.2.), copper-deficient cells probably have to devise mechanisms for bypassing this step in order to remain iron replete. The existence of such mechanisms is evident from the finding that copper-deficient cells are not iron deficient *(158)*. Indeed, *crd1* mutants behave as if they exhibit a localized iron deficiency in the plastid. Crd1 and Cth1 are hypothesized to have similar functions, whereby Cth1 is used in copper-replete cells that are not iron challenged, whereas Crd1 is used in copper-deficient cells that may be adapting to a less effective iron-assimilating pathway owing to reduced plasma membrane ferroxidase activity.

Coprogen oxidase catalyzes a step in tetrapyrrole biosynthesis, leading to heme and chlorophyll in the plastid. The increased activity of coprogen oxidase in –Cu cells has been attributed to an increased demand for heme (e.g., for cyt-c_6 synthesis) in this situation. Nevertheless, this remains an unsatisfactory explanation because the fraction of the tetrapyrrole pool (various chlorophylls and hemes) in cyt-c_6 is very small. With the discovery of a copper-requiring enzyme in iron assimilation and the logical corollary of a copper-independent backup pathway operating in copper-deficient cells, one must re-evaluate the function of *Cpx1* in –Cu cells. Increased expression of the gene does not result in relocalization to a different compartment, such as the mitochondrion *(163)*. One possibility is that increased flux through the tetrapyrrole pathway draws iron into the plastid.

The EST database has revealed several other copper metabolizing proteins (Table 3). For some of these, the function can be predicted by analogy to the function of the homologs, but for others, such as CutA and CutC homologs, genetic analysis is required. In the meantime, expression studies and subcellular localization will provide some clues.

Table 3
Homologs of Copper Transporters, Chaperones and Other Copper-Metabolizing Factors in *Chlamydomonas reinhardtii*

Gene	*Homolog*	*Putative function*	*Experimental evidence*	*Accession*
Ccc2	Arabidopsis *Ran1*	Copper transport	Based on sequence similarity. Blast score (EST): vs *A. thaliana* RAN1= 1e-8; vs Menkes protein = 2e-6	BE761354
Atx1	Arabidopsis *CCH* and *S. cerevisiae Atx1*	Copper chaperone/ delivery for Ran1/Ccc2	Rescues *S. cerevisiae atx1* mutant; transcripts induced up to 6.5× in Fe deficiency. Blast score: vs *A. thaliana* CCH =3e-13 vs *S. cerevisiae* Atx1p = 8e-4	AF280056
Cox17	Human and Arabidopsis *Cox17* homologs	Copper chaperone for cytochrome oxidase assembly	Rescues *cox17* mutant weakly. Blast score: vs human Cox17 = 1e-11 vs *A. thaliana* = 2e-11 vs *S. cerevisiae* = 2e-4	AF280543
Sco1	Related to *S. cerevisiae Sco1* and its Arabidopsis homolog	Copper delivery to cytochrome oxidase	Based on sequence similarity. Blast score (EST): vs *A. thaliana* = 2e-30; vs *S. cerevisiae*/human = 5e-22	AV620545
Cox11	Related to *S. cerevisiae Cox11* and its Arabidopsis homolog	Cytochrome oxidase Cu_B center assembly factor	Based on sequence similarity. Blast score (BE129228): vs *A. thaliana* = 9e-29; vs human = 5e-31 vs *S. cerevisiae* = 2e-29	BE129228 AV631011
CutA	Arabidopsis, human *CutA*	Copper homeostasis protein	Based on sequence similarity. Blast score (EST): vs *A. thaliana* = 3e-36 vs human = 6e-26 vs *E. coli* CutA = 2e-19	BE237847
CutC	Human, *E. coli CutC*	Copper homeostasis protein	Based on sequence similarity. Blast score (EST): vs human homolog = 2e-37 vs *E. coli* CutA = 2e-28	AV387727

4.2.1.4. Hypoxic Expression of Copper-Deficiency Targets

Cyc6, *Cpx1*, *Crd1*, and *Cth1* are each regulated also by hypoxia *(162,164)*. A simple model to explain this observation is that hypoxic cells are copper deficient, but this was ruled out by demonstrating that hypoxic cells make holoplastocyanin *(165)*. Further, the expression pattern of these genes in response to –Cu vs $-O_2$ is not the same; *Cpx1* and *Crd1* are more strongly activated by hypoxia

than by copper deficiency, whereas the converse is true for *Cyc6*. Promoter analysis of *Cpx1* identified a hypoxia-responsive element that is not required for the nutritional copper response, strengthening the argument for separation of the two responses. For the *Crd1* gene, the hypoxic response is shown to be physiologically significant because the *crd1* mutant recapitulates the copper-deficiency chlorotic phenotype, indicating that Crd1 function is required for adaptation to hypoxia. The mechanism of hypoxia perception in Chlamydomonas and its relationship to copper sensing is not known, but insights may emerge when the molecular details of Crr1 (the copper response regulator, *see* below) are revealed. Because copper deficiency and hypoxia probably co-occur in nature, it may be that the ability to respond to either signal allows the organism to anticipate and, hence, adapt more rapidly to copper deficiency.

4.2.1.5. A Master Regulator

Genetic analysis of the nutritional copper sensor led to the identification of two mutants at the *CRR1* locus that are blocked in activation of all of the above genes (Eriksson and Merchant, unpublished). Because these genes are not activated by copper deficiency or by hypoxia in these mutants, it was concluded that Crr1 must be involved in both signal transduction pathways. Whether Crr1 functions also in mediating the response to copper toxicity has not been tested.

4.2.2. Iron: Assimilation and Deficiency

4.2.2.1. Iron Assimilation

As in other organisms, iron uptake by Chlamydomonas involves reductases *(158,166–168)*. The reductases are induced in iron deficiency and may be the same enzyme as that induced in –Cu cells *(158)*. The EST database does not reveal homologs corresponding to the Saccharomyces *FRE* genes nor to the Arabidopsis *FRO* genes, but a putative homolog of *NFR* has been identified (Table 4). It should be a simple matter to assess its role in metal metabolism by testing its expression in copper- or iron-deficient cells.

