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Review

Proteins involved in maturation pathways of plant mitochondrial and plastid *c*-type cytochromes

Michal Rurek✉

Department of Molecular and Cellular Biology, Institute of Molecular Biology and Biotechnology, Faculty of Biology, Adam Mickiewicz University, Poznań, Poland

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_c_-Type cytochromes are characterized by the presence of two covalent bonds linking heme to apocytochrome and by the heme attachment motif in the apoprotein. Several molecular systems for the maturation of _c_-type cytochromes have evolved in different organisms. The best characterized are three of them: system I, system II and system III. Heme is synthesized in bacterial cytoplasm, in plastids, and in animal and fungal mitochondria. Therefore the maturation of bacterial and plastid _c_-type cytochromes involves the transport of heme and apocytochrome from the _n_-side to the _p_-side of the respective biological membranes and the formation of the covalent bond at the _p_-side. It should be underlined that the site of the _c_-type apocytochrome synthesis is also distinct from the site of its functioning. The aim of this review is to present the current state of knowledge concerning the structure and function of two systems – system I and system II – in the maturation of plant mitochondrial and plastid _c_-type cytochromes, respectively.

Keywords: *c*-type cytochromes, plant mitochondria, plastids, cytochrome *c* maturation proteins, protein–protein interactions

INRODUCTION

c-Type cytochromes are ubiquitous proteins playing an important role in almost all cells. They are present in different compartments and participate in various biochemical pathways, such as respiration, photosynthesis and apoptosis (Kranz *et al*., 1998; Turkarslan *et al*., 2006). A vast number of *c*-type cytochromes, e.g. cyt. *c*, cyt. c_1, cyt. *c*′ and cyt. c_4, are present in bacteria, where they participate in electron transfer and catalysis, especially in nitrogen metabolism (Ferguson, 2001; Allen *et al*., 2004a). *c*-Type cytochromes are also present in plant and animal cells.

The main feature of *c*-type cytochromes is the presence of a covalent bonds between heme vinyls and apoprotein cysteine thiol groups (Fig. 1). For the covalent bond formation, *c*-type apocytochromes require the conserved sequence CXXCH, which is a consensus heme-binding motif known as the *c*-type

✉Corresponding author: Michal Rurek, Department of Molecular and Cellular Biology, Institute of Molecular Biology and Biotechnology, Faculty of Biology, A. Mickiewicz University, Umultowska 89, 61-614 Poznan, Poland; phone: (48 61) 829 5968; fax: (48 61) 829 5949; e-mail: rurek@amu.edu.pl

Abbreviations: ABC, ATP-binding cassette; Bdb, *Bacillus* disulfide bond formation; BdbCD, disulfide oxidases in SII; ccb, cytochrome *c* biogenesis; ccd, cytochrome *c* defective; CCDA, disulfide reductase in plant SII; CCHL, cytochrome *c* heme lyase; CC_1HL, cytochrome c_1 heme lyase; Ccm, cytochrome *c* maturation; CcmABCD, components of transporting complex in prokaryotic SI; CcmE, heme chaperon; CcmF, heme lyase component; CcmG, periplasmic thioredoxin-like protein; CcmH, disulfide reductase; CcmI, component of *Rhodobacter* SI; Ccs, cytochrome *c* synthesis; CcsAB, proteins responsible for heme delivery in SII; Ccs1, plant homolog of prokaryotic CcsB protein; CycH(L), CcmH protein homologs; Cyc2p, accesory protein in fungal SIII; cyt., cytochrome; Dip, disulfide-isomerase-like protein; Dsb, disulfide bond formation protein; DsbAB, disulfide oxidases in prokaryotic SI; DsbD, disulfide reductase in prokaryotic SI; Erv, essential for respiration and vegetative growth; Fd, ferredoxin; FTR, ferredoxin-thioredoxin reductase; HCF, high chlorophyll fluorescence phenotype; HL, heme lyase; holocyt., holocytochrome; IMS, intermembrane space of mitochondria; OM/ IM, mitochondrial outer/ inner membrane; ORF, open reading frame; Mia, mitochondrial import and assembly; *n*/*p*-side, negatively/ positively charged membrane side; *pet*, plastid gene encoding cytochrome *f*; ResBC, components of prokaryotic SII; SI, system I; SII, system II; SIII, system III; Sec, general secretory pathway; Trx(s), thioredoxin(s).

