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Genome Analysis of *Chlamydomonas reinhardtii* Reveals The Existence of Multiple, Compartmentalized Iron–Sulfur Protein Assembly Machineries of Different Evolutionary Origins

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ABSTRACT

The unicellular green alga *Chlamydomonas reinhardtii* is used extensively as a model to study eukaryotic photosynthesis, flagellar functions, and more recently the production of hydrogen as biofuel. Two of these processes, photosynthesis and hydrogen production, are highly dependent on iron–sulfur (Fe–S) enzymes. To understand how Fe–S proteins are assembled in Chlamydomonas, we have analyzed its recently sequenced genome for orthologs of genes involved in Fe–S cluster assembly. We found a total of 32 open reading frames, most single copies, that are thought to constitute a mitochondrial assembly pathway, mitochondrial export machinery, a cytosolic assembly pathway, and components for Fe–S cluster assembly in the chloroplast. The chloroplast proteins are also expected to play a role in the assembly of the H-cluster in [FeFe]-hydrogenases, together with the recently identified HydEF and HydG proteins. Comparison with the higher plant model Arabidopsis indicated a strong degree of conservation of Fe–S cofactor assembly pathways in the green lineage, the pathways being derived from different origins during the evolution of the photosynthetic eukaryote. As a haploid, unicellular organism with available forward and reverse genetic tools, Chlamydomonas provides an excellent model system to study Fe–S cluster assembly and its regulation in photosynthetic eukaryotes.

IRON–SULFUR (Fe–S) clusters are versatile cofactors of enzymes involved in essential biological processes including photosynthesis, respiration, and metabolism (Imsande 1999; Beinert 2000). Chlamydomonas is estimated to have at least 30 enzymes that depend on Fe–S as a cofactor. This number is based on the available literature and Chlamydomonas orthologs of Fe–S proteins identified in plants and metazoa (Balk and Lobréaux 2005; Lill and Mühlenhoff 2008; and annotations in the Chlamydomonas genome). To briefly summarize, the thylakoid membranes of the chloroplast harbor components of photosynthetic electron transfer, including the [2Fe–2S] Rieske protein in the cytochrome b_6f complex and three [4Fe–4S] clusters in Photosystem I (PSI). The electrons are then passed onto [2Fe–2S] ferredoxin and ferredoxin-thioredoxin reductase, which has a [4Fe–4S] cluster. In contrast to cyanobacteria and higher plants, the NDH complex, thought to contain three clusters, is missing in Chlamydomonas (Peltier and Cournac 2002). A possible role of the NDH complex in siphoning off electrons from PSI to avoid redox damage could be equivalent to the role of the soluble [FeFe]-hydrogenases HydA1 and HydA2 in Chlamydomonas, which contain a specialized Fe–S cluster, [4Fe–4S] + [FeFe], called the H-cluster (Peters *et al.* 1998). The chloroplast stroma also contains numerous catalytic Fe–S enzymes involved in nitrogen and sulfur assimilation (nitrite reductase, sulfite reductase), amino acid biosynthesis (glutamate synthase, isopropylmalate isomerase), and cofactor biosynthesis (chlorophyll a/b oxygenase, the thiamin biosynthetic protein THIC). Mitochondria are the other cell compartment in which Fe–S proteins are abundant, such as aconitase and the respiratory complexes, especially respiratory complex I with eight Fe–S clusters. In the cytosol and nucleus, Fe–S proteins are less abundant, but nevertheless essential for transcription, translation, and DNA repair (Lill and Mühlenhoff 2008). Several novel Fe–S proteins have recently been discovered in yeast and mammals, including the ABC protein Rli1, DNA primase, and the helicase Rad3 (FancJ), and are also found in the Chlamydomonas genome. More Fe–S proteins are doubtlessly waiting to be discovered, as Fe–S binding cannot always be predicted from the primary amino acid sequence.

The importance of Fe–S clusters for photosynthesis stimulated early studies in the assembly of these metal cofactors in chloroplasts using nuclear-encoded, imported ferredoxin as a model protein (Takahashi *et al.* 1986; Li *et al.* 1990). By adding ^{35}S-labeled cysteine to lysed spinach chloroplasts, it was shown that cysteine is the source of sulfur for the [2Fe–2S] cluster of ferre-

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doxin (Takahashi *et al.* 1991b). Moreover, the process required energy in the form of ATP or GTP, and reducing equivalents in the form of NADPH (Takahashi *et al.* 1991a). However, it was not until the identification of a cysteine desulphurase activity and its corresponding gene in the nitrogen fixing *Azotobacter vinelandii* (Zheng *et al.* 1993) that the genetic components and therefore the biochemical pathways underlying Fe–S cluster assembly started to become unraveled, primarily in *Escherichia coli* and Baker's yeast *Saccharomyces cerevisiae* (Johnson *et al.* 2005; Lill and Mühlenhoff 2008).