The discovery of a multicopper oxidase encoded by *Fox1* in Chlamydomonas with significant similarity to mammalian ceruloplasmin and hephaestin was startling *(169,170)*. First, it had been assumed that iron assimilation in Chlamydomonas was independent of copper because copper-deficient cells do not appear iron deficient. Second, multicopper oxidases have not previously been implicated in iron metabolism in plants. A role for the *Fox1* gene product in iron assimilation is suggested by its pattern of expression, which shows greater than 100-fold induction under iron-deficient conditions, the parallel expression of an Ftr1-like permease *(169)*, and the localization of Fox1 to the plasma membrane by Buckhout and co-workers *(170)*. Components potentially involved in loading the oxidase with copper, such as the Ccc2 transporter and the Atx1 metallochaperone homologs (Table 3), also are found in Chlamydomonas. The latter is induced under –Fe conditions, albeit not as strongly as *Fox1* and *Ftr1*. Ccc2 and Atx1 homologs of Chlamydomonas are more related to the plant homologs RAN1 and CCH, respectively, than to the human or Saccharomyces counterparts (Table 3). Ccc2 and Atx1 of Chlamydomonas and plants probably function to load a variety of copper-containing enzymes in the secretory pathway in addition to Fox1. In plants, RAN1 is required for loading copper into the active site of the ethylene receptor *(118)* and perhaps other enzymes as well. Loss of RAN1 is not lethal in the plant, probably because of the occurrence of a homolog (Table 1). In Chlamydomonas, Ccc2/Ran1 is likely to be required for loading Fox1, but there may also be other copper proteins that are substrates for Ccc2/Ran1.

4.2.2.2. Iron Deficiency

One of the characteristic symptoms of iron deficiency in a photosynthetic organism is the development of chlorosis (chlorophyll deficiency). When heterotrophic Chlamydomonas cells are transferred from the usual medium (18 μM iron–EDTA) to iron-deficient medium (0.1 μM iron–EDTA), photosystem I and light harvesting proteins are lost (Moseley, et al., unpublished). Iron assimilation components are activated prior to development of chlorosis, which occurs only if assimilatory mechanisms remain

Table 4
Homologs of Iron Transporters and Iron-Metabolizing Enzymes in *Chlamydomonas reinhardtii*

Gene	*Homolog*	*Putative function*	*Experimental evidence*	*Reference/ accession*
Fox1	Related to mammalian ceruloplasmin and Hephaestin	Ferroxidase required for iron assimilation or distribution	Transcripts induced up to 400× in Fe deficiency; plasma membrane localized Blast score: vs hephaestin =1e-131 vs ceruloplasmin =1e-113	Unpublished; *170*
Ftr1	Related to *C. albicans* CaFtr1	Fe^{3+} transporter	Transcripts induced up to 230× in Fe deficiency; 2× RExxE motifs in cDNA Blast score (partial cDNA sequence): vs *C. albicans* CaFtr1 =4e-10	Unpublished
Fer1	Related to plant and animal ferritins	Iron storage in plastid	Based on sequence similarity; increased expression in Fe deficiency. Blast score (assembled sequence): vs *A. thaliana* ferritin 1 =7e-47 vs Xenopus ferritin H chain 2 = 4e-30	AV632653 AV619384 BE227588 AV623127 AV395232
Nramp homolog	Arabidopsis *Nramp*	Divalent metal ion transport	Based on sequence similarity. Blast score (assembled sequence): vs *A. thaliana* NRAMP1 = 4e-22	AV625564 AV389837 BE452312 BE452998
IscA1	*Azotobacter vinelandii IscA*	Iron–sulfur cluster assembly	Based on sequence similarity. Blast score (assembled sequence): vs *A. vinelandii* IscA = 2e-22	AV618923 AW721159 BE725819 BE725818 BE726308 BE726307
IscA2	*Azotobacter vinelandii IscA*	Iron–sulfur cluster assembly	Based on sequence similarity. Blast score (assembled sequence): vs *A. vinelandii* IscA = 9e-94	AV636381 AV633541 AV386551 BE725609
IscU	*Pseudomonas aeruginosa IscU*	Iron–sulfur cluster assembly	Based on sequence similarity. Blast score (assembled sequence): vs *P. aeruginosa* IscU = 2e-46 vs *A. thaliana* homolog = 1e-55	AV397853 AV619693 AV620447 AV624765 AV623500 AV623764 AV623473 AV638761 AV621762 AV625355

inadequate because of limited iron nutrition. It is possible that the threshold for cellular responses (such as activation of Fox1) and organelle responses (such as degradation of chlorophyll proteins) is different and/or that separate iron sensors occur in each compartment.

Paradoxically, the *Fer1* gene, encoding a plastid ferritin, is also induced in iron-deficient cells. Because ferritin has an iron-storage function, the increase in its mRNA in –Fe cells is counterintuitive. Two models have been offered to explain this observation; first, that increased ferritin might allow the cell to handle overload when iron is resupplied to the deficient cells. However, this model does not sit well when one considers that ferritin overexpression causes iron deficiency. It is more likely that ferritin may serve to buffer iron as it is released from degrading PSI and recycled to other compartments (such as the mitochondrion). This model is supported by the finding that iron deficiency impacts the chloroplast more severely than it does respiration. It also is compatible with the increase in *Fer1* gene expression in degreening N-starved Chlorella cells *(171)*.

4.2.2.3. Iron Metabolism Components

Based solely on BLAST searches, other candidates for iron-metabolizing components have been identified in Chlamydomonas (Table 4). These include components required for assembly of iron–sulfur clusters (*Isc* genes) and candidate metal transporters (*Nramp*). Comparison of Chlamydomonas metal-metabolizing components with those of plants and animals leads us to wonder whether Chlamydomonas is half beast/half plant. Some components have their closest homologs in mammals, such as the ferroxidase, whereas others have their closest homolog in plants, such as Ccc2/Ran1.

4.2.3. *Copper–Iron Link*

Unlike yeast cells, wild-type copper-deficient Chlamydomonas cells do not exhibit symptoms of iron deficiency (chlorosis in the case of photosynthetic organisms) when grown under copper-deficient conditions. In fact, copper-deficient and copper-supplemented Chlamydomonas cells grow equally well *(162,169)*. This suggests that either a copper–iron link equivalent to that established for yeast and mammals does not exist in Chlamydomonas or, alternatively, that this organism has backup systems that function when the copper-dependent iron uptake/metabolism is compromised. Our recent results (Section 4.2.2.) with iron-responsive expression of putative iron metabolizing proteins further supports the idea that copper-requiring components are required for iron metabolism in Chlamydomonas.

