CHAPTER 10

Transition Metal Nutrition: A Balance Between Deficiency and Toxicity

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CHAPTER CONTENTS

I. INTRODUCTION

Several transition metals[1], such as zinc (Zn)[2], iron (Fe), manganese (Mn), or copper (Cu) are essential micronutrients for all organisms. Indeed, the recruitment of transition metal ions for biochemical functions is essential as evidenced by the fact that metalloproteins represent about one third of all structurally characterized proteins with a biological activity (Finney and O'Halloran, 2003). In photosynthetic organisms, transition metals act as important cofactors for many enzymes and are essential for both mitochondrial (e.g. copper in cytochrome *c* oxidase) and plastid (e.g. copper in plastocyanin) functions. Non-essential metals such as cadmium (Cd), lead (Pb), or mercury (Hg) are generally viewed as toxic at low concentrations (Warren, 1989). However, unsuspected biological functions might be unveiled for these metals as more protein structures are elucidated and analytical techniques are improved. The discovery that Cd acts as the metal cofactor of a carbonic anhydrase isoform produced under Zn deficiency in the diatom *Thalassiosira weissflogii* (Lane and Morel, 2000a) suggests that metals previously considered to be non-essential and indeed toxic may occasionally act as a prosthetic group in biologically active metalloproteins.

Their interchangeable prevalent ionic forms and affinity for functional groups occurring in proteins are unique properties of transition metals that make them useful in biochemical redox reactions. However, the same chemical properties that make metal ions useful cofactors can also be responsible for undesired reactions in a cellular environment. Indeed, because they are able to establish and maintain several stable coordinate bonds to donor atoms of organic ligands, transition metal ions can become toxic when their interaction and binding partners are not fully controlled. Metal-induced uncontrolled redox reactions or displacement of endogenous metal cofactors from their cellular binding sites can lead to cell poisoning and endanger the organism's survival (Stohs and Bagchi, 1995; Goyer, 1997). To maintain concentrations of essential metal ions within physiological limits and to cope with the toxic effects of metal overload, photosynthetic organisms have developed a tightly controlled and sophisticated metal homeostasis network that insures the balance between metal uptake, chelation, distribution and storage processes (reviewed in Clemens, 2001; Krämer and Clemens, 2005;

[1]Commonly, "transition metal" refers to the 40 elements which belong to groups 3–12 of the periodic table. In the atomic ground state, the highest-energy electron of those elements is in a d-orbital. Most of these elements have an incomplete d sub-shell, or can give rise to cations with an incomplete d sub-shell. Zinc, cadmium, and mercury possess a complete d sub-shell in most oxidation states and constitute therefore exceptions to this definition, but are generally included in the transition metals.

[2]In the chapter, the transition metal names (e.g. zinc or Zn) refer to their biologically relevant oxidation states, and not their elemental form [i.e. zinc or Zn is equivalent to Zn(II)]. For metals that can occur in different oxidation states in biological systems [e.g. Fe(II) or Fe(III), Cu(I) or Cu(II)], the oxidation state is indicated when referring explicitly to one specific oxidation state.

Colangelo and Guerinot, 2006; Grotz and Guerinot, 2006; Merchant et al., 2006; Pilon et al., 2006; Krämer et al., 2007; Puig et al., 2007).

Important progress has been achieved in our understanding of plant metal homeostasis. The completion of the *Arabidopsis* genome sequence allowed the identification of complete protein families that constitute the metal homeostasis network (with components involved mainly in metal ion transport or biosynthesis of metal-binding chelators; Axelsen and Palmgren, 2001; Mäser et al., 2001; http://www.membranetransport.org), and the roles of individual proteins in plant metal homeostasis have begun to be elucidated (Krämer, 2005; Krämer and Clemens, 2005; Williams and Mills, 2005; Colangelo and Guerinot, 2006; Grotz and Guerinot, 2006; Pilon et al., 2006; Puig et al., 2007). At the cellular level, the contribution of each member of multigene families to uptake, chelation, distribution to organelles and storage of essential metals, as well as to the detoxification of non-essential metals remains to be deciphered. As organelles of high metal ion demand due to the involvement of metal cofactors in electron transport chains, chloroplasts represent an essential component of the metal homeostasis network in photosynthetic cells (Merchant, 2006).

Investigating metal homeostasis in plants, and more generally in photosynthetic organisms, is relevant in terms of pollution management, global primary productivity, and human health. In this context, *Chlamydomonas*, as a microorganism, has served as a useful experimental model (reviewed in Merchant, 1998; Hanikenne, 2003; Merchant et al., 2006) because it is facile to manipulate the concentration of metals in the growth medium to generate metal deficient and metal overload situations. Moreover, surveys of the *Chlamydomonas* genome identified a number of transition metal transporters (Rosakis and Köster, 2004; Hanikenne et al., 2005a; Merchant et al., 2006; http://www.membranetransport.org/).

In this chapter, we describe the known components of the metal homeostasis network in *Chlamydomonas*, as well as the physiology and the molecular mechanisms of metal tolerance and metal deficiency responses.

II. COMPONENTS OF THE METAL HOMEOSTASIS NETWORK

A. Metal transporters

1. Introduction

Chlamydomonas possesses several transition metal transporters in 13 families or subfamilies of proteins (Table 10.1), potentially involved in the uptake, exclusion and/or compartmentalization of a range of essential metals such as Zn, Cu, Fe, Mn, Co, or Ni and toxic heavy metals such as Cd. Candidate transporters have been identified on the basis of sequence similarities to known transporters in *S. cerevisiae*, human and *Arabidopsis* (Table 10.2; Rosakis and Köster, 2004; Hanikenne et al., 2005a; Merchant et al., 2006;

Table 10.1 Metal transporter family or subfamily sizes in *Chlamydomonas* compared with other eukaryotes

	Protein families														
										ABC transporters					
Organism	**CDF**	**ZIP**	**CAX**	**HMA**	**COPT CTR**	**FTR**	**NRAMP**	**PIC1**	**CCC1**	**MRP**	**ATM/ HMT**	**CoT**	**NiCoT**	**YSL**	**IREG1**
S. cerevisae	5	5	4	2	2	1	3	–	1	6	1	1	–	–	–
N. crassa	9	7	8	3	2	1	2	–	1	+	2	–	1	+?	–
H. sapiens	9	14	–	2	2	–	2	–	–	12	2	–	–	–	1
C. merolae	3	4	2	2	1	4	3	1	1	2	3	–	–	–	1
Chlamydomonas	**5**	**14**	**5**	**4**	**4**	**1**	**3**	**1**	**–**	**7**	**3**	**1**	**1**	–	–
Arabidopsis	12	17	12	8	5	–	7	1	1	15	3	1–3	2	8	2–3
O. sativa	10	12	12	9	4	–	14	2	1	17	1	3	2	18	1–3

The data used to assemble this table were collected from various sources (March 2007). S. cerevisiae: PlantsT database (http://plantst.genomics.purdue.edu/); N. crassa: Blast search of genome release 3 (http://www.broad.mit.edu/annotation/genome/neurospora/Home.html) and Kiranmayi and Mohan (2006); H. sapiens: Human transporter database (http://lab.digibench.net/transporter/Family.html); C. merolae: the released genome annotation (http://merolae.biol.s.u-tokyo.ac.jp/) and Hanikenne et al. (2005a); Chlamydomonas: genome version 3 and Hanikenne et al. (2005a), Merchant et al. (2006), and http://www.membranetransport.org; Arabidopsis: TAIR6 annotation release (http://www.arabidopsis.org) and PlantsT database; O. sativa: Blast search of TIGR release 5 (http://www.tigr.org/tdb/e2k1/osa1/index.shtml) and Baxter et al. (2003), Jasinski et al. (2003), Klein et al. (2006) and Koike et al. (2004). +: identified by BLAST search, −: not found.

Table 10.2 Putative metal and metalloid transporters

Protein name	Alternative name	Homologue(s)	Putative substrate(s)	Expression evidence[a]	Accession #	Homologue in *Volvox*[b]	References[c]
CDF Family (2.A.4)[d]							
MTP1	–	HsZnT4, AtMTP1	Zn(II)	qRT	EDP01521	Y	[1–3]
MTP2	–	AtMTP8, ShMtp1	Mn(II)	qRT	EDP00083	Y	[1–3]
MTP3	–	AtMTP8, ShMtp1	Mn(II)	qRT	EDP00084	Y	[1–3]
MTP4	–	AtMTP8, ShMtp1	Mn(II)	qRT	EDP00154	Y	[1–3]
MTP5	–	HsZnT9, AtMTP7	?	qRT	EDP01772	Y	[1–3]
ZIP Family (2.A.5)[d]							
ZRT1	ZIP1	ScZrt1 and 2	Zn(II)	qRT	EDP04087	N	[1, 2]
ZRT2	ZIP2	ScZrt1 and 2	Zn(II)	qRT	EDO98169	N	[1, 2]
ZRT3	ZIP3	ScZrt1 and 2	Zn(II)	qRT	EDP08759	N	[1, 2]
ZRT4	ZIP9	ScZrt3	Zn(II)	qRT	EDO98317	N	[1, 2]
ZRT5	ZIP4	ScZrt1 and 2	Zn(II)	qRT	EDO97271	N	[1, 2]
IRT1	ZIP11	?	Fe(II)	qRT	EDP03614	Y	[1, 2]
IRT2	ZIP10	?	Fe(II)	qRT	EDP03614	Y	[1, 2]
ZIP1	ZIP5	ScZrt1 and 2	Zn(II)	qRT	EDO97270	N	[1, 2]
ZIP2	ZIP13	ScZrt3	Zn(II)	qRT	EDP08783	N	[1, 2]
ZIP3	ZIP8	ScZrt3	Zn(II)	qRT	EDP04356	Y	[1, 2]
ZIP4	ZIP12	ScZrt3	Zn(II)	qRT	EDP02242	N	[1, 2]
ZIP6	ZIP6	HsZip1 and 3	Zn(II)	qRT	EDP05024	Y	[1, 2]
ZIP7	ZIL1	?	?	qRT	EDP06917	N	[1, 2, 4]
ZIP14	ZIL2	?	?	–	EDP01584	Y	[1, 2, 4]
CAX family (2.A.19)[d]							
CAX1	–	ScVcx1	?	EST	EDP00630	Y	[1]
CAX2	–	ScVcx1	?	EST	EDP00795	Y	[1]
CAX3	–	?	?	–	EDP03733	Y	[1]
CAX4	–	AtCAX7 to 9	?	–	EDO99207	Y	[1]
CAX5	–	?	?	EST	EDO99460	Y	[4]
HMA subfamily of P–type ATPase (3.A.3)[d]							
HMA1	–	AtHMA1	Cu(II)	EST	EDP00361	Y	[1, 2]
CTP1	HMA2	ScCcc2, Z AtRANR1	Cu(I)	qRT	EDO98907	Y	[1, 2, 5]
CTP2	HMA3	AtPAA1 and 2	Cu(I)	qRT	EDP06350	Y	[1, 2]
CTP3	–	AtPAA1 and 2	Cu(I)	qRT	EDP00070	Y	[2]
Copper transporters (9.A.12)[d]							
COPT1	–	AtCOPTs	Cu(I)	qRT	EDP07016	Y	[1, 2]
CTR1	–	DdCTRs	Cu(I)	qRT	EDP08980	Y	[2]
CTR2	–	DdCTRs	Cu(I)	qRT	EDP06249	Y	[2]
CTR3	–	DdCTRs	Cu(I)	qRT	EDP06429	Y	[4]

(Continued)

Table 10.2 *Continued*

Protein name	Alternative name	Homologue(s)	Putative substrate(s)	Expression evidence[a]	Accession #	Homologue in *Volvox*[b]	References[c]
FTR family (9.A.10.1)[d]							
FTR1	–	ScFet3	Fe(III)	RNA blot	EDP03271		[1, 2, 5]
NRAMP family (9.A.55)[d]							
NRAMP1	DMT1	ScSmf1 and 3	Mn(II) Fe(II)?	qRT	EDP04810	N	[1–3, 6]
NRAMP2	–	ScSmf1 and 3	Mn(II) Fe(II)?	qRT	EDP07304	Y	[1–3]
RET1	NRAMP3	AtEIN2	?	qRT	EDP07047	Y	[4]
PIC1 family							
PIC1	–	AtPIC1	Fe(II)?	qRT	EDP08036	Y	[1]
ABC transporter family (3.A.1)[d]							
MRP subfamily							
MRP1	HLA3	HsABCCs	bicarbonate	RNA blot	EDP07736	Y	[1, 7]
MRP2	–	ScYcf1	GSH–Cd	qRT	EDP03509	Y	[1, 8]
MRP3	–	AtMRP11	?	EST	EDP08676	Y	[1]
MRP4	–	AtMRP11	?	–	EDP06139	Y	[1]
MRP5	–	AtMRP11	?	–	EDP07482	Y	[1]
MRP6	–	AtMRP11	?	EST	EDP01107, EDP01108	Y	[1]
MRP7	–	AtMRP11	?	–	EDP09576	Y	[1]
ATM/HMT subfamily							
CDS1	ATM/HMT1	SpHmt1, HsAbcB6	Cd(II), Fe(II)?	RNA blot	EDO96584	Y	[1, 9]
ATM/ HMT2	–	SpHmt1, HsAbcB6	Cd(II), Fe(II)?	–	EDP00522	Y	[1]
ATM/ HMT3	–	AtATM3, HsAbcB7	Cd(II), Fe(II)?	–	EDP05599	Y	[1]
CoT subfamily							
COT1	–	At3g21580	Co(II)	qRT	EDP03572	Y	[4]
NiCoT family (2.A.52)[d]							
NIK1	–	AtNiCoT1 and 2	Ni(II)	qRT	EDP03691	Y	[4]
ArsAB family (3.A.4)[d]							
ARSA1	–	At3g10350	arsenite	EST	EDO97076	N	[4]
ARSA2	–	At1g01910	arsenite	–	EDP03358	N	[4]
ARSB	–	Bacterial ArsB	arsenite	–	EDP02356	Y	[4]

[a] *Expression evidence: EST (Expressed Sequence Tag), qRT (quantitative real-time RT-PCR).*

[b] *Presence of homologous sequence in Volvox carteri, vista Volvox track at http://genome.jgi-psf.org/Chlre3/Chlre3.home.html.*

[c] *References: [1] Hanikenne et al. (2005a); [2] Merchant et al. (2006); [3] Allen et al. (2007a); [4] http://genome.jgi-psf.org/Chlre3/Chlre3.home.html, [5] La Fontaine et al. (2002b); [6] Rosakis and Koster (2005); [7] Im and Grossman (2002); [8] Wang and Wu (2006); [9] Hanikenne et al. (2005b).*

[d] *Transporter Classification System (Busch and Saier, 2002). Note that the annotations provided in references 1 and 2 were based on the release 2 of the genome. At, Arabidopsis thaliana; Dd, Dictyostelium discoideum, Hs, Homo sapiens; Sc, Saccharomyces cerevisiae; Sh, Stylosanthes hamata; Sp, Schizosaccharomyces pombe.*