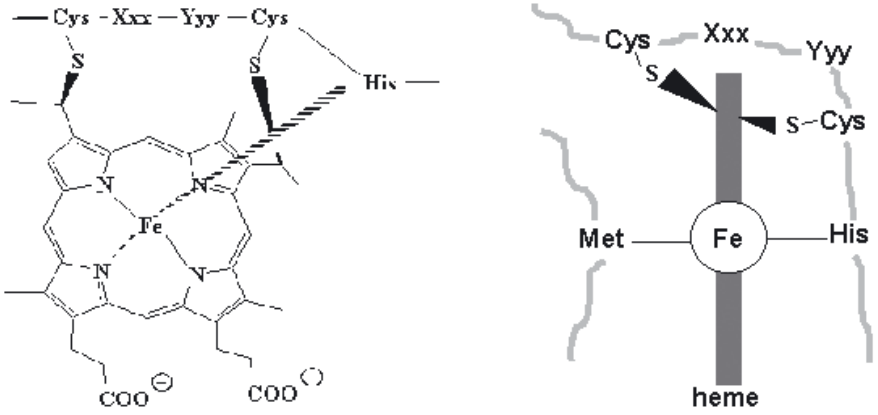

Figure 1. Covalent bonds linking heme and *c*-type apocytochromes.
After Giegé ***et al***. (2008) and Allen ***et al***. (2008), modified.

cytochrome signature. The two cysteine thiols of this sequence are covalently linked with the vinyl side chains of heme. Stevens *et al*. (2004) pointed out that the high chemical and thermal stability of *c*-type cytochromes may be the main reasons of their evolutionary success. *c*-Type cytochromes expose their functional domain outside the bacterial cytoplasmic membrane to the mitochondrial inner membrane space (IMS) or to the thylakoid lumen (Giegé *et al*., 2008).

The catalytic ligation of heme to apocytochrome *c* is the heart of the maturation pathways. This is a multistep process, mainly because the sites of heme and apoprotein synthesis and their functioning are different. The maturation of bacterial and plastid *c*-type cytochromes involves the transport of heme and apocytochrome from the *n*-side to the *p*-side of the plasma membrane and thylakoid membrane, respectively. The formation of the covalent bond proceeds at the *p*-side (i.e., in the periplasmic space or in the thylakoid lumen), where prior to the ligation of heme with apocytochrome *c*, reduction of those components is necessary. For the maturation of mitochondrial *c*-type cytochromes, the nuclear-encoded apocytochrome *c* is transported from the cytoplasm and heme biosynthesis proceeds either in mitochondria (fungi, animals) or in plastids. Maturation of *c*-type cytochromes could be treated as a model of post-translational protein modifications (Turkarslan *et al*., 2006). It should also be noted that this process requires participation of numerous proteins for its full activity.

Despite the great progress in the last 10 years, our knowledge about the pathways of *c*-type cytochrome maturation in the plant cell is still unsatisfactory. A few reviews concerning the progress in the elucidation of the *c*-type cytochrome maturation deal mainly with bacterial systems. Only one review of Nakamoto *et al*. (2000) concerns the maturation of plastid *c*-type cytochromes and the one by Giegé *et al*. (2008) characterizes the cytochrome *c* maturation in mitochondria of various organisms. Hence, the aim of the present review is to provide characteristics of the proteins involved in *c*-type cytochrome maturation pathways in plant mitochondria and plastids. Moreover, the reader will find relevant data concerning recent proteomic results obtained from the study of the maturation of *c*-type cytochromes.

GENERAL VIEW OF c-TYPE CYTOCHROME MATURATION SYSTEMS

Components of the maturation pathways and their variability

It is well known that the number of components necessary for the maturation of *c*-type cytochromes varies among different organisms. Three molecular systems are mainly in action (Kranz *et al*., 1998; Turkarslan *et al*., 2006; Feissner *et al*., 2006a): system I (SI), system II (SII) and system III (SIII). SI is present in α- and γ-proteobacteria, deinococci, plant and some protozoan mitochondria. SII was found in some Gram positive bacteria, cyanobacteria, plastids, archaeobacteria and some β-, δ- and ε-proteobacteria, while SIII is present in mitochondria of fungi, animals and surprisingly, in some green algae, apicomplexa and protozoa. Recently, Allen *et al*. (2008) speculated that SI is more ancient than SIII and was presumably present in the proto-mitochondrial endosymbiont; moreover, the main component of SIII — heme lyase (HL) — has replaced SI in many evolutionary lineages.