A survey of copper and iron proteins reveals several reactions for which both copper- and iron-containing proteins are known. Key examples are hemocyanin/hemoglobin, cytochrome oxidase/alternative oxidase, Cu,Zn-SOD/Fe-SOD, copper/heme nitrite reductases, membrane–methane monooxygenase/soluble methane monooxygenase, plastocyanin/cytochrome-c_6. The copper enzymes are believed to be more recent additions to the biochemical repertoire relative to iron enzymes *(172)*. The similarity in catalytic ability between copper and iron enzymes raises the question of whether the substitution of a copper enzyme with a "backup" iron version might represent a general metabolic adaptation to copper deficiency. Azurin and cytochrome-c_{551} in *Pseudomonas aeruginosa* form a pair analogous to plastocyanin and cytochrome-c_6, and it has been suggested, although not documented in vivo, that they might function interchangeably. A fascinating example of another pair of iron and copper proteins became known more recently following the discovery that a membrane-bound methane monooxygenase is a multicopper oxidase *(173)*. The enzyme catalyzes the hydroxylation of methane to methanol using O_2 as a substrate. A soluble version of this enzyme is well characterized and contains a diiron active site *(174)*. Some methanotrophic bacteria have both types of enzymes, and which one is used depends on copper availability in the medium, with control being effected by transcriptional regulation *(175)*. When copper is available, transcription of the gene encoding the diiron-hydroxylase is repressed, whereas synthesis of the copper enzyme is stimulated. The copper enzyme is the protein of choice when both copper and iron are available. The retention of genetic information for the diiron enzyme implies that both must be required in the natural environment; in a hypoxic environment, Cu^{1+} might be lost in insoluble precipitates, but Fe^{2+} might be more

readily available. As mentioned earlier, in Chlamydomonas, the copper-deficiency responses can be mimicked by hypoxia *(164)*. One explanation for this is that oxygen deficiency signals incipient copper deficiency and the response to hypoxia allows the organism to anticipate and, hence, survive copper-deficient conditions.

5. CONCLUDING REMARKS

Studies of copper–iron crosstalk in photosynthetic organisms are still in their infancy and many issues remain to be addressed. The full extent of the putative backup system for iron metabolism in copper-deficient Chlamydomonas must be elucidated. In an organism designed to undertake two fundamental metabolic processes (respiration and photosynthesis), the existence of such a backup system would appear to represent a shrewd evolutionary development. Does such a system also exist in vascular plants? With the current data explosion derived from complete genome sequences, EST databases, and microarray expression analysis, the resolution of such fundamental issues ought to be attainable in the very near future.

ACKNOWLEDGMENTS

The authors would like to thank Janette Kropat for helpful comments. This work is supported by grants from the National Institutes of Health (GM42143) (S.M.), the Australian Research Council International Researcher Exchange scheme (X00001622) (S.L. and S.M.), and a National Health and Medical Research Council R. Douglas Wright Fellowship (S.L.).

REFERENCES

1. Askwith, C. and Kaplan, J. (1998) Iron and copper transport in yeast and its relevance to human disease. *Trends Biochem. Sci.* **23**, 135–138.
2. Labbe, S. and Thiele, D. J. (1999) Pipes and wiring: the regulation of copper uptake and distribution in yeast. *Trends Microbiol.* **7**, 500–505.
3. Dancis, A., Haile, D., Yuan, D. S., et al. (1994) The *Saccharomyces cerevisiae* copper transport protein (Ctr1p). *J. Biol. Chem.* **269**, 25,660–25,667.
4. Pena, M. M. O., Puig, S., and Thiele, D. J. (2000) Characterization of the *Saccharomyces cerevisiae* high affinity copper transporter Ctr3. *J. Biol. Chem.* **275**, 33,244–33,251.
5. Pena, M. M. O., Lee, J., and Thiele, D. J. (1999) A delicate balance: homeostatic control of copper uptake and distribution. *J. Nutr.* **129**, 1251–1260.
6. Georgatsu, E., Mavrogiannis, L. A., Fragiadakis, G. S., et al. (1997) The yeast Fre1p/Fre2p cupric reductases facilitate copper uptake and are regulated by the copper-modulated Mac1p activator. *J. Biol. Chem.* **272**, 13,786–13,792.
7. Hassett, R. and Kosman, D. J. (1995) Evidence for Cu(II) reduction as a component of copper uptake by *Saccharomyces cerevisiae*. *J. Biol. Chem.* **270**, 128–134.
8. Eide, D. J. (1998) The molecular biology of metal ion transport in *Saccharomyces cerevisiae*. *Annu. Rev. Nutr.* **18**, 441–469.
9. Gross, C., Kelleher, M., Iyer, V. R., et al. (2000) Identification of the copper regulon in *Saccharomyces cerevisiae* by DNA microarrays. *J. Biol. Chem.* **275**, 32,310–32,316.
10. Winge, D. R., Jensen, L. T., and Srinivasan, C. (1998) Metal-ion regulation of gene expression in yeast. *Curr. Opin. Chem. Biol.* **2**, 216–221.
11. Ooi, C. E., Rabinovich, E., Dancis, A., et al. (1996) Copper-dependent degradation of the *Saccharomyces cerevisiae* plasma membrane transporter Ctr1p in the apparent absence of endocytosis. *EMBO J.* **15**, 3515–3523.
12. Hassett, R., Dix, D. R., Eide, D. J., et al. (2000) The Fe(II) permease Fet4p functions as a low affinity copper transporter and supports normal copper trafficking in *Saccharomyces cerevisiae*. *Biochem. J.* **351**, 477–484.
13. Cohen, A., Nelson, H., and Nelson, N. (2000) The family of *SMF* metal ion transporters in yeast cells. *J. Biol. Chem.* **275**, 33,388–33,394.
14. Radisky, D. and Kaplan, J. (1999) Regulation of transition metal transport across the yeast plasma membrane. *J. Biol. Chem.* **274**, 4481–4484.
15. Kampfenkel, K., Kushnir, S., Babiychuk, E., et al. (1995) Molecular characterization of a putative *Arabidopsis thaliana* copper transporter and its yeast homologue. *J. Biol. Chem.* **47**, 28,479–28,486.
16. Harrison, M., Jones, C. E., and Dameron, C. T. (1999) Copper chaperones: function, structure and coper-binding properties. *J. Biol. Inorg. Chem.* **4**, 145–153.
17. Harrison, M. D., Jones, C. E., Solioz, M., et al. (2000) Intracellular copper routing: the role of copper chaperones. *TIBS* **25**, 29–32.
18. O'Halloran, T. V. and Culotta, V. C. (2000) Metallochaperones, an intracellular shuttle service for metal ions. *J. Biol. Chem.* **275**, 25,057–25,060.