The building blocks for most Fe–S clusters are simply iron and sulfide (Beinert 2000). Intracellular free iron and sulfide concentrations are thought to be extremely low or zero, respectively, as both are toxic to the cell. In all Fe–S cluster assembly pathways studied to date, sulfide is provided by a pyridoxal phosphate-dependent cysteine desulfurase enzyme, which catalyzes the transfer of sulfur from L-cysteine in the form of persulfide to a scaffold protein (Mihara and Esaki 2002). How iron is delivered to the scaffold is less clear, but it is thought to involve the frataxin protein in nonphotosynthetic bacteria and mitochondria. After assembly on the scaffold protein, the nascent Fe–S cluster is transferred to the target apo-protein, which is facilitated by Hsp70-type chaperones and/or glutaredoxins.

In bacteria, three systems have been identified for Fe–S cluster assembly, the *ni*trogen *f*ixation (NIF), the *i*ron *s*ulfur *c*luster (ISC), and the *sul*fur mobilization (SUF) systems (Johnson *et al.* 2005). While the NIF system is dedicated to the assembly of the intricate Fe–S cofactors of nitrogenase, the ISC system is regarded as the "housekeeping" pathway of Fe–S cluster assembly. In eukaryotes, the ISC components are found predominantly in the mitochondria, most likely because this system functions better under low-oxygen conditions that exist inside this organelle. The SUF system appears to function under conditions of oxidative stress or iron limitations (Nachin *et al.* 2003; Outten *et al.* 2004). It is therefore not surprising that the SUF system is found in Arabidopsis chloroplasts (Ye *et al.* 2006b), the cell compartment in which oxygenic photosynthesis takes place.

The mitochondria also play a part in the assembly of cytosolic and nuclear Fe–S proteins, as demonstrated in yeast (Kispal *et al.* 1999), mammalian cell lines (reviewed in Lill and Mühlenhoff 2008), and Arabidopsis (J. Balk, unpublished data). The half ABC transporter Atm1 in yeast (ABCB7 in mammals) is thought to export an as yet unknown product of the ISC machinery that is required for the cytosolic Fe–S protein assembly machinery (CIA) consisting of at least four proteins, including the P-loop NTPases Nbp35 and Cfd1, the hydrogenase-like protein Nar1, and the WD40 protein Cia1 (Lill and Mühlenhoff 2008).

The Fe–S cluster assembly proteins have not only attracted the interest of biochemists, but also that of evolutionary biologists. Fe–S clusters are thought to be ancient catalysts, as reduced iron and sulfur were abundant when life emerged. Fe–S clusters are endowed with unique chemical properties required for processes that are crucial to life on earth, such as photosynthesis and nitrogen fixation. Characterization of Fe–S assembly genes in "primitive" eukaryotes has helped to identify "degenerate" mitochondria and has contributed to reshuffling these organisms in the phylogenetic tree (Van Der Giezen and Tovar 2005; Embley and Martin 2006).

The genes involved in Fe–S protein biogenesis in photosynthetic eukaryotes have thus far primarily been characterized in *Arabidopsis thaliana*. Localization studies, reverse genetics and recombinant protein techniques have enabled the construction of a working model in which Fe–S cluster assembly is highly compartmentalized in the mitochondria, plastids, and cytosol (for reviews, see Balk and Lobréaux 2005; Kessler and Papenbrock 2005; Ye *et al.* 2006b). Despite the extensive genetic resources and tools developed for Arabidopsis, the study of basic biochemical pathways is hampered by additional levels of complexity in multicellular organisms with highly specialized tissues. A single-celled photosynthetic organism like Chlamydomonas would clearly overcome these limitations and would have the additional benefit of fast-forward genetics. Indeed, Chlamydomonas has already been used successfully to find novel genes involved in Fe–S protein assembly, as demonstrated by the identification of the [FeFe]-hydrogenase maturation proteins HydEF and HydG (Posewitz *et al.* 2004). As these genes are only the tip of a specialized pathway, we have searched the recently sequenced Chlamydomonas genome (Merchant *et al.* 2007) for orthologs of Fe–S protein assembly genes known in bacteria, yeast, and Arabidopsis. We have identified a near-complete set of genes with similarity to the bacterial ISC and SUF genes, the cyanobacterial/plastid NFUs and HCF101, as well as the mitochondrial export and cytosolic cluster assembly genes found in yeast. Comparison with Arabidopsis showed that the assembly pathways are highly conserved in the green lineage, but that interesting differences exist providing insights into the evolution of photosynthetic eukaryotes. The results of this analysis form an important basis for future studies on the assembly of Fe–S proteins including PSI and [FeFe]-hydrogenases.