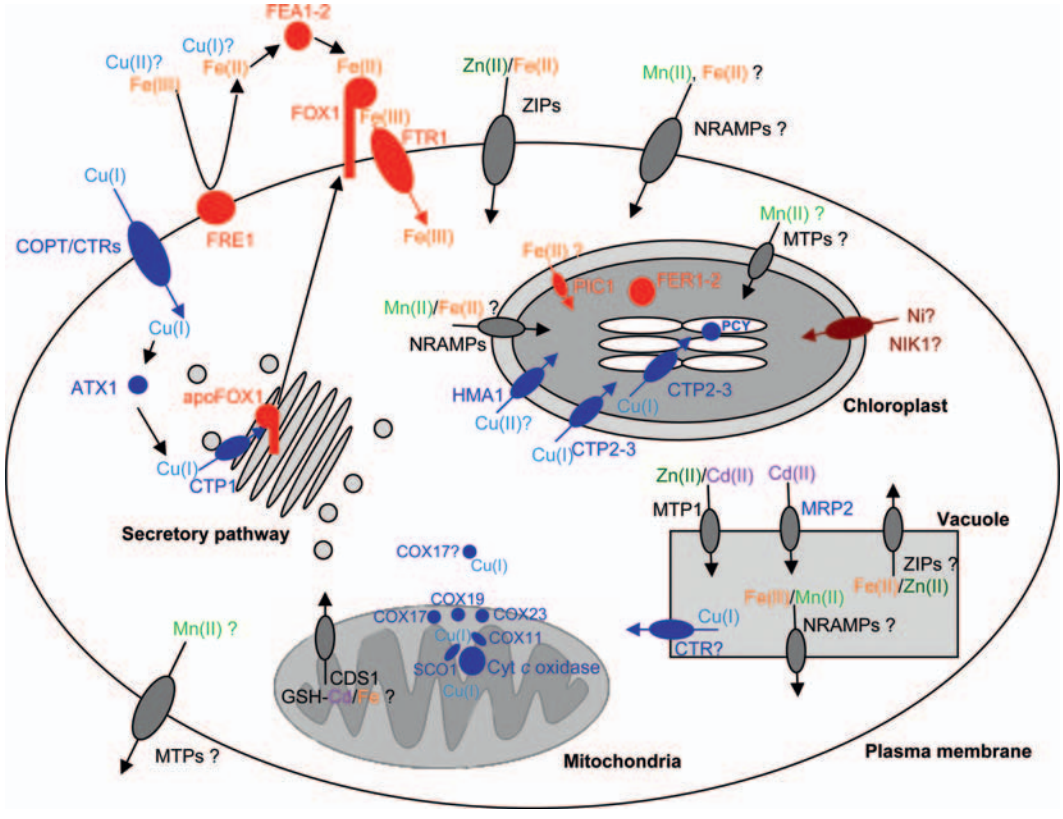

FIGURE 10.1 *Components of the metal homeostasis network. A schematic cell is represented with putative metal transporters, metal-binding proteins and chaperones (see detailed explanations in the text). Note that the nucleus is not represented. Colors are used to distinguish different metals and protein families: blue for copper, red for iron, dark green for zinc, light green for manganese, purple for cadmium, and brown for nickel. Numbers in parentheses indicate the oxidative states of the metal ions, whereas arrows show the proposed directions of transport. Uncertain components or steps are indicated with question marks. ATX1, Copper chaperone; CDS1, Cadmium-sensitive 1; COPT/CTR, Copper Transporters; COX and SCO1: chaperones for copper delivery and insertion into the cytochrome c oxidase; CTP, Copper-transporting P-type ATPases; FEA, Fe-assimilating proteins; FER, Ferritin; FOX1, Multicopper Ferroxidase 1, apoFOX1, precursor form of FOX1 prior to Cu insertion in the secretory pathway; FRE1, Ferrireductase 1; FTR1, Fe Transporter; GSH, glutathione; HMA1, Heavy Metal P-type ATPase 1; MRP2, Multidrug-Resistance associated Protein 2; MTP, Metal Tolerance Proteins; NIK, Nickel Transporter; NRAMP, Natural Resistance-Associated Macrophage Proteins; PCY, plastocyanin; PIC1, Permease in Chloroplast 1; ZIP, ZRT-, IRT-like proteins.*

http://www.membranetransport.org/). A comparison of the *Chlamydomonas* candidate transporters with the repertoire of transporters in other photosynthetic and non-photosynthetic model organisms is also given in Table 10.1. Most of the *Chlamydomonas* proteins have not been functionally characterized and at present the roles of those proteins can only be inferred from phylogenetic analysis of different families of known transporters (Hanikenne et al., 2005a), experimental data derived from functional studies with *Saccharomyces cerevisiae* and *Arabidopsis* homologues, and expression

patterns in response to nutrient deficiency (Figure 10.1; La Fontaine et al., 2002b; Merchant et al., 2006; Allen et al., 2007a,b).

2. CDFs (cation diffusion facilitators)

The CDFs are a family of ubiquitous transporters that catalyze the efflux of a range of transition metals [such as Zn(II), Cd(II), Co(II), or Mn(II)] from the cytoplasm to the extracellular milieu or into subcellular compartments (e.g. vacuole or ER; Gaither and Eide, 2001). The plant members of the CDF family have been named MTPs (Metal Tolerance Proteins; Delhaize et al., 2003), and the same nomenclature was chosen to name the five *Chlamydomonas* candidate MTPs (MTP1–MTP5, Table 10.2; Hanikenne et al., 2005a).

Chlamydomonas MTP1 is closely related to a subgroup of Zn-transporting CDFs of plants (AtMTP1–AtMTP4), *S. cerevisiae* (Zrc1 and Cot1) and humans (HsZnT2–HsZnT4 and HsZnT8; Hanikenne et al., 2005a). AtMTP1 and AtMTP3 are both involved in Zn storage in the vacuole (van der Zaal et al., 1999; Kobae et al., 2004; Desbrosses-Fonrouge et al., 2005; Krämer, 2005; Arrivault et al., 2006). The *MTP1* gene is constitutively highly expressed in metal Zn/Cd hyperaccumulators such as *Arabidopsis halleri* and *Thlaspi caerulescens*, and is proposed to play an essential role in the metal hypertolerance of these species (Dräger et al., 2004; Becher et al., 2004; Craciun et al., 2006; Talke et al., 2006; van de Mortel et al., 2006). In *S. cerevisiae*, Zrc1 and Cot1 are two vacuolar transporters involved in Zn, Co and possibly Cd storage and detoxification (Gaither and Eide, 2001). Similarly to its plant and *S. cerevisiae* homologues, *Chlamydomonas* MTP1 is predicted to localize to the vacuolar membrane (Hanikenne et al., 2005a) and is likely to contribute to Zn storage in the vacuole. The three ESTs corresponding to MTP1 originate from the stress II cDNA library, which included exposure of cells to Cd among several different stress conditions, suggesting that MTP1 might also be involved in the response to Cd toxicity (Hanikenne et al., 2005a).

Chlamydomonas MTP2–MTP4 are related to a subgroup of putative plant Mn-transporting CDFs (Hanikenne et al., 2005a). The best characterized member of this subgroup is ShMTP1 that was proposed to be involved in the vacuolar storage of Mn in the Mn-tolerant legume *Stylosanthes hamata* (Delhaize et al., 2003). The *MTP11* gene has been shown to contribute to Mn tolerance in *Arabidopsis* (Peiter et al., 2007; Delhaize et al. 2007). The *Chlamydomonas MTP2–MTP4* genes arose through presumably recent gene duplications (in the ancestor common to *Volvox* and *Chlamydomonas*) and are found in a tandem organization in the genome. The corresponding proteins might be involved in vacuolar Mn storage, and perhaps in Mn delivery to the chloroplast (Figure 10.1).

Chlamydomonas MTP5 is related to *Arabidopsis* MTP7 and human ZnT9, whose functions are unknown. The *MTP5* gene was shown to be upregulated in Mn deficiency (Allen et al., 2007a). Finally, *Chlamydomonas*

lacks homologs to (i) Zn-transporting CDFs involved in Zn delivery or sequestration in the secretory pathway, such as *S. cerevisiae* Msc2 and Zrg17 (Ellis et al., 2004, 2005) or human ZnT5 and 6 (Palmiter and Huang, 2004; Ellis et al., 2005); and (ii) to *S. cerevisiae* Mmt1 and Mmt2, two mitochondrial proteins that might be involved in Fe homeostasis (Li and Kaplan, 1997).

3. ZIPs (Zrt-, Irt-like proteins)

The ZIPs are another family of ubiquitous transporters that catalyze the movement of transition metals [such as Fe(II), Zn(II), Cd(II), or Mn(II)] from outside the cell or a subcellular compartment to the cytoplasm (Guerinot and Eide, 1999; Gaither and Eide, 2001). The ZIP proteins have been classified into four subfamilies based on sequence conservation: subfamily I groups most of the plant and *S. cerevisiae* ZIPs, subfamily II mainly includes mammalian proteins, the GufA subfamily contains prokaryotic and eukaryotic ZIPs of unknown function, and the Liv1 subfamily appears to be eukaryote-specific (Guerinot and Eide, 1999; Gaither and Eide, 2001). The *Chlamydomonas* ZIPs were first named based on their phylogenetic relationships (Hanikenne et al., 2005a), but later renamed according to their metal responsiveness at the transcriptional level (Merchant et al., 2006). Both nomenclatures are given in Table 10.2, but for clarity the latter has been adopted in this manuscript. *ZRT1–ZRT5* are up-regulated under Zn deficiency, whereas *IRT1* and *IRT2* are up-regulated under Fe deficiency, supporting a proposed function in Zn and Fe assimilation, respectively. Based on their sequences, the *ZIP1–6*, *ZIP7* and *ZIP14* genes belong to the ZIP family, but are not transcriptionally regulated by the metal nutritional status.

Chlamydomonas ZRT1–ZRT3, ZRT5, and ZIP1 belong to subfamily I and are related to most of the *Arabidopsis* (ZIP1–ZIP12 and IRT1–IRT3) and *S. cerevisiae* ZIPs (Zrt1 and Zrt2; Hanikenne et al., 2005a). Within this subfamily, transporters of the three species form distinct groups, suggesting that the diversification occurred independently in the subgroups through duplications of a common ancestral gene. The *ZRT5* and *ZIP1* genes are found in tandem in the *Chlamydomonas* genome. However, the two genes display different expression patterns, which is indicative of a diversification of function.

In *S. cerevisiae*, Zrt1 and Zrt2 are located in the plasma membrane and mediate high- and low-affinity Zn uptake, respectively (Zhao and Eide, 1996a,b). In *Arabidopsis*, ZIP1 to ZIP4 are involved in cellular Zn uptake and IRT1 constitutes the major Fe uptake system in roots where the gene is induced by Fe deficiency (Grotz and Guerinot, 2006). Several ZIP genes are constitutively expressed at high levels in *A. halleri* (Becher et al., 2004; Weber et al., 2004; Talke et al., 2006) and *T. caerulescens* (Lasat et al., 2000; Pence et al., 2000; Assunção et al., 2001; van de Mortel et al., 2006) and are postulated to function in Zn hyperaccumulation. Based on the function of their homologues and their transcriptional regulation, the

Chlamydomonas subfamily I ZIPs represent likely candidates for a role in cellular Zn uptake (Figure 10.1).

Chlamydomonas ZIP6 belongs to subfamily II and is related to the mammalian ZIP1–ZIP3, which are involved in Zn uptake (Eide, 2006), whereas *Chlamydomonas* ZRT4 and ZIP2–ZIP4 cluster with members of the GufA subfamily (Hanikenne et al., 2005a). The only characterized member of this family is *S. cerevisiae* Zrt3, which remobilizes vacuolar Zn upon Zn deficiency (MacDiarmid et al., 2000). Finally, no member of the Liv1 subfamily was found in *Chlamydomonas* (Hanikenne et al., 2005a). The human LIV1 ZIPs were shown to be capable of Zn transport, and mutations in them have been implicated in various cancers, the control of the cell cycle and cell proliferation, or the control of neurodegeneration and growth (Taylor et al., 2003, 2004).

The two *Chlamydomonas* Fe-regulated ZIPs, IRT1, and IRT2, also group with the GufA proteins (Hanikenne et al., 2005a). Although they are not related to AtIRT1, they might have similar functions. Their up-regulation under Fe deficiency suggests that they might also contribute to Fe uptake. Alternatively, they might be involved in Fe remobilization from intracellular storages (e.g. vacuoles) or between one organelle (chloroplast) and another (mitochondrion). Finally, the ZIP7 and ZIP14 proteins are very distantly related to other ZIP proteins and should be considered as putative ZIPs (Table 10.2).

Compared to other unicellular organisms, *Chlamydomonas* possesses a high number of ZIP proteins with as many proteins of this family as vascular plants and humans (Table 10.1). It is noteworthy that ZIP diversification occurred in different subfamilies in different species: in subfamily I for *Arabidopsis*, Liv1 for humans, and GufA and subfamily I for *Chlamydomonas*. This evolution from a common set of ancestral genes represents a good example of convergent adaptation where the need for a similar function (i.e. cellular Zn uptake and distribution) was achieved via the diversification of different protein subfamilies.

4. CAXs (cation exchanger)

The CAX proteins are divalent cation/proton antiporters (Shigaki and Hirschi, 2006). A *vcx1 S. cerevisiae* mutant (*v*acuolar proton/*C*a(II) exchanger) lacking a CAX protein is defective in vacuolar Ca accumulation (Cunningham and Fink, 1996). The *Arabidopsis* AtCAX1 and AtCAX2 proteins are homologues of Vcx1 and are high and low-affinity Ca(II)/proton vacuolar antiporters, respectively (Hirschi et al., 1996). AtCAX2 has also been proposed to transport Mn(II) and Cd(II) across the tonoplast of plant cells: tobacco plants overexpressing *AtCAX2* accumulate more Ca, Mn, and Cd and are more tolerant to high Mn (Hirschi et al., 2000). Root tonoplast vesicles of tobacco expressing AtCAX2 display higher transport activity of Mn and Cd (Hirschi et al., 2000). The roles of the nine other *Arabidopsis* CAXs remain unclear. Finally,

the CAX protein AtMHX1 is a proton-coupled antiporter that transports Mg and Zn across the vacuolar membrane (Shaul et al., 1999).

Five CAXs (CAX1–CAX5) are encoded in the *Chlamydomonas* genome (Hanikenne et al., 2005a): CAX1 and CAX2 are highly related to *S. cerevisiae* Vcx1 and AtCAX1–AtCAX6, whereas the other CAXs are more distantly related. *Chlamydomonas CAX1* transcripts are highly represented in the cDNA libraries Stress II and III, which include cDNAs prepared from Cd-treated cells and Cu or Fe deficient cells, respectively (Shrager et al., 2003), consistent with the idea that CAX1 is involved in transition metal homeostasis or detoxification. No homologue of AtMHX1 has been found in the *Chlamydomonas* genome. The *AtMHX1* gene is mainly expressed in the vascular tissues and might be involved in Zn(II)/Mg(II) partitioning within plant organs (Shaul et al., 1999).

5. HMAs (heavy metal-transporting P-type ATPases)

Proteins of the I_B subfamily P-type ATPases (HMAs or CPx ATPases) are polytopic proteins with eight transmembrane segments that operate in transition metal transport. Their sixth transmembrane domain contains a conserved Cys-Pro-Cys/His/Ser motif (CPx motif) believed to participate in metal cation translocation across the membrane. HMAs are found in both prokaryotes and eukaryotes and are divided in two groups based on their substrate specificity: monovalent Cu(I)/Ag(I) cations or divalent Zn(II)/Cd(II)/Co(II)/Pb(II) cations (Cobbett et al., 2003; Williams and Mills, 2005).

Four HMAs are encoded in *Chlamydomonas* (Hanikenne et al., 2005a; Merchant et al., 2006). HMA1 belongs to the divalent cation-transporting subgroup that is only found in prokaryotes and plants (Williams and Mills, 2005), and is more specifically related to AtHMA1. Like AtHMA1, *Chlamydomonas* HMA1 has an uncharacteristic Ser/Pro/Cys motif in the sixth predicted transmembrane domain instead of the common Cys-Pro-Cys/His/Ser motif. This property is shared with the Co transporter CoaT of the cyanobacterium *Synechocystis* and might determine the substrate specificity of the transporter (Cobbett et al., 2003). AtHMA1 has been shown to be a plastid Cu transporter (Seigneurin-Berny et al., 2006; Puig et al., 2007).

Chlamydomonas CTP1, CTP2, and CTP3 are putative Cu-transporting proteins from the monovalent metal transporter subfamily of HMAs (Hanikenne et al., 2005a; Merchant et al., 2006). Together with the Cu chaperone ATX1, *Chlamydomonas* CTP1 was proposed to play a role in Cu delivery to the secretory pathway (Figure 10.1; section II.B.3; La Fontaine et al., 2002b), as has been functionally demonstrated for the closely related Ccc2 in *S. cerevisiae*, RAN1 in *Arabidopsis* and the Menkes and Wilson ATP7A and B in humans (Askwith et al., 1996; La Fontaine et al., 2002a,b; Lutsenko and Petris, 2003; Puig et al., 2007). Atx1 is required in *S. cerevisiae* for delivery of cytosolic Cu to apo-ferroxidase via P-type ATPase-dependent transport of Cu in a post-Golgi vesicle (Lin et al., 1997; Yuan et al., 1997;

Himelblau et al. 1998). A *Chlamydomonas* gene encoding an Atx1 ortholog has been cloned and shown to rescue high-affinity Fe uptake when expressed in an *atx1 S. cerevisiae* mutant (La Fontaine et al., 2002b). A role for CTP1 in Cu tolerance by mediating Cu efflux from the cells, as suggested for AtHMA5, should also be considered (Andrés-Colás et al., 2006).

Chlamydomonas CTP2 and CTP3 are related to the *Arabidopsis* chloroplast proteins PAA1 and PAA2 (Hanikenne et al., 2005a; Merchant et al., 2006). AtPAA1 is localized in the outer membrane of the chloroplast envelope and has been proposed to transport Cu into the stroma (Shikanai et al., 2003), whereas AtPAA2 resides in the thylakoid membrane and is postulated to drive Cu transport to the lumen for delivery to plastocyanin (Abdel-Ghany et al., 2005b). Consistent with these hypotheses, a *paa1* mutant is defective for both stromal Cu/Zn superoxide dismutase and plastocyanin, whereas a *paa2* mutant only lacks plastocyanin (Abdel-Ghany et al., 2005b). Although it is likely that CTP2 and CTP3 play similar roles in *Chlamydomonas* (Figure 10.1), their subcellular localizations and Cu delivery activities remain to be established experimentally.