19. Valentine, J. S. (1997) Delivering copper inside yeast and human cells. *Science*, **278**, 817–818.
20. Culotta, V. C., Sturtz, L., Jensen, L., et al. (2001) Metals and oxidative stress: new insights from yeast. *J. Exp. Bot.* **52(Suppl.)**, 61.
21. Yuan, D. S., Stearman, R., Dancis, A., et al. (1995) The Menkes/Wilson disease gene homologue in yeast provides copper to a ceruloplasmin-like oxidase required for iron uptake. *Proc. Natl. Acad. Sci. USA*, **92**, 2632–2636.
22. Davis-Kaplan, S. R., Askwith, C. C., Bengtzen, A. C., et al. (1998) Chloride is an allosteric effector of copper assembly for the yeast multicopper oxidase Fet3p: An unexpected role for intracellular chloride channels. *Proc. Natl. Acad. Sci. USA* **95**, 13,641–13,645.
23. Gaxiola, R. A., Yuan, D. S., Klausner, R. D., et al. (1998) The yeast CLC chloride channel functions in cation homeostasis. *Proc. Natl. Acad. Sci. USA*, **95**, 4046–4050.
24. Glerum, D. M., Shtanko, A., and Tzagoloff, A. (1996) *SCO1* and *SCO2* act as high copy suppressors of a mitochondrial copper recruitment defect in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **271**, 20,531–20,535.
25. Rentzsch, A., Krummeck-Weiβ, G., Hofer, A., et al. (1999) Mitochondrial copper metabolism in yeast: mutational analysis of Scо1p involved in the biogenesis of cytochrome *c* oxidase. *Curr. Genet.* **35**, 10—108.
26. Martins, L. J., Jensen, L. T., Simons, J. R., et al. (1998) Metalloregulation of *FRE1* and *FRE2* homologs in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **273**, 23,716–23,721.
27. Stearman, R., Yuan, D. S., Yamaguchi-Iwai, Y., et al. (1996) A permease–oxidase complex involved in high-affinity iron uptake in yeast. *Science* **271**, 1552–1557.
28. Askwith, C., Eide, D., Van Ho, A., et al. (1994) The *FET3* gene of *S. cerevisiae* encodes a multicopper oxidase required for ferrous iron uptake. *Cell* **76**, 403–410.
29. Askwith, C. and Kaplan, J. (1997) An oxidase–permease-based iron transport system in *Schizosaccharomyces pombe* and its expression in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **272**, 401–405.
30. Hammacott, J. E., Williams, P. H., and Cashmore, A. M. (2000) *Candida albicans CFL1* encodes a functional ferric reductase activity that can rescue a *Saccharomyces cerevisiae fre1* mutant. *Microbiology* **146**, 869–876.
31. Ramanan, N. and Wang, Y. (2000) A high-affinity iron permease essential for *Candida albicans* virulence. *Science* **288**, 1062–1064.
32. Dix, D., Bridgham, J., Broderius, M., et al. (1997) Characterization of the FET4 protein of yeast. Evidence for a direct role in the transport of iron. *J. Biol. Chem.* **272**, 11,770–11,777.
33. Yun, C.-W., Ferea, T., Rashford, J., et al. (2000) Desferrioxamine-mediated iron uptake in *Saccharomyces cereivisiae*. Evidence for two pathways of iron uptake. *J. Biol. Chem.* **275**, 10,709–10,715.
34. Yun, C.-W., Tiedeman, J. S., Moore, R. E., et al. (2000) Siderophore–iron uptake in *Saccharomyces cerevisiae*. Identification of ferrichrome and fusarinine transporters. *J. Biol. Chem.* **275**, 16,354–16,359.
35. Heymann, P., Ernst, J. F., and Winkelmann, G. (1999) Identification of a fungal triacetylfusarinine C siderophore transport gene (TAF1) in *Saccharomyces cerevisiae* as a member of the major facilitator superfamily. *Biometals* **12**, 301–306.
36. Heymann, P., Ernst, J. F., and Winkelmann, G. (2000) Identification and substrate specificity of a ferrichrome-type siderophore transporter (Arn1p) in *Saccharomyces cerevisiae*. *FEMS Microbiol. Lett.* **186**, 221-227.
37. Lesuisse, E., Blaiseau, P. L., Dancis, A., et al. (2001) Siderophore uptake and use by the yeast *Saccharomyces cerevisiae*. *Microbiology* **147(Pt. 2)**, 289–298.
38. Lesuisse, E., Simon-Casteras, M., and Labbe, P. (1998) Siderophore-mediated iron uptake in *Saccharomyces cerevisiae*: the SIT1 gene encodes a ferrioxamine B permease that belongs to the major facilitator superfamily. *Microbiology* **144(Pt. 2)**, 3455–3462.
39. Klionsky, D. J., Herman, P. K., and Emr, S. D. (1990) The fungal vacuole: composition, function and biogenesis. *Microbiol. Rev.* **54**, 266–292.
40. Raguzzi, F., Lesuisse, E., and Chrichton, R.R. (1988) Iron storage in *Saccharomyces cerevisiae*. *FEBS Lett.* **231**, 253–258.
41. Urbanowski, J. L. and Piper, R. C. (1999) The iron transporter Fth1p forms a complex with the Fet5 iron oxidase and resides on the vacuolar membrane. *J. Biol. Chem*, **274**, 38,061–38,070.
42. Babcock, M., Silva, D. D., Oaks, R., et al. (1997) Regulation of mitochondrial iron accumulation by Yfh1, a putative homolog of frataxin. *Science* **276**, 1709–1712.
43. Radisky, D. C., Babcock, M. C., and Kaplan, J. (1999) The yeast frataxin homologue mediates mitochondrial iron efflux. *J. Biol. Chem.* **274**, 4497–4499.
44. Chen, O. S. and Kaplan, J. (2000) *CCC1* supresses mitochondrial damage in the yeast model of Friedreich's ataxia by limiting mitochondrial iron accumulation. *J. Biol. Chem.* **275**, 7626–7632.
45. Kispal, G., Csere, P., Prohl, C., et al. (1999) The mitochondrial proteins Atm1p and Nfs1p are essential for biogenesis of cytosolic Fe/S proteins. *EMBO J.* **18**, 3981–3989.
46. Li, J., Kogan, M., Knight, S. A. B., et al. (1999) Yeast mitochondrial protein, Nfs1p, coordinately regulates iron-sulphur cluster proteins, cellular iron uptake, and iron distribution. *J. Biol. Chem.* **274**, 33,025–33,034.
47. Leighton, J. and Schatz, G. (1995) An ABC transporter in the mitochondrial inner membrane is required for normal growth of yeast. *EMBO J.* **4**, 188–195.
48. Knight, S. A. B., Sepuri, N. B. V., Pain, D., et al. (1998) Mt-Hsp70 homolog, Ssc2p, required for maturation of yeast frataxin and mitochondrial iron homeostasis. *J. Biol. Chem.* **273**, 18,389–18,393.
49. Jensen, L. and Culotta, V. (2000) Role of *Saccharomyces cerevisiae* ISA1 and ISA2 in iron homeostasis. *Mol. Cell. Biol.* **20**, 3919–3927.
50. Robertson, L. S., Causton, H. C., Young, R. A., et al. (2000) The yeast A kinases differentially regulate iron uptake and respiratory function. *Proc. Natl. Acad. Sci. USA* **97**, 5984–5988.