RESULTS AND DISCUSSION

To analyze which pathways of Fe–S protein assembly exist in Chlamydomonas, we first searched the genome for the basic components for Fe–S cluster assembly, cysteine desulfurases (Table 1), and Fe–S scaffold proteins (Figure 1). We also searched for orthologs of other known or suspected Fe–S assembly proteins identified in *E. coli*, *S. cerevisiae*, *A. thaliana*, and Synechocystis. We

TABLE 1

Cysteine desulfurases in Chlamydomonas

Chlamydomonas model	EST evidence	Sequence in active site	Similar to	Enzyme	Organism	Group
NFS1	Yes	SSGSAC<u>C</u>TS	SSGSAC<u>C</u>TS	NifS	*Azotobacter vinelandii*	I
			SSGSAC<u>C</u>TS	IscS	*Escherichia coli*	I
			SSGSAC<u>C</u>TS	NFS1	*Saccharomyces cerevisiae*	I
			SSGSAC<u>C</u>TS	NFS1	*Arabidopsis thaliana*	I
			SSGSAC<u>C</u>SS	Slr0387	Synechocystis	I
CSD2	No	SAGAAC<u>C</u>HS	SAGAAC<u>C</u>HS	95927	*Volvox carteri*	I
			SAGAAC<u>C</u>NS	Sll0704	Synechocystis	I
SUFS	No	RSGHHC<u>C</u>A	RTGHHC<u>C</u>A	SufS/CsdB	*Escherichia coli*	II
			RSGHHC<u>C</u>A	SUFS/CpNifS	*Arabidopsis thaliana*	II
			RSGHHC<u>C</u>T	Slr0077	Synechocystis	II
CSDH	No	No conserved cysteine		ZP_00111076	*Nostoc punctiforme*	II

then tried to assign each protein to a cell compartment on the basis of (i) experimentally confirmed localization of the eukaryotic ortholog and (ii) the presence of an N-terminal targeting sequence and prediction software. The results are summarized in Table 2 and detailed below. For further information on the individual proteins, please see reviews (Balk and Lobréaux 2005; Barras *et al.* 2005; Johnson *et al.* 2005; Lill and Mühlenhoff 2008).

The core components—cysteine desulfurases and scaffolds: *Cysteine desulfurases:* Using standard BLAST tools, we identified in the Chlamydomonas genome four open reading frames with similarity to cysteine desulfurases involved in Fe–S cluster assembly in other

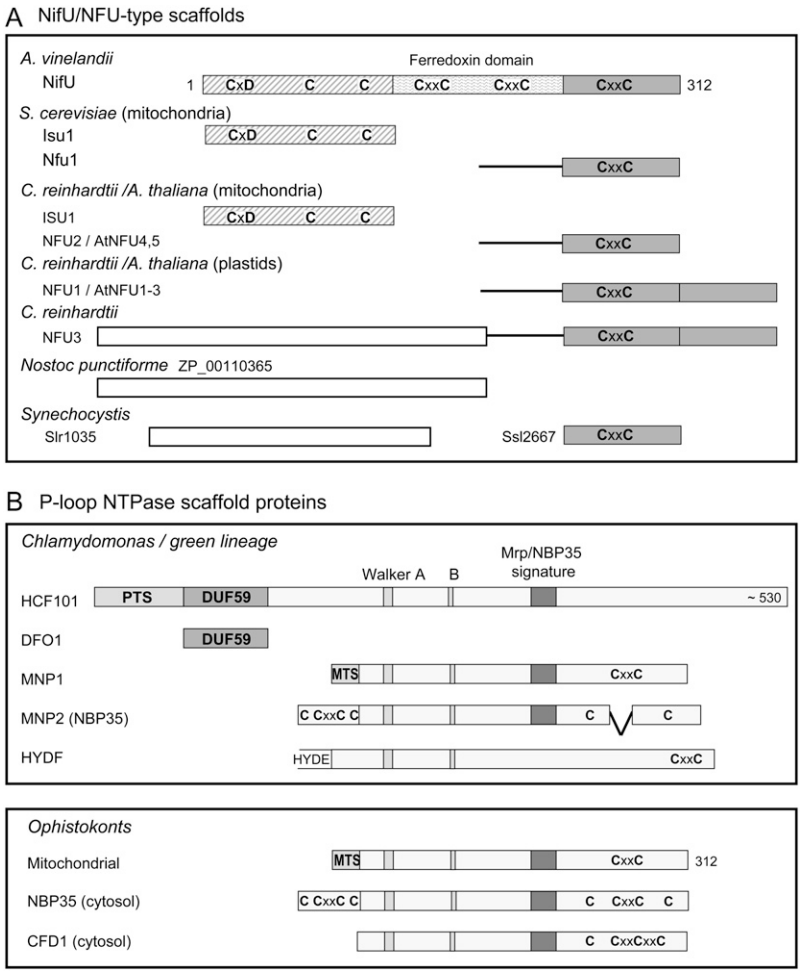

Figure 1.—Domain homology of Fe–S scaffold proteins found in Chlamyodomonas to well-studied orthologs in bacteria, plants, and yeast. Bar diagrams represent predicted scaffold proteins, specific domains, and amino acid motifs. The lengths of the bars are to scale within each box, with the length in amino acids indicated in the top bar (protein). (A) NifU orthologs; (B) P-loop NTPases. PTS, plastid targeting sequence; MTS, mitochondrial targeting sequence.