6. Copper transporters (COPTs and CTRs)

Chlamydomonas encodes four putative Cu transporters (COPT1 and CTR1–CTR3) related to *S. cerevisiae* Ctr1–Ctr3 and *Arabidopsis* COPT1–COPT5 (Hanikenne et al., 2005a; Merchant et al., 2006). In *S. cerevisiae*, Cu uptake is mediated by plasma membrane reductases that reduce Cu(II) to Cu(I) (see also section IV.B.1) and Ctr1 and Ctr3-dependent high-affinity Cu(I) transport, which uses a highly specific Cu(I)/2 K(I) antiport mechanism. The role of the vacuolar *S. cerevisiae* Ctr2 remains unclear (Eide, 1998; Puig et al., 2007). Similar proteins are involved in Cu uptake in mammalian cells as well (Petris, 2004). *Chlamydomonas* COPT1 is predicted to localize to the plasma membrane, supporting a role in Cu uptake (Figure 10.1; Hanikenne et al., 2005a).

7. FTRs (Fe transporters)

In *Chlamydomonas*, the high-affinity Fe uptake system involves a plasma membrane ferric-chelate reductase (FRE1), a multicopper ferroxidase (FOX1) and an iron permease (FTR1; Herbik et al., 2002a; La Fontaine et al., 2002b; Merchant et al., 2006; Allen et al., 2007b). This system will be described in more detail in section IV.A. FTR1 is homologous to the *S. cerevisiae* high-affinity Fe permease Ftr1 (Radisky and Kaplan, 1999) and is predicted to localize to the plasma membrane (Figure 10.1; Hanikenne et al., 2005a).

8. NRAMPs (natural resistance-associated macrophage proteins)

Proteins of the NRAMP family participate in the transport of divalent metal cations. The human NRAMP1 restricts metal availability for pathogens by transporting Mn(II) and Fe(II) across the phagosomal membranes of

macrophages, and is therefore required for natural resistance to intracellular bacterial infection (Mackenzie and Hediger, 2004). The second human NRAMP HsNRAMP2/HsDMT1 is involved in dietary Fe(II) uptake at the apical surface of enterocytes. It is also able to transport additional divalent metal cations [Cd(II), Co(II), Cu(II), Zn(II), Mn(II) or Pb(II)] in addition to Fe(II) (Mackenzie and Hediger, 2004). Out of six *Arabidopsis* NRAMPs, only NRAMP1, NRAMP3, and NRAMP4 have been functionally characterized. The three genes are up-regulated in Fe-deficiency and the corresponding proteins were shown to mediate Fe(II), Mn(II), and Cd(II) transport in *S. cerevisiae* (Curie et al., 2000; Thomine et al., 2000). AtNRAMP1 might contribute to Fe storage in the chloroplast, whereas AtNRAMP3 and AtNRAMP4 are involved in the remobilization of vacuolar Fe under deficiency (Thomine et al., 2003; Lanquar et al., 2005).

Arabidopsis possesses an additional protein related to NRAMPs called EIN2 (*E*thylene *In*sensitive 2), which is involved in ethylene responses and signaling (Alonso et al., 1999; Benavente and Alonso, 2006). The protein does not seem to be capable of metal transport activity. Surprisingly, *Chlamydomonas* RET1 shows homology to EIN2 (Table 10.2). As no ethylene signaling exists in *Chlamydomonas*, the role of RET1 is unclear, and it may be involved in some other signal transduction pathway.

The *Chlamydomonas* NRAMP1 and NRAMP2 proteins are related to *S. cerevisiae* Smf1–Smf3 (Hanikenne et al., 2005a) which are Mn, Cu and to a lesser extent Fe transporters. Smf1 localizes to the plasma membrane, whereas Smf2 resides in intracellular vesicle membranes, and Smf3 in the vacuolar membrane (Cohen et al., 2000). *Chlamydomonas* NRAMP1 was initially named DMT1, and complementation data in *S. cerevisiae* suggest that it is capable of Mn and possibly Fe transport (Rosakis and Köster, 2005). Both *NRAMP1* and *NRAMP2* genes are slightly induced by Mn deficiency (Allen et al., 2007a). The *Chlamydomonas* NRAMPs might be involved in Mn, and possibly Fe uptake, or in the remobilization of these metals from the vacuole under conditions of metal deficiency in *Chlamydomonas*. As in the case of Smf2 (Culotta et al., 2006), it is possible that one of the *Chlamydomonas* NRAMPs operates in the delivery of Mn to Mn superoxide dismutases.

9. PIC1 (permease in chloroplast1)

Chlamydomonas PIC1 is related to plant and cyanobacterial permeases (Duy et al., 2007). *Arabidopsis* PIC1 localizes to the inner chloroplastic membrane. *Arabidopsis pic1* mutants are dwarfed and chlorotic, and constitutively upregulate metal transport (*NRAMPs*, *IRT1*, *YSL1*) and homeostasis (*FER1*, *FER4*) genes. Moreover, PIC1 and its *Synechocystis* homologue complement the growth of metal uptake-defective *S. cerevisiae* mutants. Taken together, these data suggest that the PIC1 proteins participate in chloroplast Fe import (Figure 10.1; Duy et al., 2007).

10. ABC (ATP-binding cassette) transporters

a. Introduction

ABC transporters form a large and ubiquitous superfamily of transporters that participate in a wide range of physiological processes. The transport process is energized by ATP (reviewed in Holland et al., 2003). ABC transporters can be divided in several subfamilies based on structural similarities (Decottignies and Goffeau, 1997; Sánchez-Fernández et al., 2001; Dean et al., 2003; Rea, 2007). Here, presentation of the *Chlamydomonas* ABC transporter superfamily will be restricted to the members of the MRP and ATM/HMT subfamilies because members of these subfamilies contribute to metal transport in plants and *S. cerevisiae*. *Chlamydomonas* also encodes a relative of prokaryotic Co-transporting ABC proteins (Eitinger et al., 2005; Rodionov et al., 2006).

b. MRPs (multidrug-resistance associated proteins)

MRPs are full-size ABC transporters acting as glutathione-*S*-conjugate pumps (Rea et al., 1998). Two *S. cerevisiae* MRPs, namely Ycf1 (for *y*east *c*admium *f*actor) and Bpt1 (for *b*ile *p*igment *t*ransporter) are involved in heavy metal detoxification. Ycf1[3] transports glutathione (GSH)-Cd, -arsenate or -Hg complexes from the cytoplasm to the vacuole (Szczypka et al., 1994; Z.S. Li et al., 1996, 1997; Ghosh et al., 1999; Gueldry et al., 2003), and the vacuolar Bpt1 also contributes, although marginally, to Cd tolerance (Klein et al., 2002; Sharma et al., 2002). In *Arabidopsis*, MRP3 is the only MRP (out of 14) that is involved in Cd tolerance. MRP3 is upregulated by Cd and the corresponding protein complements the Cd-sensitive phenotype of a *S. cerevisiae ycf1* mutant (Bovet et al., 2003; Tommasini et al., 1998). The other characterized AtMRPs contribute to the transport of various substrates (e.g. GSH conjugates, glucuronide conjugates, or chlorophyll catabolites) and are involved in a range of physiological processes (reviewed in Klein et al., 2006; Schulz and Kolukisaoglu, 2006; Rea, 2007).

Of the seven *Chlamydomonas* MRPs, only *MRP1* and *MRP2* have been cloned and functionally characterized. MRP1 localizes to the chloroplast, and *MRP1* is regulated at the transcriptional level by light and CO_2. It is hypothesized to be involved in bicarbonate uptake (Im and Grossman, 2002; Grossman et al., 2007; see also Chapter 8). MRP2 is related to *S. cerevisiae* Ycf1 and is able to complement the Cd-sensitive phenotype of a *S. cerevisiae ycf1* mutant, which suggests a vacuolar localization. The *MRP2* gene is up-regulated by Cd. A *mrp2* mutant is Cd-sensitive and presents a modified phytochelatin (PC)-Cd[4] complex accumulation pattern, with a faster rate of low-molecular weight PC-Cd complex formation and

[3]Note that the yeast Ycf1 vacuolar protein is different from *Chlamydomonas* chloroplast Ycf1 (Boudreau et al., 1997)

[4]PC: phytochelatin. PCs are small metal binding peptides (see section II.B.1).

increased Cd sequestration by the PC-Cd complexes (Wang and Wu, 2006). These data suggest that MRP2 contributes to Cd vacuolar storage. Based on sequence similarity, it is likely that *Chlamydomonas* MRP3–MRP7 are glutathione-*S* conjugate pumps involved in xenobiotic and/or metal tolerance, but this will have to be determined experimentally.

c. ATM/HMT (ABC transporter of the mitochondria/heavy metal tolerance)

ATM/HMTs are half-size ABC transporters (Rea, 2007) that localize to the mitochondrial inner membrane or the vacuolar membrane. Mitochondrial transporters (HsABCB6 and HsABCB7, AtATM1–AtATM3 and ScAtm1) are involved in the biogenesis of iron/sulfur clusters and in Fe homeostasis (Kispal et al., 1997, 1999; Csere et al., 1998; Allikmets et al., 1999; Mitsuhashi et al., 2000; Kushnir et al., 2001; Rea, 2007), whereas vacuolar transporters (SpHMT1 and CeHMT-1) are involved in the transport of PC-Cd complexes from the cytoplasm into the vacuole and contribute to Cd tolerance (Ortiz et al., 1992, 1995; Vatamaniuk et al., 2005; Rea, 2007).

A mitochondrial ATM/HMT ortholog, named CDS1, has been studied in *Chlamydomonas* (Hanikenne et al., 2005b). The *CDS1* gene is strongly induced by Cd exposure and a *cds1* mutant is sensitive to Cd and high Fe concentrations. Two possible models can be proposed to explain the phenotype of *cds1*. First, CDS1 could be directly involved in the export of Cd out of the mitochondrial matrix, possibly as a glutathione-Cd conjugate, thereby protecting mitochondrial function from Cd toxicity. Alternatively, the Cd- and Fe-sensitive phenotype of the *cds1* mutant could be an indirect consequence of a modification to Fe homeostasis in the mitochondria. The lack of a mitochondrial CDS1 transporter could indeed result in cytosolic Fe deficiency, as suggested previously for the *S. cerevisiae* mutant *atm1*, which displays both mitochondrial Fe accumulation and cytosolic Fe deficiency phenotypes (Schueck et al., 2001). In vascular plants, Fe deficiency can often lead to an increased uptake of Cd(II) and hence sensitivity due to the induction of the Fe(II) uptake systems (Thomine et al., 2000, 2003; Connolly et al., 2002; Lombi et al., 2002).

Arabidopsis ATM2 and ATM3 are related to *Chlamydomonas* CDS1, with ATM3 being the closer homologue (Hanikenne et al., 2005a,b). D.Y. Kim et al. (2006) showed that the *Arabidopsis ATM3* gene is up-regulated by Cd and that an *atm3* mutant is Cd-sensitive. On the other hand, overexpressing plants display an increased resistance to Cd. The expression analysis of glutathione biosynthesis genes and the determination of nonprotein thiol content in the mutant support a role of AtATM3 in the export of glutathione-Cd chelates from mitochondria. The ability of mitochondrial ABC transporters to act in Cd detoxification could therefore be a newly discovered property among photosynthetic organisms.

No gene encoding a vacuolar PC-Cd-transporting ABC transporter has been identified in photosynthetic organisms, despite the fact that such a transport function has been biochemically identified in plant vacuolar membranes (Vögeli-Lange and Wagner, 1990; Salt and Rauser, 1995; Rea, 2007).

d. CoT (cobalt transporter)

Chlamydomonas COT1 is related to putative prokaryotic and plant Co-transporting ABC transporters (Eitinger et al., 2005; Rodionov et al., 2006), with the closest homologues identified to date being found in the marine green alga *Ostreococcus tauri*, *Arabidopsis*, rice, cyanobacteria, and bacteria. The plant and algal proteins are predicted to localize either to the chloroplast or mitochondrion. It is worth considering whether Co may substitute for another metal in *Chlamydomonas* as it does in marine organisms (Lane and Morel, 2000b). Although there are cobalamin-dependent enzymes in *Chlamydomonas*, the cofactor is obtained from bacteria that associate with the alga (Croft et al., 2005). In plants, Co is considered a beneficial element, but no precise function has been established (Marschner, 1995; Krämer and Clemens, 2005). Further experiments will be required to establish the substrate specificity of COT1 and its plant homologues, and to determine the function of this putative Co transporter.

11. NiCoT

In anaerobic bacteria and cyanobacteria, Ni is found in hydrogenases, dehydrogenases, and methyl reductases, whereas urease is the only known Ni-requiring enzyme in vascular plants (Eitinger et al., 2005; Krämer and Clemens, 2005). Ni is an essential micronutrient in vascular plants and accordingly, Ni deficiency induces the accumulation of toxic urea concentrations in several species (Krämer and Clemens, 2005). Although no urease homologue has been identified in the annotated *Chlamydomonas* proteins, and no Ni-Fe hydrogenase activity has been found, *Chlamydomonas* does have a putative high-affinity Ni transporter, NIK1 (Table 10.2) and a putative Ni chaperone for urease or hydrogenase (accession EDP04575) similar to the urease accessory protein UreG found in bacteria and *Arabidopsis* (Krämer and Clemens, 2005). Danilov and Ekelund (2001) have shown that high Ni concentrations had stimulatory effects on photosynthesis efficiency in *Chlamydomonas*. Moreover, Quinn et al. (2003) found that Ni treatment causes a CuRE[5]-dependent upregulation of *CYC6*, *CPX1*, and *CRD1*, mimicking their induction under Cu deficiency (see also section IV.B.3.d). The authors present two models to explain the effect of Ni, either

[5]Cu-responsive element (see section IV.B.3.d).

that the target genes and Ni metabolism might be physiologically or functionally connected, or that Ni might interfere with Cu metabolism, possibly by interacting with the Cu signaling system.

12. ArsAB family

Toxic to all living forms, the metalloid arsenic is widely distributed in water, air, and soil (reviewed in Nriagu and Pacyna, 1988; Jones, 2007; Tripathi et al., 2007). In humans, chronic arsenic exposure leads to digestive disorders and cancers (Jones, 2007). Arsenate [As(V)] and arsenite [As(III)] represent the two biologically important species of arsenic, which are interconvertible depending on the redox status. In plants as in *S. cerevisiae*, arsenate enters cells via phosphate transporters, is subsequently reduced to arsenite by arsenate reductase, and is then excluded from the cell or stored in the vacuole (Rosen, 2002; Tripathi et al., 2007).

In *Chlamydomonas*, an arsenate-resistant mutant was shown to be impaired in a putative Na^+/Pi co-transporter (see section III.B, Fujiwara et al., 2000; Kobayashi et al., 2003), suggesting that the transporter is an entry route for arsenate. Once in the algal cell, arsenate does not induce phytochelatin synthesis (see section II.B.1, Kobayashi et al., 2006). However, Cd-induced GSH and/or phytochelatin accumulation was shown to increase *Chlamydomonas* arsenate tolerance, suggesting that GSH and/or phytochelatin can interact with arsenate metabolism (Kobayashi et al., 2006). No arsenate reductase homologue has been identified in the genome. However, three genes encoding putative components of a prokaryotic ATP-dependent arsenite efflux system (Rosen, 2002; Tripathi et al., 2007) are found: *ARSA1* and *ARSA2* share homologies with arsenite-translocating ATPase, whereas *ARSB* represents a putative arsenite permease (Table 10.2). Note that *ARSA* is conserved in plastid-containing organisms, suggestive of a localization in the plastid (Merchant et al., 2007). It is also possible that at least one *Chlamydomonas* MRP (Table 10.2, section II.A.10.b) contributes to arsenite storage in the vacuole, as was shown for Ycf1 in *S. cerevisiae* (Ghosh et al., 1999).

B. Metal chelation

1. Glutathione and phytochelatins

Metal chelators are key components of the metal homeostasis network. They include various organic acids (such as malate or citrate), low-molecular weight ligands, metallochaperones, and proteins that contribute to the intracellular trafficking and storage of metals. The tripeptide GSH and the derived PCs are well-known contributors to non-essential metal tolerance in a wide range of organisms (see section II.A.10.b; Z.S. Li et al., 1996, 1997; Xiang et al., 2001; Cobbett and Goldsbrough, 2002; Clemens, 2006). Whether PCs might also have a role in essential transition metal homeostasis

has been discussed extensively and is still a matter of debate (see Clemens, 2006). In *Chlamydomonas*, GSH is the major compound induced after Hg exposure (Howe and Merchant, 1992), whereas PCs are the major metal-binding peptides induced by Cd (Howe and Merchant, 1992; Hu et al., 2001). Up to 70% of the Cd found in Cd-treated cells is bound to PCs (Hu et al., 2001). As in the fission yeast *S. pombe* and *Arabidopsis* (Howden et al., 1995a,b; Ortiz et al., 1992, 1995), low molecular weight PC-Cd complexes are formed and are rapidly converted to high molecular weight complexes that accumulate in the cells and contribute to a stable sequestration of Cd (Hu et al., 2001).