Each maturation system contains different functional modules, responsible for every step of the maturation process. They are depicted in Fig. 2. and briefly described below.

• The current model for the translocation of apocyt. *c* in prokaryotic SI and SII involves the ac-

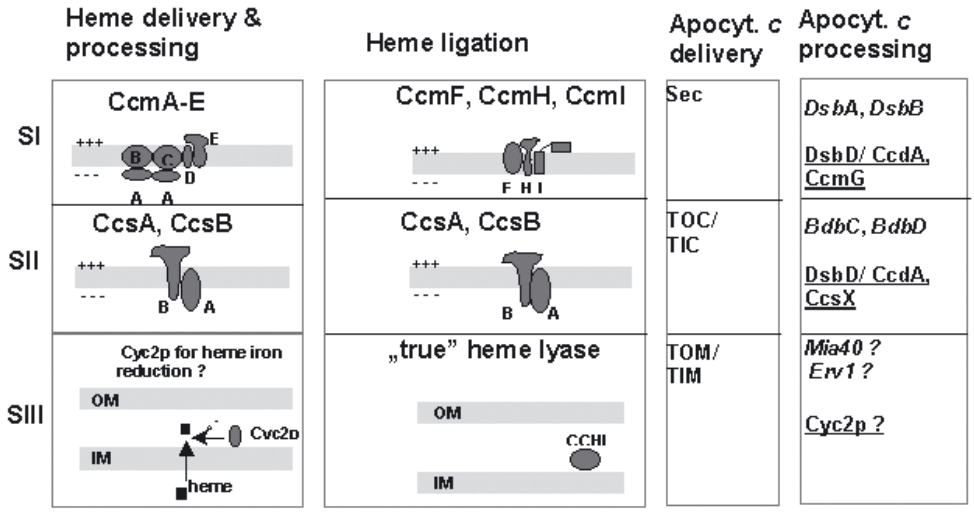

Figure 2. Comparison of the molecular components of different *c*-type cytochrome maturation systems.
After Turkarslan ***et al***. (2006), modified. Details are described in chapter "Components of the maturation pathways and their variability". The *c*-type cytochrome maturation process includes heme and apocytochrome translocation and their processing prior to ligation. The negative side of the membrane, known as *n*-side, refers to the cytoplasm in bacteria and eukaryotic cells, matrix in mitochondria and stroma in plastids. The positive side of the membrane — *p*-side — is topologically equivalent to the periplasm in Gram negative bacteria, cell outside in Gram positive bacteria, IMS in mitochondria and thylakoid lumen in plastids. Components responsible for apocyt. *c* thiooxidation are shown in italics. Proteins responsible for apocytochrome thioreduction are underlined.

tion of the Sec system, but the TOM complex is engaged in yeast SIII (Diekert *et al*., 2001). Transport of apocyt. c_1 to the yeast mitochondria, unlike that of apocyt. *c*, is potential-independent; after crossing OM and IM with the participation of the TOM and TIM complexes, apocyt. c_1 is redirected across IM. This is due to the fact that apocyt. c_1 contains two mitochondrial targeting signals functioning during the transport (Nicholson *et al*., 1989). Recently, Giegé *et al*. (2008) speculated that one of the plant mitochondrial CCM proteins (from cytochrome *c* maturation) — CCMH, might theoretically be involved in the import of apocyt. *c*. This would need to be confirmed experimentally by studying potential interactions between CCMH and apocyt. *c* during the protein import into mitochondria. It should be noted that the proposed function is not the main one ascribed to CCM proteins (see below).

• Genetic analyses of mutants lacking *c*-type cytochromes has led to the discovery that SI utilizes CcmA–E proteins for heme delivery (reviewed by Giegé *et al*., 2008). In the case of SII — CcsAB proteins are engaged in this process (see chapter "Proteins participating in heme transport and chaperoning"). It has been noticed that in the case of SIII the last steps of heme synthesis occur at the matrix side of animal and yeast mitochondria IM with the participation of protoporphyrin IX ferrochelatase. The proteins involved in the heme transport across mitochondrial IM in yeast or animal SIII are unknown, but the translocation could be achieved either by a flippase-like enzyme or it may be based on diffusion (Giegé *et al*., 2008).