TABLE 2

Orthologs of Fe–S cluster assembly proteins in Chlamydomonas, Arabidopsis, bacteria, and yeast

	C. reinhardtii	*C. r.* protein ID	*A. thaliana*	*A. t.* localization	*A. t.* AGI gene model	*E. coli*	Synechocystis	*S. cerevisiae*	Molecular functionor protein domain[a]
ISC	NFS1	118253	NFS1	Mit	At5g65720	IscS	Slr0387	Nfs1	Cysteine desulfurase
	ISD11	184200	ISD11	Mit[b]	At5g61220			Isd11	Unknown
	ISU1	187899	ISU1, -2, -3	Mit	At4g22220, At3g01020 At4g04080	IscU		Isu1, -2	Fe–S scaffold
	ISCA1	196772	Not annotated	Mit[b]	At2g16710	IscA		Isa1, -2	Fe–S scaffold
	IBA57[c]	187731[d]	Not annotated	Mit[b]	At4g12130				Unknown
	NFU2	205851[e] 205852[e]	NFU4, NFU5	Mit	At3g20970, At1g51390	NP_312283		Nfu1	Fe–S scaffold
	MFDX	154720	ADX1, -2 = MFDX1, -2	Mit	At4g05450	Fdx		Yah1	Ferredoxin
	ARH1	134840	ADXR = MFDR	Mit	At4g32360			Arh1	Reductase
	MNP1	133548	HCF101-L1	Mit[f]	At4g19540	Mrp			P-loop NTPase[a]
	Not specialized?[g]		Not specialized?[g]			HscA		Ssq1	Chaperone (HSP70)
	HSC20	144583	Not annotated	Mit[b]	At5g06410	HscB		Jac1	Cochaperone
	MGE1	132633	Not annotated	Mit[b]	At5g55200			Mge1	Nucleotide release factor
	FTX1	196778	FH	Mit	At4g03240	CyaY		Yfh1	Iron chaperone
	GRX5	195767	Not annotated	Mit[b]	At3g15660	Grx		Grx5	Glutaredoxin
Export	CDS1[h]	133289	ATM1	Mit	At4g28630			Atm1	Half ABC transporter
	ATM2	105113	ATM2	Mit	At4g28620			Atm1	Half ABC transporter
	ATM3	99736	ATM3 = STA1	Mit	At5g58270			Atm1	Half ABC transporter
	ERV1[i]	114232	ERV1	Mit	At1g49880			Erv1	Sulfhydryl oxidase
CIA	Absent		Absent			Mrp		Cfd1	P-loop NTPase[a]
	MNP2	206480	HCF101-L2	Cytosol[f]	At5g50960			Nbp35	P-loop NTPase[a]
	IOP1 (HYD3)	206492[d]	NAR1	Cytosol[b]	At4g16440			Nar1	Hydrogenase-like[a]
	CIA1	158085	Not annotated	Cytosol[b]	At2g26060			Cia1	WD40 protein
SUF	SUFA	100292	SUFA = ISCA	Plastid	At1g10500	SufA	Slr1417		Fe–S scaffold
	SUFB	132600[d]	SUFB = NAP1	Plastid	At4g04770	SufB	Slr0074		ABC/ATPase
	SUFC	289	SUFC = NAP7	Plastid	At3g10670	SufC	Slr0075		ABC/ATPase
	SUFD	154386	SUFD = NAP6	Plastid	At1g32500	SufD	Slr0076		ABC/ATPase
	SUFE	156669	SUFE1, -2, -3 (N-term.)[j]	Plastid	At4g26500, At1g67810, At5g50210	SufE	Slr1419		Unknown
	SUFS	206481	NFS2 = CpNifS	Plastid	At1g08490	SufS/CsdB	Slr0077		Cysteine desulfurase
NFU	NFU1	206476	NFU1, -2, -3	Plastid	At4g01940, At5g49940, At4g25910	NP_312283	Ssl2667		Fe–S scaffold
	NFU3	188996	NFU1, -2, -3	Plastid			Ssl2667		Fe–S scaffold

(*continued*)

TABLE 2

(Continued)

	C. reinhardtii	*C. r.* protein ID	*A. thaliana*	*A. t.* localization	*A. t.* AGI gene model	*E. coli*	Synechocystis	*S. cerevisiae*	Molecular functionor protein domain[a]
HCF101	HCF101	102098[d]	HCF101	Plastid	At3g24430		Slr0067		DUF59 + P-loop NTPase[a]
Hydrogenase maturation	HYDEF	128256	Absent						Radical SAM + GTPase[a]
	HYDG	196226	Absent						Radical SAM[a]
Other	CSD2	167884	Absent				Sll0704		Cysteine desulfurase
	DFO1	206478	Not annotated		At1g68310				DUF59 (unknown)[a]
	ISCA2	162474[d]	Not annotated	Mit/plastid[b]	At5g03905	ErpA?			Fe–S scaffold

Organisms: *C. r.*, *Chlamydomonas reinhardtii*; *A. t.*, the higher plant *Arabidopsis thaliana*; *E. coli*, the proteobacterium *Escherichia coli*; cyanobacterium Synechocystis sp. PCC 6803; and *S.* cerevisiae, Baker's yeast, *Saccharomyces cerevisiae*.