As shown in other algae (Mendóza-Cozatl et al., 2005), high molecular weight PC-Cd complexes are mainly found in the chloroplast in *Chlamydomonas* (Nagel et al., 1996), whereas PCs are stored in the vacuole in *S. pombe* and *Arabidopsis* (Vögeli-Lange and Wagner, 1990; Cobbett and Goldsbrough, 2002; Clemens and Simm, 2003). The analysis of four *Chlamydomonas* Cd-resistant mutants showed a correlation between Cd accumulation and PC production (Hu et al., 2001). Conversely, no correlation was observed between PC and Cd accumulation when analyzing two Cd-tolerant *Chlamydomonas acidophila* strains that exhibit contrasting Cd accumulation capacities (Nishikawa et al., 2006). In this case, Cd accumulation correlated with the ability to maintain high GSH levels via the induction of the γ-glutamylcysteine synthetase, the first enzyme and limiting step of the GSH synthesis. This enzyme and GSH synthetase, which together catalyze the two-step biosynthesis of GSH, are encoded by the *GSH1* and *GSH2* genes, respectively. A gene encoding a putative PC synthase is also found in *Chlamydomonas* (Table 10.3).

2. Metallothioneins

Metallothioneins are ubiquitous low molecular weight proteins rich in Cys residues that have high metal-binding capacities (Rauser, 1999; Cobbett and Goldsbrough, 2002). Surprisingly, the *Chlamydomonas* genome does not appear to encode metallothioneins. However, this might be an artifact caused by the difficulty of predicting small and low-complexity proteins (Merchant et al., 2006).

3. Metallochaperones

Intracellular trafficking of metals and their proper insertion into target proteins require proteins called metallochaperones (O'Halloran and Culotta, 2000). A few Cu metallochaperones are encoded in *Chlamydomonas* (Table 10.3). The putative Cu chaperone ATX1 was first identified from ESTs (La Fontaine et al., 2002b). By analogy to its *S. cerevisiae*, plant and animal homologues, ATX1 is proposed to handle the transfer of Cu from the uptake site at the plasma membrane to a Cu(I)-transporting HMA (possibly

Table 10.3 Genes encoding metal chelator synthesis proteins and putative metallochaperones

Gene name	Gene product and putative function	Expression evidence[a]	Accession #	Homologue in *Volvox*[b]	References[c]
GSH and PC synthesis					
GSH1	γ-glutamylcysteine synthetase, 1st step of GSH synthesis	EST	EDP06622	Y	[1]
GSH2	Glutathione synthetase, 2[d] step of GSH synthesis	EST	EDP04651	Y	[1]
PCS	PC synthase, Phytochelatin synthesis	-	EDP07275	Y	[1]
Copper chaperones					
ATX1	Cu delivery to the secretory pathway	RNA blot	EDP02326	Y	[1–2]
COX17	Cu delivery to the mitochondria	RNA blot	EDO97130	Y	[1–4]
COX19	Cu delivery to the mitochondria	EST	EDP08032	Y	[1, 3, 4]
COX23	Cu delivery to the mitochondria	EST	EDO98664	Y	[1, 4]
COX11	Cu delivery to Cu_B center of Cox1 subunit of Cyt *c* oxidase	EST	EDO98350	Y	[1, 3, 4]
SCO1	Cu delivery to Cu_A center of Cox2 subunit of Cyt *c* oxidase	EST	EDO97370	Y	[1, 3, 4]

[a] *Expression evidence: EST (Expressed Sequence Tag).*

[b] *Presence of homologous sequence in the genome of Volvox carteri, vista Volvox track at http://genome.jgi-psf.org/Chlre3/Chlre3.home.html*

[c] *References: [1] http://genome.jgi-psf.org/Chlre3/Chlre3.home.html; [2] La Fontaine et al. (2002b); [3] Merchant et al. (2006); [4] Cardol et al. (2005). Note that the annotations provided in reference 3 were based on the release 2 of the genome.*

CTP1) located in the secretory pathway, where Cu can be inserted into target proteins such as the multicopper ferroxidase FOX1 (see section II.A.5 and Figure 10.1, La Fontaine et al., 2002b).

The mitochondrial cytochrome *c* oxidase, a component of the electron transport chain, is a major Cu-requiring enzyme. Genes encoding Cu chaperones (COX17 and COX19) and assembly factors (COX11, COX23, and SCO1) required for Cu(I) insertion into cytochrome *c* oxidase (Cobine et al., 2006) are all present in *Chlamydomonas* (Table 10.3, Cardol et al., 2005; Merchant et al., 2006).

Finally, the Ccs Cu chaperone present in *S. cerevisiae*, plants and animals is required for the proper insertion of Cu and the activation of the Cu/Zn superoxide dismutase (Abdel-Ghany et al., 2005a; Culotta et al., 2006).

Chlamydomonas, like many other green algae, lacks a Cu/Zn superoxide dismutase (Sakurai et al., 1993) and consistently, also lacks a gene encoding a Ccs homologue.

C. Differences with other photosynthetic organisms

1. *Chlamydomonas* and other algae

A comparative inventory of metal transporter families in *Chlamydomonas* and the unicellular red alga *Cyanidioschyzon merolae* revealed that both algae have adapted their metal transporter complements to fit their environment (Hanikenne et al., 2005a). *Chlamydomonas* appears to be more complex than *Cyanidioschyzon* with respect to the size of the different transporter families (Table 10.1). *Chlamydomonas* is a motile organism that needs to adapt to a fluctuating environment in water and soils. It possesses a complex life cycle with distinct vegetative and sexual stages for which specialized functions may have evolved. In contrast, *Cyanidioschyzon* is an asexual alga that lives in acidic sulfur- and metal-rich (Fe, Ni, etc.) hot springs (Gross, 2000; Ciniglia et al., 2004). It is able to face stable, although extreme, conditions with a limited number of metal transporters. The metal transporter complement of *Cyanidioschyzon* can be rationalized in the context of its specific environment characterized by high Fe and low Cu availability. Among the extant genes are those encoding an IREG1-like protein for Fe efflux and a Ccc1 homologue for vacuolar Fe storage (see below in this section), whereas this alga lacks several proteins involved in Cu metabolism (the multicopper ferroxidase, a chloroplastic Cu I_B P-type ATPase, and plastocyanin; Table 10.1; Hanikenne et al., 2005a). On the other hand, most of the *Chlamydomonas* genes described here are conserved in *Volvox carteri* (Tables 10.2 and 10.3).

2. *Chlamydomonas* and vascular plants

The comparison of the *Chlamydomonas* and vascular plant genomes reveals that the alga possesses the basic tools to maintain cellular metal homeostasis (Table 10.1 and Figure 10.1). As such, it represents a powerful model to study the functions and the interactions of different metal transport and intracellular trafficking systems (Hanikenne, 2003; Hanikenne et al., 2005a; Merchant et al., 2006). However, vascular plants evolved adaptations of their metal homeostasis network, reflecting the need for long distance transport and for the proper distribution of metals between different organs and cell types.

Chlamydomonas indeed lacks several protein families that contribute to long-distance transport and metal distribution to plant tissues. Although widespread in prokaryotes, the occurrence of divalent cation-transporting HMAs in eukaryotes is apparently limited to plants (Hanikenne et al., 2005a; Williams and Mills, 2005). Unicellular algae

possess the Cu-transporting HMAs that are involved in Cu delivery to the chloroplast or the secretory pathway (see section II.A.5), but lack divalent cation-transporting HMAs such as AtHMA2 and AtHMA4. These proteins play a central role in Zn translocation from root to shoot, possibly by loading and/or unloading the xylem (Hussain et al., 2004; Verret et al., 2004; Mills et al., 2005).

Proteins of the IREG family are also potentially involved in the long-distance transport of transition metals in plants (Curie and Briat, 2003). These plant proteins are related to the mammalian IREG1 protein (or Ferroportin1) that mediates the transport of Fe(II) from the basolateral surface of the enterocytes to the bloodstream (McKie et al., 2000). The exact functions of plant IREG proteins remains unclear. AtIREG2 was shown to have a role in Ni transport to the vacuole under Fe deficiency (Schaaf et al., 2006). Absent in *S. cerevisiae*, an *IREG1*-like gene is present in *Cyanidioschyzon* (Table 10.1; Hanikenne et al., 2005a). In *Chlamydomonas*, the presence of an *IREG1*-like gene is uncertain: BLAST searches identify a protein (EDP04441) with weak similarity to human and *Arabidopsis* IREG proteins, but experimental confirmation is required to assess its putative role in metal homeostasis. This suggests that an IREG1-like protein was present in the common ancestor of photosynthetic and non-photosynthetic eukaryotes, and was maintained in mammals and vascular plants. It is hypothesized that Fe efflux function was maintained in *Cyanidioschyzon* to cope with high Fe availability in the acidic sulfur-rich hot springs (Hanikenne et al., 2005a).

Unicellular algae such as *Chlamydomonas* do not synthesize nicotianamine (NA) and lack NAS- (nicotianamine synthase) and YSL- (Yellow Stripes-like) encoding genes (Table 10.1; Hanikenne et al., 2005a). NA is a high-affinity transition metal ligand known to contribute to long-distance transport of Fe, Cu and Zn, and metal tolerance (Pich and Scholz, 1996; Ling et al., 1999; Inoue et al., 2003; Takahashi et al., 2003; Kim et al., 2005; Pianelli et al., 2005). NA is also a precursor for the synthesis of mugineic acid phytosiderophores that are involved in the strategy II Fe uptake system in grasses (Curie and Briat, 2003; Grotz and Guerinot, 2006; section III.A.1). A functional nicotianamine synthase is present in the filamentous fungus *Neurospora crassa*, where NA is suggested to be involved in the cell-to-cell distribution of Zn and other micronutrients via the incomplete septa of the hyphae (Trampczynska et al., 2006).

The YSL proteins are members of the oligopeptide transporter (OPT) family and are involved in the transmembrane transport of metal-NA chelates. Several *Arabidopsis* and rice YSLs have been functionally characterized and contribute to the distribution of transition metals (DiDonato et al., 2004; Koike et al., 2004; Le Jean et al., 2005; Schaaf et al., 2005; Waters et al., 2006). The *S. cerevisiae* Ccc1 protein is an Fe and Mn transporter and mediates the accumulation of these metals in the vacuole (Li et al., 2001). The functional homologue of Ccc1 has been characterized in

Arabidopsis and named VIT1 (Vacuolar Iron Transporter; S.A. Kim et al., 2006). The *VIT1* gene is expressed in the developing embryo and in seeds. In young seedlings, it is mainly expressed in the vasculature. Localized at the vacuolar membrane, VIT1 is essential for Fe storage in the seeds and for seedling development under Fe-limiting conditions (S.A. Kim et al., 2006). A Ccc1 homologue is not encoded in *Chlamydomonas*, but the presence of one in *Cyanidioschyzon* suggests that a *CCC1*-like gene existed early in evolution, where it was probably maintained to provide an Fe storage system.

Finally, the sizes of the transition metal transporter families increase with the complexity of the photosynthetic organisms (Table 10.1). A similar trend is also observed in fungi: the unicellular yeast *S. cerevisiae* possesses a smaller transition metal transporter complement than does the filamentous *N. crassa* (Table 10.1; Kiranmayi and Mohan, 2006). This size expansion allows cell- and tissue-specific expressions of the transporters and the fine-tuning of the metal homeostasis network in a multicellular organism. Contradicting this general trend, some large families of metal transporters are present in unicellular organisms. A striking example is the encoding of a similar number of ZIP transporters in the *Chlamydomonas* (14, Tables 10.1 and 10.2), human (14) and *Arabidopsis* (17) genomes, whereas other unicellular organisms possess only a limited number of *ZIP* genes (Table 10.1).

III. METAL TOLERANCE

A. Tolerance and toxicity

The *Chlamydomonas* cell wall possesses a high affinity for metal cations (Collard and Matagne, 1990) and constitutes a passive protection system against excess metals. Several studies have consistently shown that strains deficient in cell walls are more sensitive to metals such as Cd, Cu, Ni, and Co (Collard and Matagne, 1990; MacFie et al., 1994; Prasad et al., 1998). It could be related to the fact that some wall-deficient strains appear to be Fe-deficient (Allen et al. 2007b), possibly because of the loss of FEA proteins (see section IV.A.1.b). It could be hypothesized that the metal sensitivity of wall-deficient strains is caused by an increased uptake due to the induction of the Fe-deficiency systems.

The toxicity of excess metals has been analyzed in *Chlamydomonas* over many years (Ben-Bassat et al., 1972; Irmer et al., 1986; Collard and Matagne, 1990; Weiss-Magasic et al., 1997; Prasad et al., 1998; Danilov and Ekelund, 2001; Devriese et al., 2001; Boswell et al., 2002; Mosulén et al., 2003; Faller et al., 2005; Morlon et al., 2005; Vega et al., 2005, 2006; Wang et al., 2005; Gillet et al., 2006; ; Jamers et al., 2006; Kobayashi et al., 2006; Luis et al., 2006). Because different media have been used and different parameters have been measured to assess metal toxicity, it is difficult to draw general conclusions from these fragmentary and mainly descriptive

Table 10.4 Metal and metalloid toxicity in *Chlamydomonas*: A summary of past studies

Chemicals	Concentrations	Described effects	References[a]
Arsenate [As(V)]	125–500 μM	Growth reduction	[1]
		Reduction of photosynthesis (measure of oxygen evolution)	[1]
Arsenite [As(III)]	4 mM	Reduction of photosynthesis (measure of PSI activity) and respiration	[2]
Cadmium [Cd(II)]	100–300 μM	Growth reduction	[3–6]
	100–300 μM	Reduction of photosynthesis (measure of PSI activity) and chlorophyll content	[2–5]
	10–100 μM	Inhibition of photoactivation	[7]
	100–300 μM	Inhibition of nitrate assimilation	[8–9]
Copper [Cu(II)]	80–300 μM	Growth reduction	[4, 10–12]
	100–300 μM	Reduction of chlorophyll content	[4, 10–12]
	2–30 μM	Reduction of photosynthesis (measure of oxygen evolution)	[13]
	7–30 μM	Inhibition of external carbonic anhydrase	[14]
	100–300 μM	Inhibition of nitrate assimilation	[8–9]
Iron [Fe(II)]	250–600 μM	Growth reduction and chlorosis	[15]
Lead [Pb(II)]	1–20 μM	Reduction of photosynthesis (measure of oxygen evolution)	[16]
	1–20 μM	Ultrastructural changes (thylakoids, mitochondria, nucleus)	[16]
	2.5–10 μM	Inhibition of external carbonic anhydrase	[14]
Nickel [Ni(II)]	3–80 μM	Stimulation of photosynthesis (measure of oxygen evolution)	[13]
Mercury [Hg(II)]	1–25 μM	Reduced growth and motility	[5, 17–18]
	1–25 μM	Reduction of chlorophyll content	[5, 17–18]
Selenite [Se(IV)]	10–500 μM	Growth reduction	[19]
	10–500 μM	Ultrastructural changes (mainly in the chloroplast)	[19]
Zinc [Zn(II)]	100–300 μM	Inhibition of nitrate assimilation	[8]
	1.5–30 μM	Reduction of photosynthesis (measure of oxygen evolution)	[13]
	8–30 μM	Inhibition of external carbonic anhydrase	[14]
Thallium [Tl(I)]	0.5–2.5 μM	Growth reduction	[20]
		Reduction of chlorophyll content	[20]

[a]*References, Chlamydomonas strains and growth conditions: [1] Kobayashi et al. (2006): strain CC-125, TAP medium 1 mM Pi, 27°C, continuous light, 80 μE/m^2/s; [2] Vega et al. (2006): strain 21 gr, Sueoka medium 10 mM nitrate, 0.3 mM sulfate, 25°C, 5% CO_2, continuous light, 250 μE/m^2/s; [3] Collard and Matagne (1990): strain 137c, TAP medium 1 mM glycerophosphate, 25°C, continuous light, 80 μE/m^2/s; [4] Prasad et al. (1998): strain 2137, high salt medium, 22±2°C, continuous light, 8000 lux; [5] Howe and Merchant (1992): strains CC-124 or CC-1021, TAP medium, 22°C, continuous light, 125 μE/m^2/s; [6] Gillet et al. (2006): strain CW15, TAP medium 1 mM glycerophosphate, 24±2°C, continuous light, 150 μE/m^2/s; [7] Faller et al. (2005): strain CC-124, TAP medium; [8] Devriese et al. (2001): strain 21 gr, Sueoka medium 10 mM nitrate, 1 mM glycerophosphate, 25°C, 5% CO_2, continuous light, 250 μE/m^2/s; [9] Mosulen et al. (2003): strain 21 gr, Sueoka medium 10 mM nitrate, 0.3 mM sulfate, 25°C, 5% CO_2, continuous light, 50 W m^{-2}; [10] Boswell et al. (2002): strain CC-125, TAP medium, 25°C, continuous light, 20 μE/m^2/s; [11] Luis et al. (2006): strain CW15 (SAG 83.81), TAP medium, 20°C, 14–10 hours light-dark cycle, 20 μE/m^2/s; [12] Jamers et al. (2006): strain SAG 11-32a, TAP medium, 25±1°C, 14–10 hours light-dark cycle, 200 μE/m^2/s; [13] Danilov and Ekelund (2001): strain 137c, freshwater medium, 20°C, 16–8 hours light-dark cycle, 70 μE/m^2/s; [14] Wang et al. (2005): unknown strain, artificial fresh water medium, 25±1°C, 16–8 hours light-dark cycle, 4000 lux; [15] Hanikenne et al. (2005b): strain CW15, TAP medium, 25°C, continuous light, 80 μE/m^2/s; [16] Irmer et al. (1986): strain CCAP 11–32a, Chlorella medium, 25°C, 2.5% CO_2, continuous light, 140 μE/m^2/s; [17] Ben-Bassat et al. (1972): strain y-1, acetate, citrate and minerals medium, unknown temperature, 5% CO_2, continuous light, 1000 foot-candles; [18] Weiss-Magasic et al. (1997): strain N.C. 27215 (Carolina Biological Supply Company), modified Mauro medium, 28°C, continuous light, unknown light intensity; [19] Morlon et al. (2005): strain CCAP 11-32b, 25±1°C, continuous light, 100 μE/m^2/s; [20] Lustigman et al. (2000): strain N.C. 27215 (Carolina Biological Supply Company), modified Mauro medium, ambient temperature, continuous light, unknown light intensity. See Volume 1, Chapter 1, for more complete identification of strains.*

studies. However, Table 10.4 is an attempt to summarize these findings for As(V), As(III), Cd(II), Cu(II), Fe(II), Pb(II), Hg(II), Ni(II), Se(IV), Zn(II), and Tl(I). Most of these metals when present in excess affect growth and ultimately viability, and the photosynthetic apparatus appears as the prime target of toxicity. For example, low Cd concentrations (within the μM range) inhibit photoactivation and hence photosynthesis, most likely by competing for binding to the Ca site of Photosystem II (PS II) during the assembly of the water-splitting complex (Faller et al., 2005). A Cd-resistant mutant was shown to be impaired not in Cd uptake or detoxification but in photosynthetic activity, as revealed by reduced growth under photoautotrophic conditions, decreased – but Cd-resistant – photosynthetic oxygen evolution, reduced PS II activity and altered chlorophyll fluorescence induction kinetics in dark-adapted cells (Table 10.5; Nagel and Voigt, 1995; Voigt et al., 1998). Acetate inhibition of the water-splitting complex, which is observed in the wild type, was suppressed in the resistant mutant, suggesting that the donor side of PS II is impaired in this strain (Voigt and Nagel, 2002).