• No specific molecular factor reducing the heme iron has been described until now for SI and SII. It is known that yeast SIII utilizes the accessory flavoprotein Cyc2p for that. In addition Bernard *et al*. (2005) showed that Cyc2p indirectly promotes the CCHL-dependent ligation of apocyt. c_1 and *c* with heme. On the basis of the observations of yeast mutant strains defective in the assembly of cytochrome *c*, Bernard *et al*. (2005) suggested that yeast Cyc2p may control the redox status of the ligation reaction acting as a heme reductase and/or apocytochrome disulfide reductase (see below).

• The oxidation and reduction of apocytochrome thiols at the *p*-side of the membrane prior to ligation with heme may require the following proteins: in the case of SI — DsbAB for the thiooxidation, and for the thioreduction — DsbD and CcmG; in the case of SII — BdbCD proteins probably act for the thiooxidation and DsbD and CcdA — for the thioreduction. The participation of those proteins in such processes was proposed mainly on the basis of the experimental work done on bacterial mutants defective in the *c*-type cytochrome assembly. In yeast SIII the only known hypothetical candidates are Mia40 and Erv1 for thiooxidation (Turkarslan *et al*., 2006) and Cyc2p for thioreduction of apocyt. *c* (Bernard *et al*., 2005). Their involvement in these processes still requires clear experimental evidence. It is also known that the yeast sulfhydryl oxidase Erv1 mediates the oxidation of redox-regulated receptor Mia40 which oxidizes other proteins (Hell, 2008).

• One may only speculate that in plant mitochondria, similarly to the bacterial periplasm, ligation is most probably catalyzed by a complex containing orthologs of bacterial CcmF and CcmH proteins. Plastid SII may utilize CcsAB proteins for that process. The 'real' HL, which catalyzes the ligation of heme to the *c*-type apocytochromes, was identified in yeast and animal SIII. *Dictyostelium* and animal mitochondria contain only a single form of HL, whereas two isoforms are found in yeast (Allen *et al*., 2008). The two *c*-type apocytochrome-specific heme lyases operating in yeast and *Neurospora crassa* mitochondria are named CCHL and CC_1HL. In the absence of CC_1HL, the yeast CCHL heme lyase is active both in the maturation of cyt. c_1 and of cyt. *c*. The yeast CC_1HL activity towards apocyt. c_1 is enhanced by elevated CCHL expression or by point mutations in apocyt. c_1 or in CCHL. In contrast, yeast CC_1HL seems to be engaged only in the maturation of cyt. c_1 (Bernard *et al*., 2003). The CCHL and CC_1HL lyases contain one to three characteristic CPX motifs (where X represents V, H, I, L or S) in their N-terminal regions (Steiner *et al*., 1996).

Some organisms display interesting modifications in the presence and composition of maturation systems. In the mosquito *Anopheles gambiae*, where the main components of all maturation systems are present, some proteins of SI are absent. Genome analyses have revealed that the prokaryote *Bordetella branchioseptica* and *Desulfitobacterium hafniense* may contain some components of SI and SII. A modified Ccm protein apparatus is present in *Archaeobacteria* and *Desulfovibrio.* Allen *et al*. (2006) — on the basis of *in silico* searches — pointed out that it probably does not include CcmH; additionally, CcmE contains an altered heme-binding motif CXXXY instead of HXXXY known for *Escherichia coli*. It is tempting to speculate that archaea may contain extremely divergent Ccm proteins. Since our knowledge about the components of plant SI and SII is still limited, one could also expect the discovery of novel protein factors indispensable for the assembly process of *c*-type cytochromes.

Surprisingly, a distinct form of apocyt. *c* is present in free-living kinetoplastids (*Trypanosoma*, *Leishmania*), *Euglena* and in the flagellate *Diplonema papillatum.* It contains AX_2CH or FX_2CH motifs. This implies that such *c*-type cytochromes have only one thioether bond between heme and the apoprotein (Allen *et al*., 2008). Since there is no experimental evidence that any elements of the common maturation systems are encoded in the nuclear or mitochondrial genomes of those organisms, Allen *et al*. (2004b) suggested that a distinct maturation routes of apocyt. c_1 and apocyt. *c* may exist.