[a] Function inferred from conserved protein domain(s). DUF59, domain of unknown function 59 (Pfam PF01883); radical SAM, radical S-adenosylmethionine enzyme.

[b] Predicted.

[c] IBA57, iron–sulfur cluster assembly factor for biotin synthase- and aconitase-like mitochondrial proteins, M_r 57 kDa. (Gelling *et al.* 2008).

[d] The sequence is incomplete or not deemed correct because of annotation issues.

[e] The two models are generated by alternative splicing, which appears to be conserved in Volvox. The significance of this remains to be investigated.

[f] K. Bych and J. Balk, unpublished results.

[g] The Hsp70 proteins HscA from *E. coli* and Ssq1 from yeast are specialized for Fe–S cluster assembly, but do not share the same genetic origin. The yeast *Yarrowia lipolytica* does not have a specialized Hsp70, and this situation may be the case for most eukaryotes (Schilke *et al.* 2006).

[h] CDS1, *Cadmium sensitive 1*, is the only Chlamydomonas gene characterized experimentally (Hanikenne *et al.* 2005).

[i] Two genes with high similarity to ERV1 are present in Chlamydomonas. This may reflect the situation in *S. cerevisiae*, where ERV1 is a sulfhydryl oxidase of the mitochondrial inner membrane space, whereas ERV2 resides in the endoplasmic reticulum.

[j] In Arabidopsis, SUFE3 is a fusion protein of NADA and a SUFE domain (Murthy *et al.* 2007).

organisms (Table 1). On the basis of sequence features, in particular the amino acids around the active site in the C terminus, cysteine desulfurases can be divided into group I or group II (see Table 1) (Mihara and Esaki 2002). The two groups are also thought to differ functionally, to the extent in which oxygen is tolerated. For example, the group-II enzymes from *E. coli* and *A. thaliana* function well under conditions of oxidative stress or in proximity of oxygenic photosynthesis, respectively, whereas the group-I enzymes generally function in strict anaerobes or in low-oxygen environments like the mitochondrial matrix (Outten *et al.* 2004; Johnson *et al.* 2005; Ye *et al.* 2006b). Two of the Chlamydomonas proteins, annotated as NFS1 and CSD2, belong to group I, whereas SUFS and CSDH belong to group II. The Chlamydomonas NFS1 sequence has high similarity over its full length to AvNifS, EcIscS, and yeast NFS1 (70% identity, 85% similarity), indicating that this enzyme is part of the ISC pathway in the mitochondria. The mitochondrial enzyme is also thought to provide sulfur for biotin, thiamine, lipoate, and thiolated tRNAs (Lill and Mühlenhoff 2008). In the case of biotin and lipoate, the sulfur is not directly provided by NFS1/IscS, but by a noncatalytic Fe–S cluster in BioB and LipA, respectively (Picciocchi *et al.* 2003; Berkovitch *et al.* 2004; Cicchillo and Booker 2005). The CrSUFS enzyme, on the other hand, is similar to Arabidopsis SUFS/CpNifS (60% identity, 75% similarity) which is the major sulfur donor to Fe–S proteins in the plastids (Van Hoewyk *et al.* 2007). The Chlamydomonas CSDH protein (ID 206474) has high sequence similarity to SUFS (42%), but is lacking the conserved cysteine in the C terminus, and it is therefore not certain whether CSDH is a functional cysteine desulfurase. Interestingly, Chlamydomonas encodes a second group-I enzyme, CSD2, that is not found in the higher plants Arabidopsis and rice. It bears homology to Synechocystis Sll0704/Csd2, which can drive Fe–S cluster assembly *in vitro* (Kato *et al.* 2000). If CrCSD2 is indeed of the same evolutionary origin as Sll0704, it is probably located in the plastids and as an oxygen-sensitive group-I enzyme, may function under anaerobic conditions, which occur more frequently in algae than in land plants.

U-type scaffold proteins: Scrutiny of the Chlamydomonas genome identified four gene products with homology to domains of the *A. vinelandii* NifU Fe–S scaffold protein (Figure 1A). NifU has a modular structure, with the modules often occurring as separate, single-domain proteins in other organisms (Mühlenhoff and Lill 2000; Johnson *et al.* 2005). The Chlamydomonas ISU1 protein is similar to the N terminus of AvNifU and has three conserved cysteines that have been shown to be critical for binding a labile Fe–S cluster in bacterial IscU and yeast Isu1 proteins. The ISU scaffold proteins in yeast and Arabidopsis are localized in mitochondria, suggesting that Chlamydomonas ISU1 is also located in this organelle.