51. Dancis, A., Roman, D. G., Anderson, G. J., et al. (1992) Ferric reductase of *Saccharomyces cerevisiae*: molecular characterization, role in iron uptake, and transcriptional control by iron. *Proc. Natl. Acad. Sci. USA* **89**, 3869–3873.
52. Jungmann, J., Reins, H., Lee, J., et al. (1993) MAC1, a nuclear regulatory protein related to Cu-dependent transcription factors is involved in Cu/Fe utilization and stress resistance in yeast. *EMBO J.* **12**, 5051–5056.
53. Lin, S.-J., Pufahl, R. A., Dancis, A., et al. (1997) A role for the *Saccharomyces cerevisiae ATX1* gene in copper trafficking and iron transport. *J. Biol. Chem.* **272**, 9215–9220.
54. Yamaguchi-Iwai, Y., Stearman, R., Dancis, A., et al. (1996) Iron-regulated DNA binding by the AFT1 protein controls the iron regulation in yeast. *EMBO J.* **15**, 3377–3384.
55. Labbe, S., Pena, M. M. O., Fernandes, A. R., et al. (1999) A copper-sensing transcription factor regulates iron uptake genes in *Saccharomyces pombe*. *J. Biol. Chem.* **274**, 36,252–36,260.
56. Elvehjem, C. A. (1935) The biological significance of copper and its relation to iron metabolism. *Physiol. Rev.* **15**, 471–507.
57. Hart, E. B., Steenbock, H., Waddell, J., et al. (1928) Iron in nutrition. VII. Copper as a supplement to iron for hemoglobin building in the rat. *J. Biol. Chem.* **77**, 797–812.
58. Holmberg, C. G. and Laurell, C. B. (1948) Investigations in serum copper. II. Isolation of the copper-containing protein, and a description of some of its properties. *Acta Chem. Scand.* **2**, 550–556.
59. Eisenstein, R. S. (2000) Discovery of the ceruloplasmin homologue hephaestin: new insight into the copper/iron connection. *Nutr. Rev.* **58**, 22–26.
60. Vulpe, C. D., Kuo, Y. M., Murphy, T. L., et al. (1999) Hephaestin, a ceruloplasmin homologue implicated in intestinal iron transport, is defective in the sla mouse. *Nature Genet.* **21**, 195–199.
61. Crampton, R. F., Matthews, D. M., and Poisner, R. (1965) Observations on the mechanism of absorption of copper by the small intestine. *J. Physiol.* **178**, 111–126.
62. Van Campen, D.R. (1971) Absorption of copper from the gastrointestinal tract, in *Intestinal Absorption of Metal Ions, Trace Elements and Radionuclides* (Skoryna, S. C. and Waldron-Edwards, D., eds.), Pergamon, Oxford, pp. 211–227.
63. Zhou, B. and Gitschier, J. (1997) h*CTR1*: a human gene for copper uptake identified by complementation in yeast. *Proc. Natl. Acad. Sci. USA* **94**, 7481–7486.
64. Lee, J. and Thiele, D. J. (2001) Essential role for mammalian copper transporters in copper homeostasis and embryonic development. *J. Exp. Bot.* **52(Suppl.)**, 84.
65. Hamza, I., Schaefer, M., Klomp, L. W. J., et al. (1999) Interaction of the copper chaperone HAH1 with the Wilson disease protein is essential for copper homeostasis. *Proc. Natl. Acad. Sci. USA* **96**, 13,363–13,368.
66. Larin, D., Mekios, C., Das, K., et al. (1999) Characterization of the interaction between the Wilson and Menkes disease proteins and the cytoplasmic copper chaperone, HAH1P. *J. Biol. Chem.* **274**, 28,497–28,504.
67. Hamza, I., Chen, J., Gitlin, J. D., et al. (2001) Critical role for metallochaperone Atox1 in perinatal copper homeostasis. *J. Exp. Bot.* **52(Suppl.)**, 84.
68. Petris, M. J., Strausak, D. and Mercer, J. F. B. (2000) The Menkes copper transporter is required for the activation of tyrosinase. *Hum. Mol. Genet.* **9**, 2845–2851.
69. Petris, M. J., Mercer, J. F. B., Culvenor, J. G. et al. (1996) Ligand-regulated transport of the Menkes copper P-type ATPase efflux pump from the Golgi apparatus to the plasma membrane: a novel mechanism of regulated trafficking. *EMBO J.* **15**, 6084–6095.
70. Petris, M. J., Mercer, J. F. B., and Camakaris, J. (1999) The cell biology of the Menkes disease protein. *Adv. Exp. Biol. Med.* **448**, 53–66.
71. Petris, M. J., Camakaris, J., Greenough, M., et al. (1998) A C-terminal di-leucine is required for localization of the Menkes protein in the *trans*-Golgi network. *Hum. Mol. Genet.* **7**, 2063–2071.
72. Petris, M. J. and Mercer, J. F. B. (1999) The Menkes protein (ATP7A; MNK) cycles via the plasma membrane both in basal and elevated extracellular copper using a C-terminal di-leucine endocytic signal. *Hum. Mol. Genet.* **8**, 2107–2115.
73. Linder, M. C., Wooten, L., Cerveza, P., et al. (1998) Copper transport. *Am. J. Clin. Nutr.* **67**, 965S–971S.
74. Hung, I. H., Suzuki, M., Yamaguchi, Y., et al. (1997) Biochemical characterization of the Wilson disease protein and functional expression in the yeast *Saccharomyces cerevisiae*. *J. Biol. Chem.* **272**, 21,461–21,466.
75. Schaefer, M., Hopkins, R. G., Failla, M. A., et al. (1999) Hepatocyte-specific localization and copper-dependent trafficking of the Wilson's disease protein in the liver. *Am. J. Physiol.* **276**, G639–G646.
76. Suzuki, M. and Gitlin, J. D. (1999) Intracellular localization of the Menkes and Wilson's disease proteins and their role in intracellular copper transport. *Pediatr. Int.* **41**, 436–442.
77. Terada, K., Aiba, N., Yang, X.-L. et al. (1999) Biliary excretion of copper in LEC rat after introduction of copper transporting P-type ATPase, ATP7B. *FEBS Lett.* **448**, 53–56.
78. Terada, K., Nakako, T., Yang, X.-L., et al. (1998) Restoration of holoceruloplasmin synthesis in LEC rat after infusion of recombinant adenovirus bearing WND cDNA. *J. Biol. Chem.* **273**, 1815–1820.
79. Sato, M. and Gitlin, J. D. (1991) Mechanisms of copper incorporation during the biosynthesis of human ceruloplasmin. *J. Biol. Chem.* **266**, 5128–5134.
80. Holmberg, C. G. and Laurell, C. B. (1948) Investigations in serum copper. II. Isolation of the copper containing protein and a description of some of its properties. *Acta Chem. Scand.* **2**, 550–556.
81. Winzerling, J. J. and Law, J. H. (1997) Comparative nutrition of iron and copper. *Annu. Rev. Nutr.* **17**, 501–526.
82. Linder, M.C. (1991) *Biochemistry of Copper*, Plenum, New York.
83. Evans, G. W. (1973) Copper homeostasis in the mammalian system. *Physiol. Rev.* **53**, 535–569.
84. Dameron, C. T. and Harrison, M. D. (1998) Mechanisms for protection against copper toxicity. *Am. J. Clin. Nutr.* **67(Suppl.)**, 1091S–1097S.