Table 10.5 Metal-resistant and metal-sensitive mutants

Metal	No of mutants	R/S[a]	Mechanisms	Genes	References[b]
Arsenate	12	R	Lower As content, higher Pi[a] content	Unknown	[1, 2]
	1	R	Lower As content Defective in a putative Pi transporter	*PTB1*	[1–3]
	2	S	Higher As content, unchanged Pi content	Unknown	[1, 2]
	1	S	Unchanged As content, impaired in the conversion of inorganic arsenic to dimethylarsinic acid	Unknown	[1, 2]
Cadmium	2	R	Lower Cd content, putative permeability mutants	Unknown	[4, 5]
	1	R	Impaired photosynthesis	Unknown	[6–9]
	2	R	Increased Cd accumulation and higher phytochelatin content	Unknown	[10]
	2	R	Reduced Cd accumulation and lower phytochelatin content	Unknown	[10]
	5	S	Unknown	Unknown	[11]
	1	S	Unknown	Unknown	[12, 13]
	5	S	Higher Cd and phytochelatin content Fe^{2+} sensitivity Defective in a mitochondrial ABC transporter	*CDS1*	[12, 13]
	1	S	Defective in a MRP ABC transporter with putative vacuolar localization	*MRP2*	[14]
Copper	5	S	Unknown	Unknown	[15]

[a]*Abbreviations: R: metal-resistant mutants, S: metal-sensitive mutants, Pi: inorganic phosphate.*

[b]*References: [1] Fujiwara et al. (2000); [2] Kobayashi et al. (2005); [3] Kobayashi et al. (2003); [4] Collard and Matagne (1990); [5] Collard and Matagne (1994); [6] Nagel and Voigt (1989); [7] Nagel and Voigt (1995); [8] Voigt et al. (1998); [9] Voigt and Nagel (2002); [10] Hu et al. (2001); [11] McHugh and Spanier (1994); [12] Hanikenne et al. (2001); [13] Hanikenne et al. (2005b); [14] Wang and Wu (2006); [15] Hanikenne (2003).*

The resistance phenotype might result from a reduced affinity for Cd of a cation-binding site, possibly for Ca or Mn, in PS II.

In *Chlamydomonas*, Cd mainly accumulates in the chloroplast (Nagel et al., 1996). The dramatic effect of Cd on photosynthesis was revealed in a proteomic study showing that about 1/3 of the soluble proteins whose abundance is decreased upon Cd treatment are involved in photosynthesis, including Rubisco, ferredoxin, ferredoxin-NADP-reductase and several enzymes of the Calvin cycle and chlorophyll biosynthesis (Gillet et al., 2006). It is possible that Cd(II) exerts its effects through interference with Fe(II) uptake and/or thiol metabolism (Howe and Merchant, 1992; Rubinelli et al., 2002).

Arsenite, Cu, and to a lesser extent Zn, were also shown to inhibit photosynthesis efficiency in *Chlamydomonas*. On the other hand, relatively high Ni and Pb concentrations had stimulatory effects (see also section II.A.11; Danilov and Ekelund, 2001, Vega et al., 2006). The effects of Cu excess on photosynthesis and the regulation of photosynthetic genes were analyzed in detail by Luis et al. (2006). High Cu causes a decrease in chlorophyll and carotenoid contents, whereas α-tocopherol synthesis is stimulated to protect the photosynthetic apparatus from the Cu-induced oxidative stress. With increasing Cu concentrations, functionality of both photosystems is gradually lost and the *psaA* and *psaB* genes are up-regulated, to cope with an increased demand for replacement of damaged PS I subunits. While the genes *FSD1* and *MSD1*, encoding Fe and Mn superoxide dismutases, are induced in response to Cu, the corresponding enzymatic activities are independent of metal status. Based on the Cu concentration at which the onset of toxicity is observed, the study suggests that stromal photosynthetic functions are more sensitive to Cu-induced reactive oxygen species than the membrane-located reactions (Luis et al., 2006). Jamers et al. (2006) performed a complementary analysis of the transcriptional response to Cu toxicity using microarrays. In presence of 125 μM Cu, the expression of genes involved in oxidative stress responses (a putative GSH-*S*-transferase, a GSH peroxidase, a mitochondrial thioredoxin and a putative peroxiredoxin), the detection and repair/proteolysis of damaged proteins (26S proteasome subunit, a DnaJ homologue, and HSP70B), and the mitochondrial electron transport chain (Rieske protein, cytochrome *c* peroxidase, and cytochrome c_1) is increased. Two thioredoxin (*TRX*) genes are also induced by Cd and Hg in *Chlamydomonas* (Lemaire et al., 1999, 2002), suggesting that Trx might play a general role in metal toxicity (Howe and Merchant, 1992). Several genes (e.g. *CRD1* and *CTH1*) involved in the Cu deficiency response (see section IV.B.3.c) are down-regulated upon exposure to high Cu concentrations (Jamers et al., 2006). Note that this study probably reflects only part of the transcriptional response to Cu overload, because only about 3000 genes were represented on the microarray.

In addition to photosynthesis, other components of cellular metabolism are affected by toxic concentrations of metals. Arsenite was shown

to inhibit respiration (Vega et al., 2006). The external carbonic anhydrase of *Chlamydomonas*, which is involved in the CO_2-concentrating mechanism (see Chapter 8), is inhibited by Cu, Pb and Zn, whereas Cd has a slight stimulatory effect (Wang et al., 2005). Moreover, an excess of Cd, Cu, and Zn has also an impact on the assimilation of macronutrients such as nitrate or sulfate. These three metals inhibit nitrate uptake and synthesis of glutamine synthetase (Devriese et al., 2001; Mosulén et al., 2003; Gillet et al., 2006), whereas Cd increases the sulfate assimilation rate (Domínguez et al., 2003; Mosulén et al., 2003). Cd also induces the accumulation of enzymes of glutamate and cysteine metabolism (Vega et al., 2005; Gillet et al. 2006). The abundance of RNA encoding the *S*-adenosylmethionine (SAM) synthetase was increased by Cd, SAM being a precursor of cysteine (Rubinelli et al., 2002). Higher sulfate assimilation and increased glutamate and cysteine synthesis would support the Cd-induced accumulation of GSH and PCs (see section II.B.1).

To cope with metal-induced oxidative stress, catalase and ascorbate peroxidase activities are also induced upon Cd treatment, whereas arsenite induces catalase and GSH reductase activities (Vega et al., 2005). Cd also increases abundance of an ascorbate peroxidase and an MnSOD (product of the *MSD2* gene; Gillet et al., 2006). Similarities can be found in the response to Cd at the proteome level, and the RNA abundance changes occurring upon Cu treatment (Gillet et al., 2006; Jamers et al., 2006; see above in this section). For example, a GSH-*S*-transferase, a peroxiredoxin and HSP70 are up-regulated in both cases. Cd also has an effect on proteins of carbohydrate metabolism and fatty acid, amino acid, and protein biosynthesis (Gillet et al., 2006).

Finally, phosphates also play a role in *Chlamydomonas* metal tolerance. Cd accumulation with phosphate in the vacuole of *Chlamydomonas acidophila* is correlated with a decreased polyphosphate content (Nishikawa et al., 2003). Moreover, Cd and Cu tolerance depends on ambient phosphate concentrations (Wang and Dei, 2006), and arsenate resistance in mutants was shown to correlate with high intracellular phosphate in *C. reinhardtii* (Kobayashi et al., 2005). These mechanisms might be supported by the induction of an inorganic pyrophosphatase that catalyzes the hydrolysis of pyrophosphate into two orthophosphates (Gillet et al., 2006). It is additionally relevant that Allen et al. (2007a) described an interaction between Mn and phosphate metabolism with respect to Mn deficiency (see section IV.C).

To summarize, it appears that photosynthesis is the primary target of metal toxicity, and that the photosynthetic apparatus undergoes numerous adaptations upon metal excess in *Chlamydomonas*. Response to metal overload also involves the induction of oxidative stress protection mechanisms and of protein chaperones, and requires metabolic adaptations to support the synthesis of the metal chelators GSH and PC.

B. Tolerance mutants

To gain a better understanding of the metal tolerance mechanisms in *Chlamydomonas*, several groups have attempted to isolate metal-resistant or metal-sensitive mutants (Table 10.5; Nagel and Voigt, 1989, 1995; Collard and Matagne, 1990; Collard and Matagne, 1994; McHugh and Spanier, 1994; Voigt et al., 1998; Fujiwara et al., 2000; Hanikenne et al., 2001, 2005b; Hu et al., 2001; Kobayashi et al., 2003, 2005; Voigt and Nagel, 2002; Wang and Wu, 2006). For most of these mutants, the molecular identity of the corresponding genes is unknown. However, improving molecular and informatic tools will facilitate their identification.

A number of Cd and arsenate tolerant mutants were shown to be permeability mutants (Collard and Matagne, 1990, 1994; Fujiwara et al., 2000; Hu et al., 2001) probably resulting from an alteration of metal transport across the plasma membrane. Other Cd-resistant mutants were shown to accumulate PC-Cd high molecular weight complexes of increased size (section II.B.1; Hu et al., 2001) or to be impaired in photosynthesis (section III.A; Nagel and Voigt, 1995; Voigt et al., 1998; Voigt and Nagel, 2002).

An arsenate-resistant mutant was found to accumulate less arsenate than the corresponding wild-type strain (Fujiwara et al., 2000) and subsequently shown to be defective in a putative Na(I)/Pi co-transporter named PTB1 (Kobayashi et al., 2003). *ptb1* cells display arsenate resistance due to low arsenate uptake, suggesting that PTB1 is a major entry route for arsenate. It was also shown that *ptb1* cells accumulate higher levels of inorganic phosphate and possesses increased high-affinity inorganic phosphate transport activity relative to the wild type, which might compensate for the loss of PTB1 (Kobayashi et al., 2005).

Cd-sensitive mutants allowed the identification and the cloning of ABC transporter genes (*CDS1* and *MRP2*, Table 10.5) involved in Cd tolerance (Hanikenne et al., 2001, 2005b; Wang and Wu, 2006). CDS1 is a mitochondrial protein possibly involved in the export of Cd (section II.A.10.c), whereas MRP2 is a putative GSH-Cd vacuolar transporter (section II.A.10.b).

IV. METAL NUTRITION AND DEFICIENCY RESPONSE

A. Iron

1. *Iron assimilation*

a. An animal-, fungal-like iron uptake system in *Chlamydomonas*

Considering that Fe is among the most abundant elements in the earth's crust (Taylor, 1964), it is surprising that Fe deficiency is the most common nutritional deficiency in the world's population (Adamson, 2004). The poor bioavailability of Fe due to the low solubility of ferric [Fe(III)] oxides, in particular in alkaline environments, is likely to account for this paradox (Lindsay and Schwab, 1982; Marschner, 1995). However, Fe is an essential

micronutrient in its role as metal cofactor for many enzymes and redox reactions. Organisms have therefore developed efficient homeostatic mechanisms for Fe assimilation and distribution within cells and organs. In photosynthetic organisms, Fe deficiency induces metabolic adaptations that allow the maintenance of both photosynthetic and respiratory functions.

Organisms possess high- and low-affinity systems for the uptake of Fe depending on its extracellular concentration and chemical state (oxidized/reduced, free or chelated, etc.). High-affinity systems are usually highly selective for Fe and operate in Fe-deficient conditions, while low-affinity systems are less selective and are active in Fe-replete conditions. Plants have developed two strategies for high-affinity Fe uptake (reviewed in Curie and Briat, 2003; Colangelo and Guerinot, 2006; Grotz and Guerinot, 2006). On the one hand, dicots and non-graminaceous monocots use strategy I (or reduction strategy) where the soil is acidified by H^+ ATPases to solubilize iron, Fe(III) is reduced by ferric chelate reductases, and Fe(II) uptake is mediated by IRT1, a protein of the ZIP family. On the other hand, grasses use strategy II (or chelation strategy) where phytosiderophores [i.e. high-affinity Fe(III) chelators] are released, and Fe(III) chelates are subsequently taken up by the YS1 transporter, a member of the OPT family (Curie et al., 2001). The chelation strategy is more efficient than the reduction strategy and allows grasses to survive on soils with very low iron availability (Curie and Briat, 2003; Grotz and Guerinot, 2006). In addition, it has been shown that in rice (a strategy II plant) iron can also be taken up by an IRT1-like protein (Bughio et al., 2002; Ishimaru et al., 2006), allowing rice to directly absorb Fe(II) which is more available in submerged conditions. These observations suggest a more general role of ZIP proteins in plant Fe uptake.

In *Chlamydomonas* the high-affinity Fe uptake system is localized to the plasma membrane and is postulated to require a ferrireductase (FRE1), a multicopper ferroxidase (FOX1), and an iron permease (FTR1), akin to the systems that operate in Fe nutrition in yeast and mammals but distinct from either of the two pathways operating in plants (Figure 10.1; Table 10.6; Herbik et al., 2002a; La Fontaine et al., 2002b; Merchant et al., 2006).

Because the complexation properties of Fe(II) and Fe(III) ions are distinct, the control of the redox state of Fe is central to its transport/mobilization in the cell. In vascular plants and algae, the involvement of ferrireductase(s) in Fe assimilation at the plasma membrane is well documented (Eckhardt and Buckhout, 1998; Lynnes et al., 1998; Schmidt, 1999; Weger et al., 2002). Ferrireductase activity is induced by Fe deficiency, and depends upon intracellular reducing power in the form of NADH or NADPH for the reduction of extracellular Fe(III) and Fe(III) chelates (Xue et al., 1998; Weger and Espie, 2000). Sequence similarities identify four ferrireductase-like proteins encoded by the *FRE1, CBR1, RBOL1*, and *RBOL2* genes in *Chlamydomonas* (Merchant et al., 2006), although their functions remain

Table 10.6 Genes encoding proteins involved in iron assimilation and storage

Gene name	Putative function	Expression evidence[a]	Accession #	Homologue in *Volvox*[b]	References[c]
FRE1	Ferrireductase, reduction of Fe(III) and Fe(III)-chelates	qRT	EDP04183	Y	[1–3]
FOX1	Multicopper ferroxidase, reoxidation of Fe(II)	RNA blot, immunoblot	EDP02580	Y	[1–3]
FOX2	Putative multicopper oxidase	RNA blot	EDP03605	Y	[1, 3]
FTR1	Iron permease, Fe(III) uptake	RNA blot	EDP03271	Y	[1–3]
FEA1	Fe-assimilating protein, Periplasmic Fe(II)-binding	RNA blot, immunoblot, purified protein	EDP02900	Y	[1, 3, 4]
FEA2	Fe-assimilating protein, Periplasmic Fe(II)-binding	RNA blot, immunoblot, purified protein	EDP02901	N	[1, 3]
FER1	Ferritin, Iron storage	RNA blot, immunoblot	EDP02528	N	[1–3]
FER2	Ferritin, Iron storage	qRT, immunoblot, RNA blot	ABW87266	Y	[1, 3]

[a] *Expression evidence: qRT (quantitative real-time RT-PCR).*

[b] *Presence of homologous sequence in the genome of Volvox carteri, vista Volvox track at http://genome.jgi-psf.org/Chlre3/Chlre3.home.html (March 2007).*

[c] *References: [1] http://genome.jgi-psf.org/Chlre3/Chlre3.home.html; [2] La Fontaine et al. (2002b); [3] Merchant et al. (2006); [4] Rubinelli et al. (2002). Note that the annotations provided in reference 3 were based on the release 2 of the genome.*

to be established experimentally. The *FRE1* gene was the most dramatically regulated by iron-deficiency (Allen et al., 2007b). The *FRE1* gene product was identified as a 100-kD plasma membrane protein in Fe-deficient cells (Reinhardt et al., 2006). This further supports a role of FRE1 as an iron reductase in Fe assimilation. *CBR1* encodes a cytochrome b_5 reductase-like protein, and the *RBOL* genes encode polypeptides with similarity to plant respiratory burst oxidase proteins, but none of these has been tested experimentally (Allen et al. 2007b).