The NFU proteins share sequence similarity to the C terminus of AvNifU. The Arabidopsis NFU proteins are found in mitochondria and plastids and can be distinguished by domain structure: the plastid proteins have a C-terminal duplication of the entire NFU domain that lacks the CxxC motif (Figure 1A) (Léon *et al.* 2003). In Chlamydomonas, the single-domain NFU2 is therefore likely to reside in the mitochondria, whereas NFU1 and NFU3 are predicted to localize to the chloroplast. The function of the mitochondrial NFUs is not clear, as the deletion of the yeast *NFU1* gene has no phenotype, unless in combination with mutations in *SSQ1*, encoding the HSP70-type chaperone specifically adapted to Fe–S cluster transfer. In contrast, the plastid NFU2 protein in Arabidopsis has been demonstrated to play a key role in Fe–S protein assembly in chloroplasts. *nfu2* knock-out mutants had decreased protein levels and/or activities of PSI, ferredoxin, and sulfite reductase, although glutamate synthase and the Rieske protein were unaffected (Touraine *et al.* 2004; Yabe *et al.* 2004). The CrNFU3 protein carries an extra N-terminal domain that is found as a separate protein in cyanobacteria. This N-terminal domain has no recognizable protein motifs, but does have weak similarity to monothiol glutaredoxins, also implicated in Fe–S cluster assembly (Herrero and De La Torre-Ruiz 2007). A similar two-domain version of NFU3 is found in the red alga *Cyanidioschyzon merolae* (gene CMP295C).

A-type scaffold proteins: In addition to the U-type scaffolds ISU1 and NFU1–3, the Chlamydomonas genome contains three so-called A-type scaffolds, annotated as SUFA, ISCA1, and ISCA2 (Table 2). Members of this protein family have three conserved cysteines and are usually associated with Fe–S cluster assembly operons in bacteria, but their function has remained elusive, as deletion mutants do not have a clear phenotype. Sequence alignments of the Chlamydomonas A-type proteins with (cyano)bacterial and Arabidopsis orthologs showed that CrSUFA clustered with the Arabidopsis SUFA and Synechocystis Slr1417 proteins, whereas the CrISCA1 protein clustered with *E. coli* IscA and the mitochondrial Isa1 in yeast. The latter protein is required for the function of biotin synthase (Mühlenhoff *et al.* 2007). The CrISCA2 protein appears to represent a third group together with the uncharacterized Arabidopsis protein AT5G03905. These proteins may be required for Fe–S assembly on IspG (Protein ID 55268, HDS), an enzyme of isoprenoid biosynthesis in the chloroplast, as suggested by the recent identification and characterization of the ErpA scaffold protein in *E. coli* (Loiseau *et al.* 2007).

P-loop NTPase scaffold proteins: A recent study (Netz *et al.* 2007) has provided evidence for another type of scaffold protein in yeast. The P-loop NTPases Nbp35 and Cfd1 were shown to transfer Fe–S clusters *in vitro*, supporting *in vivo* data that the proteins are required for the assembly of cytosolic and nuclear Fe–S clusters (Roy

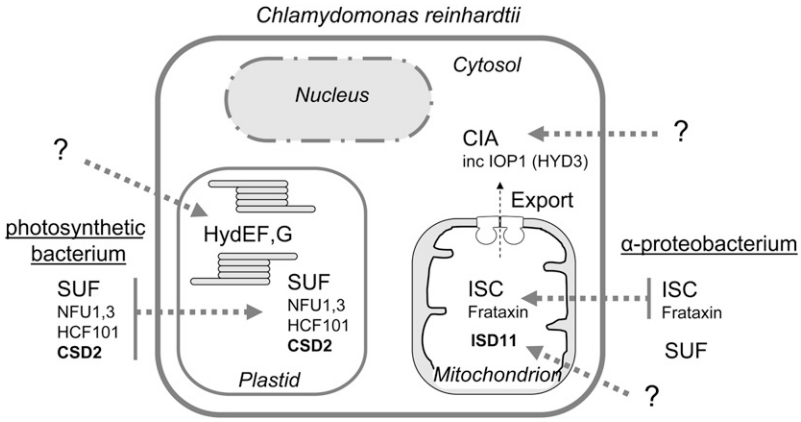

FIGURE 2.—Fe–S protein assembly machineries in Chlamydomonas based on genome analysis and their evolutionary origin. See text and Table 2 for details.