85. Miles, A. T., Hawksworth, G. M., Beattie, J. H., et al. (2000) Induction, regulation, degradation, and biological significance of mammalian metallothioneins. *Crit. Rev. Biochem. Mol. Biol.* **35**, 35–70.
86. Vasak, M. and Hasler, D. W. (2000) Metallothioneins: new functional and structural insights. *Curr. Opin. Chem. Biol.* **4**, 177–183.
87. Labbe, S., Simard, C., and Seguin, C. (1997) Metallothionein gene regulation in mouse cells, in *Metal Ions in Gene Regulation* (Silver, S. and Walden, W., eds.), Chapman & Hall, New York, pp. 231–249.
88. Remondelli, P., Moltedo, O., Pascale, M. C., et al. (1999) Metal regulation of metallothionein gene transcription in mammals. *Adv. Exp. Med. Biol.* **448**, 223–236.
89. Andrews, N. C. (1999) The iron transporter DMT1. *Int. J. Biochem. Cell Biol.* **31**, 991–994.
90. Aisen, P., Wessling-Resnick, M., and Leibold, E. A. (1999) Iron metabolism. *Curr. Opin. Chem. Biol.* **3**, 200–206.
91. Schneider, B. D. and Leibold, E. A. (2000) Regulation of mammalian iron homeostasis. *Curr. Opin. Clin. Nutr. Metab. Care* **3**, 267–273.
92. Tandy, S., Williams, M., Leggett, A., et al. (2000) Nramp2 expression is associated with pH-dependent iron uptake across the apical membrane of human intestinal Caco-2 cells. *J. Biol. Chem.* **275**, 1023–1029.
93. Bennett, M. J., Lebron, J. A., and Bjorkman, P. J. (2000) Crystal structure of the hereditary haemochromatosis protein HFE complexed with transferrin receptor. *Nature* **403**, 46–53.
94. Riedel, H. D., Muckenthaler, M. U., Gehrke, S. G., et al. (1999) HFE downregulates iron uptake from transferrin and induces iron-regulatory protein activity in stably transfected cells. *Blood* **94**, 3915–3921.
95. Han, O., Fleet, J. C., and Wood, R. J. (1999) Reciprocal regulation of HFE and Nramp2 gene expression by iron in human intestinal cells. *J. Nutr.* **129**, 98–104.
96. Nelson, N. (1999) Metal ion transporters and homeostasis. *EMBO J.* **18**, 4361–4371.
97. Haile, D. J. (1999) Regulation of genes of iron metabolism by the iron-response proteins. *Am. J. Med. Sci.* **318**, 230–240.
98. Conrad, M. E., Umbreit, J. N., and Moore, E. G. (1999) Iron absorption and transport. *Am. J. Med. Sci.* **8**, 213–229.
99. Donovan, A., Brownlie, A., Zhou, Y., et al. (2000) Positional cloning of zebrafish *ferroportin 1* identifies a conserved vertebrate iron exporter. *Nature* **403**, 776–781.
100. McKie, A. T., Marciani, P., Rolfs, A., et al. (2000) A novel duodenal iron-regulated membrane protein (Ireg1) implicated in basolateral transfer of iron to the circulation. *Mol. Cell*, **5**, 299–309.
101. Abboud, S. and Haile, D. J. (2000) A novel mammalian iron-regulated protein involved in intracellular iron metabolism. *J. Biol. Chem.* **275**, 19,906–19,912.
102. Kaplan, J. and Kushner, J. P. (2000) Mining the genome for iron. *Nature* **403**, 712–713.
103. Andrews, N. C., Fleming, M. D., and Gunshin, H. (1999) Iron transport across biologic membranes. *Nutr. Rev.* **57**, 745–748.
104. Tabuchi, M., Yoshimori, T., Yamaguchi, K., et al. (2000) Human NRAMP2/DMT1, which mediates iron transport across endosomal membranes, is localized to late endosomes and lysosomes in HEp-2 cells. *J. Biol. Chem.* **275**, 22,220–22,228.
105. Rolfs, A. and Hediger, M. A. (2001) Intestinal metal ion absorption: an update. *Curr. Opin. Gastroenterol.* **17**, 177–183.
106. Eisenstein, R. S. and Blemmings, K. P. (1998) Iron regulatory proteins, iron responsive elements and iron homeostasis. *J. Nutr.* **128**, 2295–2298.
107. Kühn, L. C. (1998) Iron and gene expression: molecular mechanisms regulating cellular iron homeostasis. *Nutr. Rev.* **56**, S11–S19.
108. Thomson, A. M., Rogers, J. T., and Leedman, P. J. (1999) Iron-regulatory proteins, iron-responsive elements and ferritin mRNA translation. *Int. J. Biochem. Cell Biol.* **31**, 1139–1152.
109. Fox, T. C. and Guerinot, M. L. (1998) Molecular biology of cation transport in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **49**, 669–696.
110. Hirayama, T., Kieber, J. J., Hirayama, N., et al. (1999) *RESPONSE TO ANTAGONIST1*, a Menkes/Wilson disease-related copper transporter, is required for ethylene signaling in *Arabidopsis*. *Cell*, **97**, 383–393.
111. Tabata, K., Kashiwagi, S., Mori, H., et al. (1997) Cloning of a cDNA encoding a putative metal-transporting P-type ATPase from *Arabidopsis thaliana*. *Biochim. Biophys. Acta* **1326**, 1–6.
112. Pilon, M., Burkhead, J., and Pilon-Smits, E. A. H. (2001) Metal homeostasis in Arabidopsis chloroplasts: the roles of the copper binding protein AtCutAp and the P-type ATPase Paa1p. *J. Exp. Bot.* **52(Suppl.)**, 65.
113. Kaneko, T., Kato, T., Sato, S., et al. (2000) Structural analysis of Arabidopsis thaliana chromosome 3. II. Sequence features of the regions of 4,251,695 bp covered by ninety P1, TAC and BAC clones. *DNA Res.* **7**, 217–221.
114. Zhu, H., Shipp, E., Sanchez, R. J., et al. (2000) Cobalt(2+) binding to human and tomato copper chaperone for superoxide dismutase: Implications for metal ion transfer mechanism. *Biochemistry* **39**, 5413–5421.
115. Himelblau, E., Mira, H., Lin, S. J., et al. (1998) Identification of a functional homolog of the yeast and human homeostasis gene *ATX1* from *Ardabidopsis*. *Plant Physiol.* **117**, 1227–1234.
116. Lin, X., Kaul, S., Rounsley, S. D., et al. (1999) Sequence and analysis of chromosome 2 of the plant *Arabidopsis thaliana*. *Nature* **402**, 761–768.
117. Bleecker, A. B. and Kende, H. (2000) Ethylene: a gaseous signal molecule in plants. *Annu. Rev. Cell Dev. Biol.* **16**, 1–18.
118. Hirayama, T. and Alonso, J. M. (2000) Ethylene captures a metal! Metal ions are involved in ethylene perception and signal transduction. *Plant Cell Physiol.* **41**, 548–555.
119. Woeste, K. E. and Kieber, J. J. (2000) A strong loss-of-function mutation in *RAN1* results in constitutive activation of the ethylene repsonse pathway as well as a rosette-lethal phenotype. *Plant Cell* **12**, 443–455.
120. Williams, L. E., Pittman, J. K., and Hall, J. L. (2000) Emerging mechanisms for heavy metal transport in plants. *Biochim. Biophys. Acta* **1465**, 104–126.