In *Chlamydomonas* the presumed second step of Fe uptake involves a ferroxidase, that is a Cu-containing enzyme (multicopper oxidase) that catalyzes the oxidation of Fe(II) to Fe(III) ions prior to their transport by the Fe(III) permease (La Fontaine et al., 2002a). This re-oxidation step confers selectivity and specificity to high affinity Fe uptake and avoids the production of reactive oxygen species in the presence of Fe(II) (Askwith and Kaplan, 1998; Herbik et al., 2002a). A ferroxidase-like protein, originally called FLP1, was

identified in a proteomic study of *Chlamydomonas* plasma membranes (Herbik et al., 2002a, b). FLP1 shares sequence similarity with *S. cerevisiae* Fet3 and mammalian multicopper oxidases such as hephaestin and ceruloplasmin, which function in Fe homeostasis (Herbik et al., 2002a, b; La Fontaine et al., 2002a). The gene encoding FLP1 was identified independently as *FOX1* on the basis of the presence of multicopper oxidase motifs (La Fontaine et al., 2002b). *FOX1* is up-regulated under Fe deficiency (La Fontaine et al., 2002b), and predicted to encode an integral membrane protein with a N-terminal hydrophobic membrane anchor. FOX1/FLP1 is believed to be glycosylated based on its electrophoretic behavior in non-denaturing gels (Herbik et al., 2002b). This is consistent with a plasma membrane localization and routing through the secretory pathway where loading of Cu into apoferroxidase also takes place (Figure 10.1; for review see Sargent et al., 2005). In *S. cerevisiae*, this copper-requiring step is essential for the complete maturation of the ferroxidase/Fe permease complex (see section IV.B.2) and its translocation to the plasma membrane (Stearman et al., 1996).

FOX1 has a paralog called FOX2 (with 55% protein sequence identity to FOX1) whose involvement in metal nutrition is unclear based on the observation that *FOX2* was both reported not to be regulated by metal availability (Merchant et al., 2006), and also described as induced upon prolonged Fe deficiency (Reinhardt et al., 2006). It is possible that the genetic background differences account for the disparity. The predicted FOX2 protein retains the copper-binding motifs found in FOX1, but lacks the hydrophobic N-terminal anchor. It is therefore likely to be localized to a different cell compartment than FOX1.

The final step of Fe uptake involves the delivery of Fe(III) across the membrane by Fe(III) permeases or transporters that are usually physically associated with multicopper oxidases. The *FTR1* gene was postulated to encode the Fe(III) permease in *Chlamydomonas* (Figure 10.1) based on the facts that the predicted FTR1 polypeptide contains characteristic Fe-binding motifs that are present in other FTR1-like proteins, and that the *FTR1* mRNA is up-regulated coordinately with *FOX1* under Fe deficiency (La Fontaine et al., 2002b). FTR1 represents the only putative Fe(III) transporter identified so far in *Chlamydomonas*.

In yeast and mammals, Fe uptake is Cu-dependent and Cu deficiency leads to Fe deficiency (Askwith et al., 1994; Askwith and Kaplan, 1998). In *Chlamydomonas*, the existence of another Fe uptake pathway that functions independently of Cu nutritional status is suspected. Indeed, Cu deficiency does not lead to Fe deficiency and even under conditions where Fe nutrition is limited, copper deficiency does not exacerbate the Fe-deficient phenotype (La Fontaine et al., 2002b). Herbik et al. (2002a) showed, however, a reduction of Fe uptake rates when Cu supply is limited. Again, this discrepancy might be attributed to strain-specific differences between laboratories or different experimental conditions. A genetic dissection of metal nutrition

has demonstrated the existence of such a Cu-independent pathway through the isolation of the *crd2-1* mutant (for *c*opper *r*esponsive *d*efect), which is impaired in Fe assimilation (Eriksson et al., 2004). The Fe nutritional defect is conditional: Fe homeostasis in *crd2-1* is not affected under Cu-replete conditions, but when Cu availability decreases, *crd2* manifests a distinct chlorotic and growth inhibition phenotype. The up-regulation of the genes involved in Fe assimilation indicates that *crd2* cells experience Fe deficiency as a response to low Cu availability in the medium. As would be expected, provision of excess Fe rescues the *crd2* Cu-conditional growth phenotype. The *CRD2* gene has not been cloned yet but three alternative hypotheses can be proposed for the function of the CRD2 protein. One is that CRD2 is a Cu-independent ferroxidase that operates with FTR1 in Fe assimilation. In this context, it worth mentioning that *Cyanidioschyzon* lacks a FOX1-like multicopper oxidase (Hanikenne et al., 2005a). Because *Cyanidioschyzon* grows in an Fe- and S-rich milieu with low Cu availability, it is conceivable that this organism has evolved a Cu-independent ferroxidase associated with the FTRs. A similar situation is found in the moss *Physcomitrella patens*, which also lacks a FOX1 homologue but possesses an FTR-related protein. A second possibility is that CRD2 is a high affinity transporter of a novel type. Finally, CRD2 might be recruited to ensure that Cu is preferentially allocated to ferroxidase in situations where its availability is limited. The fact that the *crd2* mutant is unable to accumulate normal amounts of ferroxidase supports the proposal that CRD2 functions in a pathway for copper delivery to ferroxidase (Eriksson et al., 2004). The phenotype of *fox1-kd* strains, which display a growth defect in both copper-supplemented and copper-deficient cells, argues against the first two possibilities (Chen et al., 2008). The strains do not show a phenotype in iron-replete medium (20 μM), suggesting that alternative low affinity transporters (see below) may operate in this situation.

In summary, it appears that *Chlamydomonas* uses an animal/fungal-like system that is dependent on Cu availablility for high-affinity iron uptake. Multicopper oxidases have also been identified in other algae such as *Dunaliella salina*, a halotolerant unicellular green alga (see section IV.A.1.b; Paz et al., 2007), and various diatoms (Peers et al., 2005; Maldonado et al., 2006). Moreover, *Chlamydomonas* and *Cyanidioschyzon* possess Cu-independent Fe uptake mechanisms. Several genes encoding putative Fe transporters have been identified in *Chlamydomonas* including 2 NRAMPs and several ZIPs (such as IRT1 and IRT2), but there is little direct evidence for their involvement in Fe assimilation (see section II.A).

The coexistence of copper-dependent and copper-independent Fe uptake systems might be an ancestral feature of photosynthetic cells. Plants have lost the seemingly ancestral and highly specific Cu-dependent system and have adopted the two Cu-independent strategies, described above, which allow them to cope with the range of iron availabilities and oxidation

states characteristic of their habitats (reviewed in Curie and Briat, 2003; Colangelo and Guerinot, 2006; Grotz and Guerinot, 2006). The actual distribution of Fe uptake systems in photosynthetic organisms might reflect past or present differences in metal ion availability in their environments.

b. Extracellular proteins involved in iron assimilation

The extracellular space of *Chlamydomonas* cells is known to harbor proteins which participate in nutrient assimilation (e.g. the arylsulfatase involved in sulfur scavenging; de Hostos et al., 1988; Fett and Coleman, 1994; Quisel et al., 1996). FEA1 and FEA2 are two related proteins that are secreted in the medium by wall-deficient cells grown under Fe deficiency (Allen et al., 2007b). The major protein, FEA1, corresponds to the previously identified *H43* gene product (Rubinelli et al., 2002), which is a homologue of the high CO_2 and Fe deficiency-inducible *Chlorococcum littorale HCR1* gene (Sasaki et al., 1998; Hanawa et al., 2007). The level of FEA1 secreted in the medium of Fe-deficient *Chlamydomonas* cultures is proportional to the level of Fe deficiency. Furthermore, *FEA1* is transcriptionally activated in response to Fe deficiency, as are *FOX1* and *FTR1* (see section IV.A.1.a). Together, these observations support a function of FEA1 in the Fe assimilation pathway (Figure 10.1). The abundance of FEA2 is lower than that of FEA1 and not as influenced by Fe availability in the medium, although *FEA2* RNA abundance increases coordinately with *FEA1* mRNA in response to Fe deficiency. *FEA2* expression is not affected by CO_2 availability. This suggests a diversification of FEA2 versus FEA1 function. The *FEA1* and *FEA2* genes are adjacent and their gene products contain conserved Cys, Asp, and Glu acid residues that could serve as Fe-binding sites.

FEA-like proteins are found in other green algae (*Scenedesmus*, *Volvox*) and also in a dinoflagellate, but are not found in vascular plants. Expression of FEA1 in *S. cerevisiae* increases the Fe content of a *fet3 fet4* mutant, which lacks ferroxidase and the low-affinity Fe transporter (Rubinelli et al., 2002), giving further credence to a role of FEA1 in Fe assimilation. Rubinelli et al. (2002) showed that *Chlamydomonas FEA1* is induced by Cd exposure, an expected finding as Cd induces Fe deficiency by competing for the same uptake systems.

A possible role for FEA1 is to increase the local concentration of Fe at the vicinity of FOX1/FTR1, to optimize uptake (Figure 10.1). It appears that algae have evolved unique mechanisms for metal acquisition as evidenced by the discovery of a transferrin-like protein in *Dunaliella salina*. A transferrin-like protein (TTF for triplicated transferrin) was found to accumulate in the plasma membrane of Fe-deficient *Dunaliella* cells (Sadka et al., 1991; Fisher et al., 1997, 1998). Previously, transferrins had been reported to be involved in Fe uptake only in animal cells (reviewed in Sargent et al., 2005), and none is apparently encoded in *Chlamydomonas*. The increased accumulation of TTF under Fe deficiency correlates with enhanced Fe uptake

activity, and it has been postulated that the protein allows the cells to overcome the limited Fe availability under high salinity. The discovery that a multicopper oxidase (D-FOX) and a glycoprotein (p130B) form a complex with TTF at the plasma membrane in *Dunaliella*, and that this complex exhibits Fe binding and uptake activity, suggests that the TTF-mediated Fe assimilation pathway is of a novel type (Paz et al., 2007).

2. Metabolic adaptations to iron deficiency

a. Photosynthetic apparatus

Chlamydomonas cells are able to adapt to changes in the nutritional Fe status, and the first notable response at the molecular level is up-regulation of the genes encoding Fe assimilation components. In fact, the expression of *FOX1*, *FTR1*, and *FEA1* is significantly increased when the Fe content is reduced in the culture medium before any phenotypic symptoms of Fe deficiency are visible (La Fontaine et al., 2002b). Manifestations of Fe deficiency such as chlorosis and inhibition of cell division occur only when the Fe supply does not meet the demand for the biogenesis of Fe-requiring enzymes (i.e. Fe concentration in the sub-micromolar range; La Fontaine et al., 2002b).

Because of the central role of Fe in photosynthesis and respiration, photosynthetic organisms have evolved intracellular mechanisms to adapt to Fe deficiency in addition to the activation of the Fe assimilation pathway, which constitutes the first adaptative response to Fe deficiency. One example of such an adaptation has been described in cyanobacteria where ferredoxin, an abundant FeS protein involved in photosynthesis can be substituted by flavodoxin, a flavin mononucleotide-containing protein (Laudenbach et al., 1988; La Roche et al., 1996).

Metabolic adaptation to Fe deficiency in *Chlamydomonas* has been documented using two approaches. In one approach, the impact of Fe on the photosynthetic apparatus was examined, in particular on PS I which, with four Fe_4S_4 clusters, accounts for half of the Fe content in the photosynthetic apparatus (Moseley et al., 2002a; Naumann et al., 2005). Transition of *Chlamydomonas* cells from Fe-replete to mild and then severe Fe deficiency is accompanied by progressive modification of the interaction of light-harvesting proteins with PS I (Moseley et al., 2002a). Moseley et al. (2002a) defined several states of iron nutrition and distinguished, on the basis of the activity and composition of the photosynthetic apparatus, the iron-deficient from the iron-limited state. In particular, they noted the impact of iron deficiency on PS I and its associated light harvesting proteins, and they suggested that loss of the PSAK subunit is an early event in the remodeling of photosystem I in response to iron deficiency. The LHCA subunits of LHCI, which are involved in supplying PS I with excitation energy via chlorophyll pigments, are in tight association with PS I and form a LHCI–PSI

supercomplex (Takahashi et al., 2004; see Chapter 14). In contrast to vascular plants where the LHCI–PSI complex consists of only four LHCA polypeptides (Ben-Shem et al., 2003), *Chlamydomonas* LHCI is composed of seven major and two minor LHCA polypeptides found in a complex with an estimated size of 300–400 kD (Stauber et al., 2003). Under Fe deficiency, PS I undergoes a structural modification and loses its connection to part of the LHCI pigments. PSAK, a connecting subunit between LHCI and PS I (Fromme et al., 2001; Fang and Wang, 2002; Ben-Shem et al., 2003; Kargul et al., 2003), dissociates from PS I. This disconnection results in a drop in the excitation energy transfer efficiency between PS I and LHCI and is believed to be necessary to prevent photooxidative stress resulting from the loss of FeS clusters, a consequence of Fe deficiency. Remodeling of the photosynthetic apparatus in response to Fe deficiency includes changes in the subunit composition of LHCI (and also LHCII, the LHC associated with PS II) (Moseley et al., 2002a).

In subsequent work, Naumann et al. (2005) analyzed the changes in the LHC proteins as iron-replete cells acclimate to the iron-limited state. Analysis of thylakoid membranes by 2-D separation followed by mass spectrometric identification of spots showed that Fe deficiency induces the degradation of LHCA5 and increased accumulation of LHCA4 and LHCA9 and the N-terminal processing of LHCA3, a key event that results in the dissociation of LHCI–PSI supercomplexes by physical separation of LHCI from PS I reaction centers (Naumann et al., 2005). It is conceivable that the change in LHCA subunit composition leads to the production of an antenna complex that acts as a better sink for energy dissipation. Progression to severe Fe deficiency results in the sequential proteolytic degradation of the LHCI subunits and both photosystems (Moseley et al., 2002a). Quantitative proteomics and spectroscopic data revealed that the antenna size of PS II is significantly increased under iron deficiency (Naumann et al., 2007). This enlargement in PS II antenna size results in a pronounced photoinhibition of PS II, which is believed to constitute an efficient protective mechanism against photo-oxidative damage. The photoinhibition can be explained by the fact that electron transfer is compromised under low iron because the absorbed light energy exceeds the capacity of the photosynthetic apparatus. Concomitantly with PS II antenna remodelling, stress related chloroplast polypeptides, like 2-cys peroxiredoxin and a stress-inducible light-harvesting protein, LHCSR3, as well as a novel light-harvesting protein and several proteins of unknown function were found to be induced in response to Fe deprivation (Naumann et al., 2007). The retention of PS II might also serve the purpose of chlorophyll storage so that the photosystems can be rapidly re-synthesized when iron supply is restored. Surprisingly, subunits of Fe-containing respiratory complexes in the mitochondria are not decreased suggesting that under photoheterotrophic conditions, there might be a hierarchy of iron allocation to respiratory versus photosynthetic complexes under Fe-deficient growth conditions.