In plant cells only a limited number of monoheme cytochromes is present. It seems that bacteria containing more monoheme and additionally some multiheme cytochromes, have gained a broader adaptability to environmental conditions. Unlike in plants, in bacterial cells there are various forms of the respiratory chain. The presence of various monoheme proteins in prokaryotic cells may be a consequence of the evolutionary development of the particular system of maturation. It is evident that different organisms and organelles utilize various systems for the maturation of *c*-type cytochromes, characterized by broad complexity and sometimes quite a surprising composition (Allen *et al*., 2003; Stevens *et al*., 2004). One may conclude that the potential new molecular systems for the maturation of diverse *c*-type cytochromes are awaiting characterization.

Utilization of different maturation pathways

What is the substrate specificity of the different systems of *c*-type cytochrome maturation? It was shown that SI of eubacteria expressed from constructs containing *ccm* genes could be involved in the maturation of a wide variety of *c*-type cytochromes (Allen & Ferguson, 2006). Recent findings indicate that heme levels may discriminate the participation of SI and SII in the maturation of *c*-type cytochromes. Engineered *E. coli* strains producing recombinant SI used fivefold lower heme concentrations than those using SII from *Helicobacter pylori*, which is more abundant in heme. None of the known *c*-type cytochrome maturation systems uses non-iron-protoporphyrins for the maturation; they sometimes display even an inhibitory effect on the maturation of cyt. *c* (Richard-Fogal *et al*., 2007). Feissner *et al*. (2006a) speculated that low endogenous heme levels could promote the evolution of SI. Moreover, they pointed out that SII has a lower affinity for heme than SI, and therefore SII may require a higher heme concentration to assemble cyt. *c* than SI.

It was also shown that in a Ccm knocked-out strain of *E. coli*, SI could be entirely replaced by a fused CcsB-CcsA protein of *H. pylori* SII (Feissner *et al*., 2006b). This means that at least heme delivery and periplasmic ligation are interchangeable between the two *c*-type cytochrome maturation systems in *Prokaryota*. However, system II lacks some vital elements of system I, for example the heme chaperone known as the CcmE protein (Feissner *et al*., 2006b). Moreover, SI contains an associated ABC transporter that could transport heme present at a low concentration (Goldman *et al*., 1998; Kranz *et al*., 1998). Thus, the maturation pathway of distinct *c*-type cytochromes seems to be more flexible in SII than in SI.

c-TYPE CYTOCHROMES IN PLANT MITOCHONDRIA AND PLASTIDS

Plant mitochondria contain two *c*-type cytochromes present in the respiratory chain, both encoded in the nuclear genome: cyt. c_1 and cyt. *c*. A common feature of all *c*-type cytochromes is the prevalence of α-helical regions. Plastidal cyt. *f*, composed almost entirely of β-sheet barrels, is the only exception (Stevens *et al*., 2004).

As regards plastids, cyt. *f* is encoded by the plastid *pet*A gene (however, nuclear in *Euglena*) and cyt. c_6 — by the nuclear *Cyc*6 gene. The latter is — in *Chlamydomonas* — known to be transcribed during abiotic stress conditions, namely in response to copper deficiency and during hypoxia (Quinn *et al*., 2000). Cyt. c_6 represents a soluble cytochrome, found only in cyanobacteria and algae (Nakamoto *et al*., 2000). Recently, a novel *c*-type cytochrome — cytochrome c_6-like (known also as cyt. c_x) was discovered in the plastid lumen of vascular plants and green algae (Weigel *et al*., 2003; Howe *et al*., 2006). It appeared that *Chlamydomonas* plastids contain both cyt. c_6 and cyt. c_6-like. The structural data of *Arabidopsis* cyt. c_6-like show that its surface properties are different from those of plastocyanin. Therefore, it is believed (Howe *et al*., 2006) that cyt. c_6-like, unlike cyt. c_6, cannot serve as a simple substitute for plastocyanin.

PROTEIN SYSTEM FOR THE MATURATION OF *c*-TYPE CYTOCHROMES IN PLANT MITOCHONDRIA

Plant mitochondria follow the pathway of SI for the maturation of *c*-type cytochromes, which resembles the system of α-proteobacteria and which is distinct from the fungal and animal SIII. The essential features of SI based on prokaryotic data are presented in Fig. 3.