121. Kanamaru, K., Kashiwagi, S., and Mizuno, T. (1994) A copper-transporting P-type ATPase found in the thylakoid membrane of the cyanobacterium *Synechococcus sp.* PCC7942. *Mol. Microbiol.* **13**, 369–377.
122. Phung, L. T., Ajlani, G. and Haselkorn, R. (1994) P-type ATPase from the cyanobacterium Synechococcus 7942 related to the human Menkes and Wilson disease gene products. *Proc. Natl. Acad. Sci. USA* **91**, 9651–9654.
123. Rutherford, J. C., Cavet, J. S., and Robinson, N. J. (1999) Cobalt-dependent transcriptional switching by a dual-effector MerR-like protein regulates a cobalt-exporting variant CPx-type ATPase. *J. Biol. Chem.* **274**, 25,827–25,832.
124. Thelwell, C., Robinson, N. J., and Turner-Cavet, J. S. (1998) An SmtB-like repressor from Synechocystis PCC 6803 regulates a zinc exporter. *Proc. Natl. Acad. Sci. USA* **95**, 10,728–10,733.
125. Tottey, S., Rich, P. R., Rondet, S. A. M., et al. (2001) Two Menkes-type ATPases supply copper for photosynthesis in *Synechocystis* PCC6803. *J. Biol. Chem.* **276**, 19,999–20,004.
125a. Tottey, S., Rondet, S. A., Borrelly, G. P., Robinson, P. J., Rich, P. R. and Robinson, N. J. (2002) A copper metallochaperone for photosynthesis and respiration reveals metal-specific targets, interaction with an importer, and alternative sites for copper acquisition. *J. Biol. Chem.* **277**, 5490–5497.
126. Mira, H., Martinez-Garcia, F., and Penarrubia, L. (2001) Evidence for the plant-specific intercellular transport of the Arabidopsis copper chaperone CCH. *Plant J.* **25**, 521–528.
127. Himelblau, E. and Amasino, R. M. (2000) Delivering copper within plant cells. *Curr. Opin. Plant Biol.* **3**. 205–210.
128. Zhou, J. and Goldsbrough, P. B. (1994) Functional homologues of fungal metallothionein genes from *Arabidopsis*. *Plant Cell* **6**, 875–884.
129. Dykema, P. E., Sipes, P. R., Marie, A., et al. (1999) A new class of proteins capable of binding transition metals. *Plant Mol. Biol.* **41**, 139–150.
130. Robinson, N. J., Procter, C. M., Connolly, E. L., et al. (1999) A ferric-chelate reductase for iron uptake from soils. *Nature* **397**, 694–697.
131. Yi, Y. and Guerinot, M. L. (1996) Genetic evidence that induction of root Fe(III) chelate reductase activity is necessary for iron uptake under iron deficiency. *Plant J.* **10**, 835–844.
132. Guerinot, M. L., Grotz, N., Hibbard, S., et al. (2001) Molecular characterization of the uptake of iron and other divalent cations in *Arabidopsis*. *J. Exp. Bot.* **52(Suppl.)**, 62.
133. Eide, D., Broderius, M., Fett, J., et al. (1996) A novel iron-regulated metal transporter from plants identified by functional expression in yeast. *Proc. Natl. Acad. Sci. USA* **93**, 5624–5628.
134. Korshunova, Y., Eide, D., Clark, W. G., et al. (1999) The IRT1 protein from *Arabidopsis thaliana* is a metal transporter with a broad substrate range. *Plant Mol. Biol.* **40**, 37–44.
135. Guerinot, M. L. (2000) The ZIP family of metal transporters. *Biochim. Biophys. Acta* **1465**, 190–198.
136. Curie, C., Alonso, J. M., Jean, M. L., et al. (2000) Involvement of NRAMP1 from *Arabidopsis thaliana* in iron transport. *Biochem. J.* **347**, 749–755.
137. Thomine, S., Wang, R., Ward, J. M., et al. (2000) Cadmium and iron transport by members of a plant metal transporter family in *Arabidopsis* with homology to *Nramp* genes. *Proc. Natl. Acad. Sci. USA* **97**, 4991–4996.
138. Bagnaresi, P., Thoiron, S., Mansion, M., et al. (1999) Cloning and characterization of a maize cytochrome-b5 reductase with Fe3+-chelate reduction capability. *Biochem. J.* **338(Pt. 2)**, 499–505.
139. Curie, C., Panaviene, Z., Loulergue, C., et al. (2001) Maize yellow stripe1 encodes a membrane protein directly involved in Fe(III) uptake. *Nature* **409**, 346–349.
140. Bagnaresi, P., Mazars-Marty, D., Pupillo, P., et al. (2000) Tonoplast subcellular localization of maize cytochrome b5 reductases. *Plant J.* **24**, 645–654.
141. Briat, J.-F. and Lobreaux, S. (1997) Iron transport and storage in plants. *Trends Plant Sci.* **2**, 187–193.
142. Bell, W. D., Bogorad, L., and McIlrath, W. J. (1958) Response of the yellow-stripe maize mutant (ys1) to ferrous and ferric iron. *Bot. Gaz.* **120**, 36–39.
143. Theil, E. C. (1987) Ferritin: structure, gene regulation, and cellular function in animals, plants and microorganisms. *Annu. Rev. Biochem.* **56**, 289–315.
144. Briat, J. F., Lobreaux, S., Grignon, N., et al. (1999) Regulation of plant ferritin synthesis: how and why. *Cell. Mol. Life Sci.* **56**, 155–166.
145. Van Wuytswinkel, O., Vansuyt, G., Grignon, N., et al. (1999) Iron homeostasis alteration in transgenic tobacco overexpressing ferritin. *Plant J.* **17**, 93–97.
146. Briat, J. F. and Lobreaux, S. (1998) Iron storage and ferritin in plants. *Metal Ions Biol. Syst.* **35**, 563–584.
147. Wei, J. and Theil, E. C. (2000) Identification and characterization of the iron regulatory element in the ferritin gene of a plant (soybean). *J. Biol. Chem.* **275**, 17,488–17,493.
148. Petit, J.-M., Wuytswinkel, O. V., Briat, J.-F., et al. (2001) Characterization of an iron dependent regulatory sequence (IDRS) involved in the transcriptional control of *AtFer1* and *ZmFer1* plant ferritin genes by iron. *J. Biol. Chem.* **276**, 5584–5590.
149. Cohen, C. K., Norvell, W. A., and Kochian, L. V. (1997) Induction of root cell plasma membrane ferric reductase. *Plant Physiol.* **114**, 1061–1069.
150. Welch, R. M., Norvell, W. A., Schaefer, S. C., et al. (1993) Induction of iron (III) and copper (II) reduction in pea (*Pisum sativum* L.) roots by Fe and Cu status: does the root-cell plasmalemma Fe(III)-chelate reductase perform a general role in regulating uptake? *Planta* **190**, 555–561.
151. Harris, E. H. (1989) *The Chlamydomonas Sourcebook: A Comprehensive Guide to Biology and Laboratory Use*, Academic, San Diego, CA.