et al. 2003; NETZ *et al.* 2007). Chlamydomonas has four P-loop NTPases that could be involved in Fe–S cluster assembly: three proteins of the Mrp/NBP35 class and HydF (fused to HydE, Figure 1B). The presence (or absence) of targeting sequences suggests that MNP2 (NBP35) is localized in the cytosol, MNP1 in the mitochondria, and HCF101 in the plastid. Both MNP2 and HCF101 lack the conserved CxxC motif that is usually found in the C terminus of Mrp/NBP35 proteins. Mutants in *h*igh *c*hlorophyll *f*luorescence (HCF)101 have been characterized in Arabidopsis and have a defect in the assembly of PSI, as well as instability of ferredoxin-thioredoxin reductase, but not ferredoxin (LEZHNEVA *et al.* 2004; STÖCKEL and OELMÜLLER 2004). HCF101 has an extra domain at its N terminus, *d*omain of *u*nknown *f*unction (DUF)59 (pfam PF01883) not found in the other Mrp/NBP35-like proteins. This domain is also found as a separate, single-domain Chlamydomonas protein, *DUF*59-*o*nly (DFO)1 protein.

The HydF protein, as part of the HydEF fusion protein, was identified in screens for Chlamydomonas mutants defective in hydrogenase maturation (POSEWITZ *et al.* 2004). The HydF ortholog in *Thermotoga maritima* has been characterized at the biochemical level (BRAZZOLOTTO *et al.* 2006). The recombinant TmHydF protein displayed GTPase activity and bound an Fe–S cluster, but its molecular function in the maturation of the H-cluster and its CO/CN ligands remains to be determined.

Fe–S protein assembly machineries and compartmentalization: The above inventarization of cysteine desulfurases and scaffold proteins yielded a wealth of well-conserved Fe–S protein assembly components. Grouping these proteins on the basis of sequence and predicted localization resulted in a complex model for Fe–S cluster assembly in Chlamydomonas, compartmentalized in the mitochondria, cytosol, and chloroplast (Table 2 and Figure 2).

The mitochondrial ISC system and export: Chlamydomonas is anticipated to contain a complete ISC system in the mitochondria, including the group I cysteine desulfurase NFS1, its helper protein ISD11, the scaffold protein ISU1, the ferredoxin MFDX, the A-type scaffold ISCA1, and the NFU2 protein. The ISC system in bacteria and Baker's yeast contains a dedicated Hsp70-type chaperone, but it is not certain whether this is the case in all eukaryotes (SCHILKE *et al.* 2006). The mitochondria are also predicted to harbor the frataxin protein, FTX1, thought to deliver iron to the ISU1 scaffold protein; a glutaredoxin related to Grx5 in yeast which is involved in cluster transfer from ISU1 to target Fe–S proteins; and the P-loop NTPase MNP1, which is required for the assembly of respiratory complex I (K. BYCH, S. KERSCHER, D. J. A. NETZ, A. J. PIERIK, K. ZWICKER, M. A. HUYNEN, R. LILL, U. BRANDT and J. BALK, unpublished results).

The mitochondrial ISC pathway in Chlamydomonas is likely to be required for both mitochondrial as well as cytosolic Fe–S proteins, because of the presence of several ATM transporters. One of these mitochondrial half ABC transporters, CDS1, was identified in a screen for cadmium-sensitive mutants, and its mitochondrial localization was confirmed (HANIKENNE *et al.* 2005). A link between cadmium detoxification and the mitochondrial ABC transporters has also been observed for Arabidopsis (KIM *et al.* 2006). Cadmium may interfer with transport of the ISC-dependent compound required for cytosolic Fe–S proteins, either by binding to crucial thiol groups or by depleting glutathione, which is involved in the transport process (SIPOS *et al.* 2002).

The cytosolic CIA system: Of four cytosolic proteins known to be involved in Fe–S protein maturation in Baker's yeast, three could be identified in Chlamydomonas. These are the P-loop NTPase MNP2 (Nbp35 in yeast), the WD40 protein CIA1, and the hydrogenase-like protein IOP1 (previously annotated as HYD3, similar to Nar1 in yeast; BALK *et al.* 2004; HUANG *et al.* 2007). The three yeast proteins form protein interactions *in vivo*, with Nbp35 acting upstream of Nar1, which acts upstream of Cia1 (BALK *et al.* 2005; NETZ *et al.* 2007), but their precise molecular functions are as yet unknown. An ortholog of the yeast P-loop NTPase Cfd1 was not found in Chlamydomonas, nor in any of the other

green lineage organisms sequenced to date (Figure 1B). As a result, MNP2 is likely to function as a homodimer, rather than a Nbp35-Cfd1 heterotetramer (Netz *et al.* 2007).