Lee *et al*. (2007) have suggested that duplications, deletions and fusions of transmembrane segments occurred during evolution of some SI proteins, like CcmC and CcmF as well as ResC, the member of SII. Nevertheless, there is similarity in the structure among those components of the two maturation systems as they have evolved from an ancestor protein containing six putative transmembrane segments.

Terminology, organization and expression of genes encoding plant mitochondrial components of system I

The initial terminology of genes encoding components of the plant mitochondrial system I was based on the bacterial gene homologs. Currently they are frequently called *ccm* (from cytochrome *c* maturation). This term has replaced the previous one — ccb (from cytochrome *c* biogenesis) used for

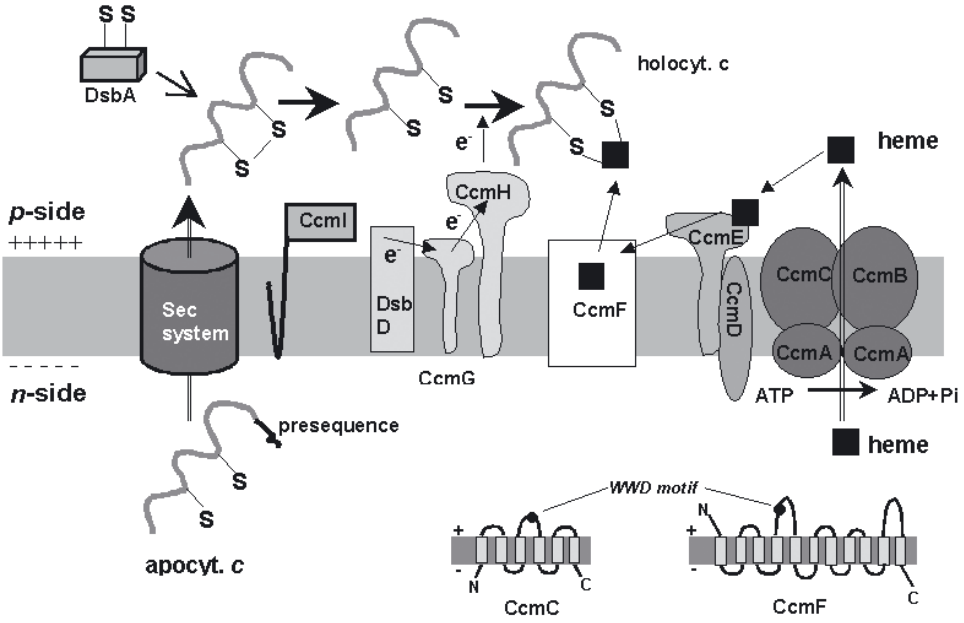

Figure 3. Model for system I of *c*-type cytochrome biogenesis based on prokaryotic data.
After Feissner *et al.* (2006b), modified. Detailed description of the presented pathways is provided in chapters "Components of the maturation pathways and their variability", "Proteins participating in heme transport and chaperoning", "Thiooxidation/thioreduction pathway" and "Components of system I responsible for the heme lyase activity". Apocytochrome import is shown at the left, the thioreduction pathway in the middle and heme translocation and delivery route at the right. Topology of bacterial CcmC and CcmF proteins with the localization of the WWD motif is also shown.

mitochondrial genes. Plant mitochondrial proteins necessary for the maturation pathway of *c*-type cytochromes are known as CCM proteins. CcmB, CcmC, $CcmF_c$, $CcmF_{N1}$ and $CcmF_{N2}$ proteins are encoded in the *Arabidopsis thaliana* mitochondrial genome, while CCMA, CCMH and CCME proteins — in the nucleus. The different terminology systems of *c*-type cytochrome maturation genes are summarized in Table 1.

Notably, the plant *ccmF* gene is divided into distinct ORFs encoding the N- (*ccm*F_N) and C- (*ccm*F_C) terminal domains of the protein; in *Brassica napus* and *Arabidopsis* mitochondria, *ccm*F_N ORF is further divided into *ccm*F_{N1} and *ccm*F_{N2}. In *B. napus* 'Polima' those genes are located on two separate subgenomic molecules (Menassa *et al*., 1997). On the other hand, mitochondrial DNA of *Nicotiana tabacum*, *Beta vulgaris* and *Oryza sativa* contains a single *ccm*F_N gene. The *ccm*F_C gene is present in all mitochondrial genomes of *Embryophyta* investigated so far. In the mitochondrial genome of *Marchantia polymorpha*, though, it is divided into two separate genes: *ccm*F_{C1} and *ccm*F_{C2}.