152. Wood, P. M. (1978) Interchangeable copper and iron proteins in algal photosynthesis. Studies on plastocyanin and cytochrome *c*-552 in *Chlamydomonas*. *Eur. J. Biochem.* **87**, 8–19.
153. Li, H. H. and Merchant, S. (1995) Degradation of plastocyanin in copper-deficient *Chlamydomonas reinhardtii*. *J. Biol. Chem.* **270**, 23,504–23,510.
154. Quinn, J. M. and Merchant, S. (1995) Two copper-responsive elements associated with the Chlamydomonas Cyc6 gene function as targets for transcriptional activators. *Plant Cell* **7**, 623–638.
155. Hill, K. L. and Merchant, S. (1992) In vivo competition between plastocyanin and a copper-dependent regulator of the *Chlamydomonas reinhardtii* cytochrome c_6 gene. *Plant Physiol.* **100**, 319–326.
156. Li, H. H., Quinn, J., Culler, D., et al. (1996) Molecular genetic analysis of plastocyanin biosynthesis in *Chlamydomonas reinhardtii*. *J. Biol. Chem.* **271**, 31,283–31,289.
157. Merchant, S. and Bogorad, L. (1987) Metal ion regulated gene expression: use of a plastocyanin-less mutant of *Chlamydomonas reinhardtii* to study the Cu(II)-dependent expression of cytochrome *c*-552. *EMBO J.* **6**, 2531–2535.
158. Hill, K. L., Hassett, R., Kosman, D., et al. (1996) Regulated copper uptake in *Chlamydomonas reinhardtii* in response to copper availability. *Plant Physiol.* **112**, 697–704.
159. Howe, G. and Merchant, S. (1992) Heavy metal-activated synthesis of peptides in *Chlamydomonas reinhardtii*. *Plant Physiol.* **98**, 127–136.
160. Cobbett, C. S. (2000) Phytochelatin biosynthesis and function in heavy-metal detoxification. *Curr. Opin. Plant Biol.* **3**, 211–216.
161. Hill, K. L. and Merchant, S. (1995) Coordinate expression of coproporphyrinogen oxidase and cytochrome c6 in the green alga *Chlamydomonas reinhardtii* in response to changes in copper availability. *EMBO J.* **14**, 857–865.
162. Moseley, J., Quinn, J., Eriksson, M., et al. (2000) The *Crd1* gene encodes a putative di-iron enzyme required for photosystem I accumulation in copper deficiency and hypoxia in *Chlamydomonas reinhardtii*. *EMBO J.* **19**, 2139–2151.
163. Quinn, J. M., Nakamoto, S. S., and Merchant, S. (1999) Induction of coproporphyrinogen oxidase in *Chlamydomonas* chloroplasts occurs via transcriptional regulation of *Cpx1* mediated by copper-response elements and increased translation from a copper-deficiency-specific form of the transcript. *J. Biol. Chem.* **274**, 14,444–14,454.
164. Quinn, J. M., Barraco, P., Eriksson, M., et al. (2000) Coordinate copper- and oxygen-responsive *Cyc6* and *Cpx1* expression in *Chlamydomonas* is mediated by the same element. *J. Biol. Chem.* **275**, 6080–6089.
165. Quinn, J. M., Eriksson, M., Moseley, J. L., and Merchant, S. (2002) Oxygen deficiency responsive gene expression in *Chlamydomonas reinhardtii* through a copper-sensing signal transduction pathway. *Plant Physiol.* **128**, 463–471.
166. Eckhardt, U. and Buckout, T. J. (1998) Iron assimilation in *Chlamydomonas reinhardtii* involves ferric reduction and is similar to Strategy I higher plants. *J. Exp. Bot.* **49**, 1219–1226.
167. Lynnes, J. A., Derzaph, T. L. M., and Weger, H. G. (1998) Iron limitation results in induction of ferricyanide reductase and ferric chelate reductase activities in *Chlamydomonas reinhardtii*. *Planta* **204**, 360–365.
168. Weger, H. G. (1999) Ferric and cupric reductase activities in the green alga *Chlamydomonas reinhardtii*: experiments using iron-limited chemostats. *Planta* **207**, 377–384.
169. Quinn, J. M., LaFontaine, S., Gohre, V., et al. (2000) Copper-dependent and copper-independent iron metabolism in *Chlamydomonas reinhardtii*. *Plant Physiol.* S961.
170. Herbik, A. and Buckhout, T. J. (2001) Is a ferroxidase involved in the high-affinity iron uptake in Chlamydomonas? *J. Exp. Bot.* **52(Suppl.)**, 80.
171. Hortensteiner, S., Chinner, J., Matile, P., et al. (2000) Chlorophyll breakdown in *Chlorella protothecoides*: characterization of degreening and cloning of degreening-related genes. *Plant Mol. Biol.* **42**, 439–450.
172. Osterberg, R. (1974) Origins of metal ions in biology. *Nature* **249**, 382–383.
173. Nguyen, H. H., Shiemke, A. K., Jacobs, S. J., et al. (1994) The nature of the copper ions in the membranes containing the particulate methane monooxygenase from *Methylococcus capsulatus* (Bath). *J. Biol. Chem.* **269**, 14,995–15,005.
174. Lipscombe, J. D. (1994) Biochemistry of the soluble methane monooxygenase. *Annu. Rev. Microbiol.* **48**, 371–399.
175. Neilsen, A. K., Gerdes, K., and Murrell, J. C. (1997) Copper-dependent reciprocal transcriptional regulation of methane monooxygenase genes in *Methylococcus capsulatus* and *Methylosinus trichosporium*. *Mol. Microbiol.* **25**, 399–409.