In a second approach, Reinhardt et al. (2006) used electrophoretic separation and mass spectrometry to identify proteins whose synthesis and/or degradation were modified when *Chlamydomonas* cells experience Fe deficiency. Metabolic adaptation of *Chlamydomonas* cells to Fe deficiency may include the increased accumulation of PEP carboxykinase and isocitrate lyase, two cytosolic non-plastid enzymes of general metabolism that function in gluconeogenesis and the glyoxalate cycle, respectively (Reinhardt et al., 2006). The authors' interpretation of this finding, which requires validation by quantitative methods or immunoblot analysis of specific proteins, is that carbon metabolism may be redirected from lipid molecules to glucose synthesis as a result of impaired photosynthesis and respiration in Fe limited cells.

b. Ferritin

Ferritin is a multimeric molecule that binds Fe(III) (reviewed by Briat et al., 1999). Fe is mineralized within the ferritin core via oxidation of the Fe(II) form by the intrinsic ferroxidase activity of ferritin (reviewed in Chasteen and Harrison, 1999). Ferritin resides in the cytoplasm of mammalian cells (Eisenstein, 2000) but is found in the plastid stroma in plants, where it acts as a Fe supply source during plastid biogenesis (Briat and Lobréaux, 1998). Ferritin functions as an iron buffer in plastids: when the intracellular Fe is high, ferritin synthesis is increased to provide enhanced Fe chelating capacity but in situations where the intracellular demand for Fe is augmented or when facing extracellular Fe deficiency, Fe can be mobilized from ferritin-bound Fe(III). The pattern of ferritin expression in plants is consistent with these functions (reviewed in Briat et al., 1999). In *Chlamydomonas* a ferritin-like protein is encoded by *FER1*, whose expression is increased in Fe deficient cells (La Fontaine et al., 2002b). Increased abundance of ferritin was also noted in *Chlamydomonas* cells grown in Fe-deficient medium (Reinhardt et al., 2006). One explanation is that increased ferritin is needed to increase the Fe buffering capacity in the chloroplast as Fe is released following the degradation of PS I and ferredoxin in Fe-limited cells (see above). A second ferritin-encoding gene (*FER2*) is present in *Chlamydomonas* whose expression pattern differs from that of *FER1* (Table 10.6; Merchant et al., 2006). Its function remains to be elucidated.

c. Superoxide dismutases

In plants, three forms of superoxide dismutase are found which use different metal cofactors and have distinct intracellular localizations: Cu/ZnSOD is in the cytosol, plastid and peroxisome, MnSOD is in the mitochondria, and FeSOD is in the plastid (Kliebenstein et al., 1998). In *Chlamydomonas*, biochemical studies have identified one FeSOD and two MnSODs (Sakurai et al., 1993; Kitayama et al., 1999; Allen et al., 2007a). The FeSOD is encoded by *FSD1*, whereas there are five genes encoding MnSODs (*MSD1–MSD5*;

Merchant et al., 2006; Allen et al., 2007a). MnSOD activity was found not only in mitochondria as expected, but also in the chloroplast (Allen et al., 2007a). An MnSOD in diatoms is associated with PS II on the lumenal side of the thylakoids (Wolfe-Simon et al., 2005), suggesting that the occurrence of MnSOD in the chloroplast may be more common than previously suspected. The *MSD3* gene is greatly induced in Fe-deficient *Chlamydomonas* cells, presumably to compensate for the loss of FeSOD activity in the chloroplast (Allen et al., 2007a), but the correspondence between the gene products and measurable activities in isolated cell fractions has not been established. The presence of both Fe- and Mn-containing SODs in the chloroplast raises the interesting question of how specific loading of the active sites may be accomplished *in vivo* (discussed in Yang et al., 2006).

d. Iron distribution and signaling

Distributive Fe transporters to organelles such as mitochondria, plastids, and vacuoles are also expected to participate in Fe homeostasis, and candidate proteins include members of the ZIP family with a putative vacuolar localization (Figure 10.1; Hanikenne et al., 2005a; Merchant et al., 2006) and members of the ATM family with predicted organellar localization (see section II.A.10.c).

Metabolic adaptation to Fe deficiency must depend on the operation of a signaling pathway from sensing mechanism to target genes/proteins and the sensed signal is likely to be the Fe nutritional status. The involvement of a global regulator controlling Fe homeostasis is also suspected based on other regulatory circuits that control metal nutrition (Rutherford and Bird, 2004; Kropat et al., 2005). At present the molecular identities of these components are unknown, but they could be revealed through genetic approaches. The use of reporter genes established that the Fe-responsive transcriptional activity of the *FEA1*, *FOX1*, and *FTR1* genes requires Fe-responsive *cis*-elements (or FeRE) that lie in the promoter region (Allen et al. 2007b). FeRE-mediated regulation occurs via transcriptional activation of target genes whose mRNA abundance is increased when Fe is deficient. It is likely that the FeREs are the binding sites of a yet-to-be discovered transcriptional activator that stimulates the transcription of the *FEA*, *FOX1*, and *FTR1* genes (and additional target genes such as *FER1* or *MSD3*) in response to Fe deficiency. Molecular dissection of the *FOX1* promoter by mutagenesis identified two separate FeREs, FeRE1 (CACACG) and FeRE2 (CACGCG) which are both required for Fe-dependent transcriptional stimulation of the *FOX1* gene (Deng and Eriksson, 2007). A similar study using the *ATX1* promoter led to the definition of a distinct FeRE sequence (GNNGCNNTGGCATNT) (Fei and Deng, 2007). It seems that at least two types of FeREs are involved in the Fe-dependent transcriptional regulation of genes in *Chlamydomonas*. It is conceivable that the two types of FeREs are recognized by distinct transcriptional factors in response to

the Fe nutritional status of the cell. Note that neither FeRE bears any similarity to *cis*-elements that were defined in Fe regulatory circuits in other organisms.

B. Copper

1. Involvement of a reductase and a transporter in Cu assimilation

Cu is an essential micronutrient because of its role as a metal cofactor in enzymes that catalyze reactions involving redox chemistry or oxygen. In *Chlamydomonas*, cytochrome *c* oxidase in the mitochondria, plastocyanin in the thylakoid lumen, and the multicopper oxidase FOX1 at the plasma membrane are the three most abundant Cu-utilizing proteins.

In *Chlamydomonas* Cu uptake is regulated in response to Cu availability (Hill et al., 1996). Investigation of Cu nutrition has led to the definition of a saturable and temperature-dependent Cu uptake pathway. This high-affinity uptake system is more active in cells that are adapted to Cu deficiency than in cells grown in Cu-replete conditions (Hill et al., 1996). *Chlamydomonas* grown in Cu-deficient medium are more sensitive to Ag(I)-induced toxicity relative to cells grown in Cu-replete conditions (Howe and Merchant, 1992). This observation was attributed to the increased capacity for Ag(I) uptake in Cu-deficient cells that are responding to Cu starvation by increasing the Cu uptake components. The identity of the Cu transporter is not established but four candidate proteins of the COPT/CTR family with selectivity for Cu(I) (Petris, 2004) are encoded in *Chlamydomonas* (see section II.A.6 and Table 10.2). One of these transporters is likely to constitute the major Cu assimilatory transporter at the plasma membrane, but this awaits experimental demonstration.

A Cu(II) reductase activity is also increased in Cu-deficient compared with Cu-replete cells (Hill et al., 1996). These findings support the involvement of a Cu(II) reductase in the Cu uptake pathway, analogous to what is observed for Fe assimilation (see section IV.A). The identity of the Cu(II) reductase is unknown and it is also unclear if the reductase is Cu-specific, or is of broad metal specificity. Biochemical studies have shown, however, that that the Cu(II) reductase activity is also induced under Fe deficiency (Weger, 1999) and Fe(II) uptake is inhibited by excess Cu(II) (Eckhardt and Buckhout, 1998), suggesting that the same enzyme might be responsible for both Cu(II) and Fe(III) reductase activities (Figure 10.1). Nevertheless it appears that Cu(II) ions need to be reduced to Cu(I) prior to uptake by the assimilatory transporter (COPT1 and/or CTR1 to 3). In summary, the Cu uptake pathway in *Chlamydomonas* is defined by a cell surface Cu(II) reductase and a Cu(I) transporter, similarly to Cu assimilation routes operating in other eukaryotes (Hassett and Kosman, 1995; Rees and Thiele, 2004).

2. Intracellular Cu distribution

Following its uptake, Cu needs to be distributed and incorporated into specific apoprotein targets such as apoplastocyanin, apoforms of Cox1 and COX2 (the two Cu-containing subunits of cytochrome *c* oxidase), and apoferroxidase. The mechanisms of Cu delivery to apoprotein substrates that reside in different compartments are controlled by distributive components such as Cu chaperones and Cu transporters. The mechanism for the incorporation of Cu into apoferroxidase is likely to be similar to the one proposed for mammalian and fungal ferroxidases, depending on the cytosolic Cu chaperone ATX1 and the Cu-transporting P-type ATPase CTP1 (see section II.A.5).

It can be predicted that two of the other candidate P-type ATPases, CTP2 and CTP3, correspond to *Arabidopsis* PAA1 and PAA2 and cyanobacterial CtaA and PacS, which distribute Cu across the plastid envelope (or cyanobacterial membrane) and thylakoid membrane, respectively (Kanamaru et al., 1994; Phung et al., 1994; Tottey et al., 2001). Definitive subcellular localization and functional dissection is required to distinguish the site of action of these P-type ATPases and confirm their involvement in Cu distribution. The recruitment of a metal chaperone in the delivery of Cu to apoplastocyanin in the thylakoid lumen has been postulated for the *Chlamydomonas PCY2* locus, whose function is required for stable holoplastocyanin accumulation (H.H. Li et al., 1996). The *pcy2-1* mutant displays a weak non-photosynthetic phenotype in Cu-supplemented medium (with apoplastocyanin accumulation), but not in Cu-deficient medium (where plastocyanin no longer participates in photosynthesis). By analogy to functions required for Cu insertion into cytochrome *c* oxidase (see below), one possible explanation is that the *PCY2* gene product acts as a Cu/apoprotein chaperone in the thylakoid lumen. The fact that metal insertion into apoplastocyanin is metal-selective *in vivo* supports the view that holoplastocyanin assembly is a catalyzed process (Hill et al., 1991), but this catalysis could occur at the level of copper delivery to the lumen via PAA1 and PAA2 homologues rather than via a metallochaperone in the lumen.

Incorporation of three Cu atoms into cytochrome *c* oxidase occurs in the mitochondrial intermembrane space and requires the activity of both Cu chaperones COX17 and COX19, and assembly factors COX11, COX23, and SCO1/2 (for reviews see Carr and Winge, 2003; Cobine et al., 2006; Fontanesi et al., 2006). While COX17 and SCO1 define a pathway for the delivery of two Cu atoms to COX2 (CuA site), COX17 and COX11 are in a pathway for CuB site formation (one Cu atom) in COX1. The actual roles of COX19 and COX23 remain unresolved. Homologues of these Cu distributing factors are encoded in *Chlamydomonas*, suggesting that the mechanisms for Cu delivery to cytochrome *c* oxidase are similar to those in fungi and mammals (Table 10.3; La Fontaine et al., 2002b; Cardol et al., 2005).

3. Metabolic adaptations to Cu deficiency

a. *Chlamydomonas* as a model for Cu deficiency

Studies of metabolic responses to Cu nutritional state in other organisms have historically focused on the toxicity aspect of Cu overload. Indeed, Cu deficiency is much more difficult to establish under laboratory conditions, since Cu is needed only at trace levels. Nevertheless, cases of Cu deficiency are not uncommon in natural environments and can also occur as a result of a genetic disease (reviewed by Rossi et al., 2006). *Chlamydomonas* has been a good model to dissect metabolic adaptation to Cu deficiency because the majority of the copper in the cell is found in just a few proteins and its nutritional status is easily manipulated (Quinn and Merchant, 1998). Moreover *Chlamydomonas* sustains photosynthesis under Cu deficiency when holoplastocyanin no longer accumulates, suggesting the existence of regulatory circuits and backup mechanisms (Wood, 1978; Quinn and Merchant, 1998).

b. Backup enzymes

Plastocyanin is an essential electron carrier which shuttles the electrons between cytochrome b_6f and PS I. Loss-of-function mutations in *PCY1*, the apoplastocyanin-encoding gene, result in the loss of photoautotrophic growth when *Chlamydomonas* is grown under Cu-replete conditions, but not under Cu deficiency (Gorman and Levine, 1965; Wood, 1978; H.H. Li et al., 1996; Quinn et al., 1993; Quinn and Merchant, 1998). Cells grown in Cu-deficient conditions remain photosynthetically competent because they are able to substitute plastocyanin with cytochrome c_6, a heme-containing protein that is functionally equivalent (Wood, 1978; Merchant and Bogorad, 1987b). The *CYC6* gene, which encodes cytochrome c_6, is transcriptionally responsive to Cu status (Table 10.7), with its expression being undetectable except under Cu deficiency (Merchant and Bogorad, 1987b; Quinn and Merchant, 1995). Transcriptional activation of *CYC6* is paralleled by loss of holoplastocyanin, resulting from induced proteolysis of the apoprotein in the thylakoid lumen (Li and Merchant, 1995). The replacement of plastocyanin with cytochrome c_6 under Cu deficiency is not restricted to *Chlamydomonas*, also occurring in many algae and cyanobacteria (Sandmann et al., 1983).

c. Transcriptional activation under Cu deficiency

In addition to plastocyanin loss and cytochrome c_6 gain, Cu deficiency in *Chlamydomonas* elicits a change in the transcriptional activities of three other genes: *CPX1*, *CRD1*, and *CTH1* (Table 10.7). All three genes are involved in tetrapyrrole metabolism, but the biological significance of this regulation is not clearly understood.

CPX1 encodes a soluble plastid coproporphyrinogen III oxidase whose accumulation increases in Cu-deficient medium (Hill and Merchant, 1995),

Table 10.7 Genes involved in metabolic adaptation to copper deficiency

Gene name	Gene product and function	Regulation in -Cu	Expression evidence[a]	Accession #	Homologue in *Volvox*[b]	References[c]
PCY1	Plastocyanin, electron transfer in plastid	-	RNA blot, protein purified	AAA33078	Y	[1–6]
CYC6	Cytochrome c_6, back-up of plastocyanin in Cu deficiency	Up[d]	RNA blot, purified protein	AAB00729	N	[1, 5–6, 7]
CPX1	Coproporphyrinogen III oxidase, plastid heme synthesis	Up[d]	RNA blot, protein sequence	AAD28475	Y	[1, 6, 8]
CPX2	Coproporphyrinogen III oxidase, plastid heme synthesis	-	EST	EDP06766	Y	[1, 6]
CRD1/ CHL27A	Aerobic oxidative cyclase, tetrapyrrole biosynthetic pathway	Up[d]	RNA blot, immunoblot	AAF65221	Y	[1, 6, 9–10]
CTH1/ CHL27B	Aerobic oxidative cyclase, tetrapyrrole biosynthetic pathway	Down[d]	RNA blot, immunoblot	AAK32149	Y	[1, 6, 10]
CRR1	Transcription factor, regulation of Cu deficiency response	-	RNA blot	AAZ81510	Y	[1, 6, 11–12]

[a] *Expression evidence: EST (Expressed Sequence Tag).*

[b] *Presence of homologous sequence in the genome of Volvox carteri, vista Volvox track at http://genome.jgi-psf.org/Chlre3/Chlre3.home.html (March 2007).*

[c] *References: [1] http://genome.jgi-psf.org/Chlre3/Chlre3.home.html; [2] Gorman and Levine (1966); [3] Merchant et al. (1990); [4] Quinn et al. (1993); [5] Quinn and Merchant (1998); [6] Merchant et al. (2006); [7] Merchant and Bogorad (1987); [8] Hill and Merchant (1995); [9] Moseley et al. (2000); [10] Moseley et al. (2002b); [11] Eriksson et al. (2004); [12] Kropat et al. (2005). Note that the annotations provided in reference 3 were based on the release 2 of the genome.*

[d] *Regulation mediated via CuRE* cis *elements and Crr1. Note that the Cu transporters (Copt1, Ctr1–Ctr3, Hma1 and Ctp1Ctp3) described in Table 10.2 are also likely to contribute to metabolic adaptation to copper deficiency in Chlamydomonas (see details in the text).*

and whose enzymatic activity increases accordingly (Quinn et al., 1999). A second coproporphyrinogen oxidase is encoded by *CPX2*. This isoform is closely related to the mammalian mitochondrial enzyme, whereas the *CPX1* product shows greatest sequence similarity to the plant plastid enzyme (Quinn et al., 1999). The subcellular localization of coproporphyrinogen oxidase 2 is unknown and *CPX2* mRNA abundance is not regulated by Cu nutrition (Merchant et al., 2006). In many organisms, coproporphyrinogen oxidase can become the rate-limiting step when there is a high demand for heme or under low oxygen conditions. Under Cu deficiency, cytochrome c_6 synthesis is induced in order to cope with loss of plastocyanin and there is an increased demand for its heme cofactor. The Cu deficiency-induced activation of *CPX1* might be interpreted as a response to meet this requirement.