The organization of *ccm* genes in different mitochondrial genomes is variable because of their evolutionary shuffling and splitting in the course of recombination.

For instance, the *ccm*F_N and *ccm*F_C genes in *B. vulgaris* and *O. sativa*, *ccm*F_C and *ccmB* in *Tripsacum*

Table 1. Terminology of genes encoding components of cytochrome *c* biogenesis system I

	E. c.	*B. j.*	*R. c.*	*R. a.*	*C. m.*	*M. p.*	*O. b.*	*A. t., B. n.*	*B. v., N. t., O. s., S. b., B. v., T. a., T. d., Z. m.*
	ccmA	*cycV*	*helA*	*yejW*	*yejW*			*A. t.* CCMA *(nuclear)*	
	ccmB	*cycW*	*helB*	*yejV*	*yejV*	*orf277*	*orf206*	*ccmB (ccb2, ccb206)*	*ccmB (orf206)*
	ccmC	*cycZ*	*helC*	*yejU*	*yejU*	*orf228*	*orf250*	*ccmC (ccb3, ccb256)*	*ccmC (orf240);* (*B. v.*-3′ *orf518*)
	ccmD	*cycX*	*helD*						
	ccmE	*cycJ*						*A. t.* CCME *(nuclear)*	
	ccmF	*cycK*	*ccl1*	*ccl1*	*yejR*				
						ccmF_N *(orf509)*	*ccm*F_N *(orf577)*	*ccm*F_{N1} *(ccb6n1, ccb382)*	*ccm*F_N *(ccb574, orf589)*
								ccmF_{N2} *(ccb6n2, ccb203)*	
						ccmF_{C1} *(orf169)*	*ccm*F_C *(orf454)*	*ccm*F_C *(ccb6c, ccb452)*	*ccm*F_C *(ccb438, orf437)*
						ccmF_{C2} *(orf322)*			
	ccmG	*cycY*	*helX*						
	ccmH	*cycL*	*ccl2*					*A. t.* CCMH *(nuclear)*	
Main references	Thöny-Meyer, *et al.* (1995)	Ramseier *et al.* (1991), Ritz *et al.* (1995)	Beckman *et al.* (1992)	Lang *et al.* (1997)	Ohta *et al.* (1998)	Oda *et al.* (1992)	Schuster *et al.* (1993), Jekabsons & Schuster (1995)	Unseld *et al.* (1997), Handa (2003), Giegé & Brennicke (1999), Rayapuram *et al.* (2007), Spielewoy *et al.* (2001), Meyer *et al.* (2005)	Kubo *et al.* (2000), Sugiyama *et al.* (2005), Notsu *et al.* (2002), Ogihara *et al.* (2005), Clifton *et al.* (2004), Gonzalez *et al.* (1993), Bonnard & Grienenberger (1995)

The included species are: α proteobacteria: *Bradyrhizobium japonicum (B. j.)* and *Rhodobacter capsulatus (R. c.)*; γ proteobacterium *Escherichia coli (E. c.)*; the protist *Reclinomonas americana (R. a.)* and the following plant representatives: *Cyanidioschyzon merolae* (*C. m.*), *Marchantia polymorpha (M. p.), Arabidopsis thaliana (A. t.), Triticum aestivum (T. a.), Brassica napus (B. n.), Oenothera berteriana (O. b.), Beta vulgaris (B. v.), Oryza sativa (O. s.), Nicotiana tabacum (N. t.), Sorghum bicolor (S. b.), Tripsacum dactyloides (T. d.)* and *Zea mays (Z. m.)*.

dactyloides, *ccmB* and $ccmF_N$ in *Zea luxurians*, $ccmF_C$ and *ccmC* in *Cycas taitungensis* and all *ccm* genes in *M. polymorpha* and *Physcomitrella patens* mitochondrial genomes are located close to one another. The *T. dactyloides* and *Z. luxurians* $ccmF_C$ gene, *T. dactyloides* $ccmF_N$ gene or *Oenothera bertheriana* and *Daucus carota ccmB* and *ccmF* genes are duplicated (Schuster *et al*., 1993; Schuster, 1994).