Chloroplast–SUF plus additional scaffolds: A complete set of six SUF proteins can be found in the Chlamydomonas genome. By similarity with Arabidopsis the SUF proteins are likely to reside in the chloroplast, where they facilitate Fe–S cluster assembly under aerobic conditions. The cysteine desulfurase activity of SUFS/CpNifS is enhanced by SUFE, which facilitates persulfide transfer (Outten *et al.* 2003; Ye *et al.* 2006a). SufBCD forms a nonintrinsic ABC protein, and also stimulates the activity of SUFS (Outten *et al.* 2003). The Arabidopsis SUFE1 has been reported to be dual-targeted to mitochondria and chloroplasts (Xu and Møller 2006), but it remains to be seen whether the same holds true for other SUFE proteins in the green lineage. In addition to the SUF pathway, the Chlamydomonas chloroplast is predicted to harbor several scaffold proteins, NFU1, NFU3, and HCF101. On the basis of characterization of the Arabidopsis *nfu2* and *hcf101* mutants, these proteins may serve specific Fe–S proteins (see above).

Chloroplast–HydEFG: Chlamydomonas contains at least two proteins specifically dedicated to the maturation of [FeFe]-hydrogenases (Posewitz *et al.* 2004). Although the localization of HydEF and HydG gene products has not been confirmed to date, they are likely to reside in the chloroplast, colocalizing with the HydA1 and HydA2 hydrogenases. HydEFG constitute a complex molecular machinery, consisting of two radical SAM enzymes (HydE and HydG), and an Fe–S-containing P-loop GTPase (HydF). The proteins are conserved in organisms with functional Fe-only hydrogenases and are required for assembly of the intricate H-cluster, consisting of a di-iron center liganded by CN and CO groups. The di-iron center is bridged to a [4Fe–4S] cluster via a cysteine sulfur (Böck *et al.* 2006). The latter cluster, as well as the clusters on HydE, -F, and -G, is likely to be provided by the generic Fe–S cluster assembly machinery in the chloroplast.

Evolution: Fe–S clusters are ancient cofactors that are key to the activity of a number of highly conserved enzymes. It is therefore not surprising that the assembly pathways of Fe–S clusters are also conserved from bacteria to higher eukaryotes. The mitochondrial ISC pathway is most likely derived from its α-proteobacterial ancestor, having lost the SUF pathway (present in *E. coli*). The cyanobacterial ancestor would have possessed an assembly pathway more adapted to oxygenic photosynthesis (*i.e.*, no ISU proteins), which has survived in the plastids of the photosynthetic eukaryotes. The origin of the CIA proteins is unknown. IOP1 bears striking sequence similarity to [FeFe]-hydrogenases, but is not thought to function as such (Meyer 2007). First of all, it lacks a critical double cysteine motif. Second, our Chlamydomonas genome analysis suggests that the HydEFG proteins are not in the same cell compartment as IOP1, excluding a role for HydEFG in the assembly of an H-cluster on IOP1. The eukaryotic IOP1 (Nar1) proteins have a monophyletic origin, whereas the HydEFG and the HydAs have been acquired later in separate events by only a handful of eukaryotes (Meyer 2007).

Comparing Chlamydomonas and Arabidopsis reveals a remarkable conservation of Fe–S protein maturation in the green lineage. The organisms contain almost the same set of components of the ISC, export, CIA, and chloroplast machinery. Exceptions are the cysteine desulfurase CSD2 and the N-terminal domain of the NFU3 protein in Chlamydomonas, which are not found in higher plants. Another difference between the two green organisms is a difference in copy number (Table 2). In several cases Chlamydomonas has one gene copy, whereas Arabidopsis has two to three, for instance for ISU, MFDX, mitochondrial, and plastid NFUs, and SUFE. These gene copies may be specialized for specific tissues, which is corroborated by data on the Arabidopsis ISUs and SUFEs (Léon *et al.* 2005; Murthy *et al.* 2007). Of course, additional copies may still be found in the remaining part of the Chlamydomonas genome that has not yet been sequenced/assembled. In Chlamydomonas, multiple copies were found for the A-type scaffolds, NFUs, and ABC transporters of the mitochondria (ATMs). For the A-type scaffolds and NFUs, there are good reasons to assume that they are targeted to different organelles and/or perform different functions (discussed above). Whether the same is true for the three mitochondrial ABC transporters remains to be investigated.

Comparison of Chlamydomonas and Arabidopsis with nongreen eukaryotes shows a few features that appear to be specific for the green lineage. These include the absence of a cytosolic Cfd1 ortholog and alterations to the C-terminal domain of Nbp35, as well as duplication of the NFU domain (without the CxxC motif) in plastids. It will be interesting to investigate what the significance of these adaptations is.

Future research questions: From our comparative genome analysis, it is clear that Chlamydomonas has a very elaborate and interesting set of >30 genes involved in Fe–S assembly. Other than the HydEFG genes, only the ABC transporter CDS1 has been investigated experimentally (Hanikenne *et al.* 2005), but not in the context of Fe–S protein maturation. The current set of putative Fe–S cluster assembly genes (Table 2) provides a working model for future investigations including (i) to confirm the proposed function of each gene; (ii) to identify the chloroplast proteins that assist HydEFG in the maturation of [FeFe]-hydrogenase, such that they can be targeted for increased hydrogen production; and (iii) to investigate the coordination and regulation of the compartmentalized pathways, as well as iron distribution.

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