CRD1 (*CHL27A*) and *CTH1* (*CHL27B*) encode isoforms of the aerobic oxidative cyclase (Moseley et al., 2000, 2002b). This di-iron enzyme catalyzes

the oxidative cyclization of Mg-protoporphyrin IX monomethylester in aerobic chlorophyll synthesis (Tottey et al., 2003). CRD1 and CTH1 accumulation is reciprocal: CRD1 abundance is increased in Cu-depleted cells, whereas CTH1 accumulates in Cu-replete cells. In *Arabidopsis*, a single *CHL27* gene specifies a protein with dual localization to the plastid envelope and the thylakoid membrane (Tottey et al., 2003). The localizations of *Chlamydomonas* CRD1 and CTH1 have not been established. *crd1* mutants display a Cu nutrition-dependent pale/chlorotic phenotype characterized by absence of PS I and LHCI, and a reduced level of PS II and LHCII (Moseley et al., 2002a). The fact that particular chlorophyll-binding proteins are lost as a consequence of the *crd1* mutation suggests that the photosynthetic electron transfer chain undergoes remodeling to adapt to Cu deficiency. One possible explanation is that a new antenna complex with a modified subunit composition is required to accommodate the reduced catalytic efficiency of cytochrome c_6 relative to plastocyanin (Sommer et al., 2004).

The fact that Cu nutrition regulates the genes for two key oxygen-dependent steps in the tetrapyrrole pathway suggests a connection between Cu and tetrapyrrole metabolism. Indeed, *CYC6*, *CPX1*, and *CRD1* respond to oxygen deprivation as well as Cu starvation. This response is physiologically relevant and occurs through the same transcription factor (CRR1) that regulates Cu homeostasis (see section IV.B.3.d; Moseley et al., 2000; Eriksson et al., 2004; Kropat et al., 2005).

Microarray analysis confirmed the up-regulation of *CYC6*, *CPX1*, and *CRD1* in response to Cu deficiency, but also revealed induction of several genes encoding enzymes involved in photosynthesis, the pentose phosphate pathway and the mitochondrial electron transfer chain (Jamers et al., 2006). Although the results need to be validated by RNA blots or quantitative PCR, the work suggests that examination of energy metabolism in Cu-deficient cells is warranted.

d. Cu-responsive signal transduction

A *cis*-element named CuRE (Cu Responsive Element) responsible for transcriptional activation of target genes in response to Cu deficiency was defined molecularly through dissection of the *CYC6* and *CPX1* promoters (Quinn and Merchant, 1995; Quinn et al., 2000). The CuREs are able to confer Cu-dependent expression to a reporter gene and consist of a core sequence GTAC, which is absolutely necessary but not sufficient for Cu-dependent transcriptional activity. The *CYC6* promoter includes two CuREs, each of which is necessary on its own for Cu responsiveness, while a single CuRE mediates Cu-dependent transcriptional activation of *CPX1*. Putative CuREs are present in the *CRD1* promoter but remain to be experimentally tested. CuREs are required for responses to both hypoxia and Cu-deficiency (Quinn et al. 2000), although their corresponding DNA binding proteins may be different.

CRR1 is a regulatory gene whose involvement in nutritional Cu homeostasis was established genetically through a screen for mutants that display Cu-deficiency conditional phenotypes (Table 10.7; Eriksson et al., 2004). *crr1* mutations result in impaired growth under Cu deficiency that can be alleviated by addition of Cu to the medium. *CPX1*, *CYC6*, and *CRD1* transcription no longer respond to the Cu nutritional status in *crr1* mutants, indicating that *CRR1* controls their transcription, and possibly that of other genes induced by Cu deficiency. Apoplastocyanin is no longer degraded in *crr1*, suggesting that the gene encoding the protease responsible for apoplastocyanin proteolysis is one of those under *CRR1* regulation.

The CRR1 protein sequence is unique but includes a Zn-dependent DNA-binding domain called SBP (Squamosa Binding Protein; Klein et al., 1996), and a nuclear localization signal (Kropat et al., 2005). The presence of ankyrin repeats suggests that CRR1 might interact with other protein partners, and a Cys-rich domain (with similarity to metallothionein) indicates possible redox or metal sensing. The presence of metal-binding motifs such as the SBP domain and the Cys-rich segment are consistent with a role as a Cu sensor, but it is also possible that the sensing activity resides in a protein interacting with CRR1, for example via the ankyrin repeats. The CRR1 SBP domain displays Zn-dependent binding activity to a CuRE sequence *in vitro*, and competition experiments indicate that the SBP domain is 10-fold more selective toward a wild-type CuRE sequence versus a version carrying a point mutation in the GTAC core, a mutation which also confers loss of Cu responsiveness *in vivo* (Kropat et al., 2005).

Each of the known target genes of Cu deficiency is also transcriptionally activated by Ni or Co ions (Quinn et al., 2003), and excess Cu cannot reverse the effect of Ni on the transcriptional response. The response to Ni ions requires at least one CuRE and is *CRR1*-dependent, suggesting that Ni interferes with a component of the copper status signal transduction pathway. One hypothesis is that Ni and Co bind at the Cu-sensing site (CRR1 or another Cu-binding molecule) in an irreversible manner that prevents the access of Cu, and therefore the Cu-dependent regulation of CRR1 activity. In such a model, Ni or Co binding to the metal sensing site results in constitutive activation of CRR1.

The reciprocal Cu-responsive accumulation patterns of CRD1 and CTH1 are also under control of CRR1 (Moseley et al., 2002b). In Cu-replete cells, *CRD1* is expressed at a low basal level and *CTH1* produces a 2-kb mRNA, leading to the preferential accumulation of CTH1. In Cu-deficient conditions, while increased CRD1 abundance can be explained by CRR1-mediated transcriptional activation, decreased accumulation of CTH1 is due to the activity of an upstream CuRE-containing promoter in the *CTH1* transcription unit. This promoter drives the CRR1-dependent transcription of a 5′-extended 3-kb mRNA that does not lead to a translated gene product because of the presence of small ORFs in all frames in the 5′ UTR. It is

likely that expression of the functional *CTH1* 2-kb transcript is blocked by occlusion of the promoter under Cu-deficient conditions.

Note that the SPB domain present in CRR1 is a plant-specific DNA binding domain that is also detected in several proteins from vascular plants. In *Arabidopsis*, some SPB domain-containing proteins also display motifs found in CRR1. It is conceivable that the activity of these proteins is controlled by Cu nutrition, similarly to *Chlamydomonas* CRR1.

C. Manganese and selenium

The study of Mn and Se nutrition is technically challenging because both metals are essential for growth in most organisms, yet required only at trace levels. Mn is an essential micronutrient because of its participation as a redox cofactor or an activator at the metal-binding sites of several enzymes (reviewed by Christianson, 1997). For instance, Mn is the redox-active cofactor of MnSOD, and it activates bound water for nucleophile-based hydrolysis of the guanidinium group of arginine in arginase, an arginine catabolic enzyme (van Loon et al., 1986; Lebovitz et al., 1996). In photosynthetic organisms, Mn occurs as a Mn_4Ca cluster involved in water splitting (reviewed by Merchant and Sawaya, 2005). *Chlamydomonas* has a higher demand for Mn as compared to non-photosynthetic organisms. Indeed, cells growing heterotrophically in Mn-deficient medium display a PS II-deficient phenotype accompanied by decreased abundance of D1, the PS II subunit that binds the Mn_4Ca cluster, and release of the oxygen evolving enhancer proteins from the thylakoid membrane (Allen et al., 2007a). Mn deficiency also impacts MnSOD activity proportionally. Mn-deficient cells experience specific peroxide-sensitive stress and the *GPX*s, *APX*, and *MSRA2* genes (encoding the anti-oxidant enzymes glutathione peroxidase, ascorbate peroxidase, and methionine sulfoxide reductase) are slightly up-regulated. The Mn, Fe, and P contents of cells grown in Mn-deficient medium are also reduced proportionately. As expected, Mn-deficient cells with reduced Fe content display a chlorotic phenotype that can be relieved by provision of extra Fe in the medium. It is conceivable that the Fe deficiency phenotype of Mn-depleted cells is the consequence of a defense mechanism aimed at reducing intracellular Fe content in order to prevent oxidative stress.

The lowered P content of Mn-deficient cells suggests that Mn starvation causes reduced P uptake. Phosphate uptake (see Chapter 6) occurs through PHO84-type transporters, which use a divalent cation as the counterion for symport (van Veen et al., 1994; Persson et al., 1999). The *PTA3* and *PTA4* genes encode such transporters and are up-regulated in response to Mn deficiency, and it is likely that Mn^{2+} is their preferred counterion. The induction of *NRAMP1* and *MTP5*, which encode candidate transporters (see section II.A.2; Table 10.2 and Figure 10.1) in Mn- but not Fe-deficient cells, suggests that the corresponding gene products may also operate in a

Mn^{2+}-selective assimilation pathway. It remains to be established which transporters function in the distribution of Mn to plastids and mitochondria.

Selenium is a rare element, present in the form of selenocysteine, a modified amino acid incorporated in proteins found in bacteria, archaea and many eukaryotes (reviewed by Rother et al., 2001; Kyriakopoulos and Behne, 2002). In bacteria, selenoproteins are recruited for catabolic reactions, whereas in eukaryotes they can have both biosynthetic and antioxidant functions. The discovery of selenoproteins and components for selenocysteine incorporation in *Chlamydomonas*, a member of the plant kingdom, came as a surprise because plants do not contain selenoproteins (Novoselov et al., 2002; Rao et al., 2003). A survey of the *Chlamydomonas* genome led to the identification of the complete selenoproteome defined by 12 selenoproteins representing 10 families (Grossman et al., 2007; Lobanov et al., 2007), among which 5 have homologues in other species that function in redox metabolism, with the functions of the remaining seven currently unknown. The algae *Ostreococcus* and *Cyanidioschyzon* also use selenoenzymes (Matsuzaki et al., 2004; Derelle et al., 2006; Palenik et al., 2007). *Chlamydomonas* growth does not appear to depend on added Se, presumably because sufficient Se is present as a trace contaminant in other media components. This is reasonable considering that the abundance of selenoproteins in *Chlamydomonas* (and other organisms) is low. However, it is conceivable that the demand for Se increases under stress conditions where redox metabolism and hence participation of selenoproteins is stimulated.

V. CONCLUSIONS AND PERSPECTIVES

For several decades, *Chlamydomonas* has been used as a model to study the physiology, biochemistry and molecular genetics of transition metal homeostasis and tolerance at the cellular level. As these data are integrated with genome information (Merchant et al., 2007), our understanding of the metal homeostasis network of a model photosynthetic cell will continue to improve.

Based on previous work (Rosakis and Köster, 2004; Hanikenne et al., 2005a; Merchant et al., 2006) and genome annotation, Table 10.2 presents a compilation of the *Chlamydomonas* metal transporter complement. Putative functions are proposed on the basis of their homology and phylogenetic relationship to functionally characterized yeast, human and plant proteins. Further experimental work will be necessary to establish their functions, metal specificities and localizations, and will probably rely in part on developing reverse genetic techniques (Rohr et al., 2004; Schroda, 2006).

The predicted metal transporter complement of *Chlamydomonas* reveals both animal- and plant-like features. *Chlamydomonas* possesses several transporters that are present in plants but absent in animals such as subfamily

I ZIPs, a putative HMA1 protein for Cu delivery to the plastid, the RET1 NRAMP protein homologous to the plant EIN2 protein and Ni/Co transporters (section II.A, Tables 10.1 and 10.2). On the other hand, high-affinity Fe uptake is driven by an animal-like Cu-dependent system, although plant-like Cu-independent systems might also contribute (section IV.A.1). Another example is the presence of selenoproteins in *Chlamydomonas*, as discussed above. Although present in prokaryotes and many eukaryotes, selenoproteins are absent in vascular plants. Thus, it appears that *Chlamydomonas* has retained various metal-related systems that have undergone specialization and/or gene loss in the animal versus the plant lineages.

Physiological studies, genetic screens and functional analyses of candidate genes have advanced our understanding of the cellular adaptations to metal deficiency or overload in *Chlamydomonas*. Transcriptomic (Eberhard et al., 2006), proteomic (Stauber and Hippler, 2004; Allmer et al., 2006; Muck et al., 2006) and metabolomic (Bölling and Fiehn, 2005) tools are starting to be applied to study metal homeostasis (Gillet et al., 2006; Jamers et al., 2006; Reinhardt et al., 2006). In the future, the use of these techniques will certainly increase our understanding of the global metabolic adaptations to metal stress in photosynthetic cells.

Chlamydomonas species live in a range of environments and geographic locations (Harris, 1989). For example, Aguilera and Amils (2005) described three *Chlamydomonas* strains isolated from an acidic- and heavy-metal rich river in Spain. This natural diversity could be exploited to analyze the genetic variation associated with the metal homeostasis network within the species and genus (Borevitz and Ecker, 2004; Koornneef et al., 2004; Weigel and Nordborg, 2005).

Future research is likely to tackle key unresolved questions, some of which are:

1. Is there a hierarchy of metal distribution to cellular compartments depending on metal availability and/or physiological needs? This question is of particular relevance in the context of metal deficiency, where it might be necessary to recycle metal from abundant metalloproteins for re-allocation to other metal-utilizing pathways. For instance, it is conceivable that Cu liberated by the degradation of plastocyanin in the thylakoid lumen is re-allocated to cytochrome *c* oxidase in the mitochondria.

2. How does deficiency or overload of one metal (e.g. Zn) influence the homeostasis of other micronutrients (e.g. Fe, Mn, or Cu) at the cellular level? Complex interactions are likely to occur between the homeostasic networks of different metals. It is well known that Fe deficiency is accompanied by secondary Cd toxicity in both plant and animal systems (Thomine et al., 2000, 2003; Connolly et al., 2002; Lombi et al., 2002; Bressler et al., 2004). Another example of such

interactions is found in the Zn hyperaccumulating plant species *A. halleri* and *T. caerulecens*, where adaptation to high Zn accumulation in shoot tissues requires the constitutive overexpression of genes associated with Fe and Cu homeostasis (Chiang et al., 2006; Filatov et al., 2006; Talke et al., 2006; van de Mortel et al., 2006).

3. What are the components involved in metal sensing, signaling and regulation, and what is the cross-talk between the homeostatic network of metals and other micro- or macronutrients? These regulatory mechanisms remain largely unknown in photosynthetic cells with a few exceptions. For example, the basic helix-loop-helix transcription factor FIT1/FER in vascular plants partly co-ordinates the iron-deficiency response in *Arabidopsis* and tomato (Colangelo and Guerinot, 2004; Brumbarova and Bauer, 2005). In *Chlamydomonas*, the central roles of the CuRE *cis* element and the CRR1 transcription factor in the regulation of the Cu deficiency response were described in section IV.B.3. Further progress could be made by comparing promoter regions of homologous genes in *Chlamydomonas* and *Volvox* to identify conserved regulatory motifs. In addition, a systematic structure-function analysis of algal and plant metal transporters will allow the identification of specific sequence motifs essential for interaction with partners, regulators, or chaperones.

4. Can we find "chaperones" for Zn, Fe, or Mn in *Chlamydomonas*? Although intracellular trafficking of metals and their proper insertion into target proteins require metallochaperones (O'Halloran and Culotta, 2000), only a few Cu metallochaperones have been identified so far in *Chlamydomonas* (Table 10.3) and more generally, in photosynthetic cells. The roles of low molecular weight metal chelators such as organic acids, GSH, or PC in metal trafficking remain to be elucidated.

5. Finally, several attempts have been made to use *Chlamydomonas* as a biotechnological tool for bioremediation. The biosorption capacities for Hg, Cd, Pb, and Cr of *Chlamydomonas* biomass or of *Chlamydomonas* immobilized on alginate beads have been studied in detail (Arica et al., 2005; Tüzün et al., 2005; Bayramoglu et al., 2006). Several genes have been expressed in *Chlamydomonas* to increase metal tolerance and metal accumulation capacities. For example, the expression of a chicken metallothionein gene enhances Cd tolerance and binding capacity (Hua et al., 1999). Moreover, the overexpression of a gene encoding the enzyme catalyzing the first step of Pro biosynthesis from Glu leads to increased Cd tolerance and accumulation (Siripornadulsil et al., 2002). The data suggested that the free Pro could act as an antioxidant in the Cd-treated cells,

resulting in a more reducing cellular environment (measured as an increased GSH/GSSH ratios) that in turn facilitates PC synthesis (Siripornadulsil et al., 2002).

The identification of genes encoding transporters for a range of transition metals provides additional targets to engineer metal accumulation. Such approaches could represent efficient and cost-effective solutions to clean-up polluted waters and soils.

ACKNOWLEDGMENTS

Research projects in the authors' laboratories are funded by the "Fonds de la Recherche Scientifique-FNRS, Belgium" (Grants No. 2.4638.05, 2.4540.06 and 2.4583.08) (M.H.), the "Fonds spéciaux pour la Recherche, University of Liège, Belgium" (M.H.), the National Institutes of Health GM42143 (SM), the Department of Energy DE-FG02-04ER15529 (SM), and the College of Biological Sciences, the College of Medicine and the Dorothy M. Davis Heart and Lung Institute at The Ohio State University (P.H.). P.H. wishes also to acknowledge the Institute of Mitochondrial Biology at The Ohio State University for intellectual input. M.H. thanks Prof. P. Motte and Dr. D. Baurain for helpful discussions. M.H. is a Postdoctoral Researcher of the "Fonds de la Recherche Scientifique-FNRS" (Belgium).

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