The transfer of mitochondrial *ccm* genes to the nucleus represents one of the known phenomena connected with the evolution of organellar genomes. *E. coli ccm* genes are clustered in a single operon: *ccmABCDEFGH*; however, during the acquisition of the mitochondrial ancestor by the eukaryotic cell, the operon was shuffled by recombination; some *ccm* genes were transferred to the nucleus (Thöny-Meyer *et al*., 1995). The nuclear *CCMA* and *CCME* plant genes are transcribed at a low, but detectable level in roots, rosette leaves, stems, stem leaves and flowers of *Arabidopsis* (Spielewoy *et al*., 2001; Meyer *et al*., 2005; Rayapuram *et al*., 2007).

Some surprising data concerning the plant mitochondrial *ccm* gene transcription came from the analysis of the structure and expression of the $ccmF_{N1}$ and $ccmF_{N2}$ genes. For *Brassica* mitochondria, where despite the distinct transcriptional units the two ORFs the $CcmF_N$ protein is present, Handa *et al.* (1996) proposed, without any conclusive experiment, that either so called '*ribosome hopping*' or protein *trans*-splicing are responsible for that phenomenon. However, up to now no decisive experimental data supporting one of the proposed views has been published.

The mitochondrial *ccm* transcripts are among the most extensively edited plant mitochondrial mRNAs (Giegé & Brennicke, 1999). Recently, Hazle and Bonen (2007) examined *in silico* the 5′ UTRs of different protein-coding mitochondrial genes of a few flowering plants in order to identify the essential sequence features related to the initiation of translation. The maize and wheat $ccmF_N$ gene transcripts may utilize only the proximal (out of two putative ones) start codon for the translation. The mature *ccm* transcripts may also be interrupted before stop codons, like in the case of *Brassica ccmC* mRNA (Handa, 2003). Moreover, such interrupted *Arabidopsis* and cauliflower *ccmC* transcripts were translated. The CcmC protein which accumulates in *Arabidopsis* mitochondria is therefore truncated by only few less important residues (Raczynska *et al*., 2006).

Proteins participating in heme transport and chaperoning

The heme delivery in SI is probably connected with the activity of a few proteins CCMA–E: in plants the best characterized are: CCMA, CcmB, CcmC and CCME.

It is difficult to describe precisely the function of each of these proteins in plant mitochondria because of the limited experimental data and due to the fact that the vast majority of published data concerning the functionality of SI bases on prokaryotic models. Moreover, plant CCM proteins are of highly hydrophobic and low-abundant. There are suggestions that in the prokaryotic SI, the CcmA, CcmB, CcmC and CcmD proteins form a transporter complex which contains an ATP-binding cassette. Nevertheless, it is still under discussion whether prokaryotic CcmAB proteins are really required for the translocation of heme across the cytoplasmic membrane (Goldman & Kranz, 2001; Feissner *et al*., 2006a; Christensen *et al*., 2007). It has been shown that membrane vesicles depleted of CcmA display an efficient heme uptake. From those experiments one may conclude that CcmA and CcmB proteins may not be necessary for heme transport through the membrane and their real transported ligand remains to be identified (Cook & Poole, 2000).

The globular CcmA polypeptide belongs to the classical ATP-binding protein family because it contains the Walker A (GX_4GKS/T) and Walker B ($R/KX_3GX_3LX_3D$) motifs (Walker *et al*., 1982) and the ABC signature unique to ABC transporters. Mutation in this motif may result in a total loss of cytochrome *c* maturation efficiency *in vivo* (Christensen *et al*., 2007). Some minor differences between bacterial and plant CCMA orthologs were discovered (Rayapuram *et al*., 2007). The plant CCMA protein was immunodetected at a low level in *Arabidopsis* cell cultures, roots, leaves and flower mitochondria (Rayapuram *et al*., 2007). This suggests its housekeeping function. A striking feature of the plant CCMA protein is the presence of a short N-terminal extension without properties of a typical mitochondrial targeting α-helical sequence. Nevertheless, CCMA is imported into cauliflower mitochondria in a membrane-potential dependent way. Rayapuram *et al*. (2007) also showed that CCMA is associated with the inner membrane and oriented towards the matrix and this association could be modulated by the binding and hydrolysis of ATP, inducing a conformational change in the protein. Therefore the *Arabidopsis* CCMA protein has properties of the ATP-binding domain of the ABC transporter, like its prokaryotic homolog. The authors also identified a 480-kDa complex containing CCMA in the *Arabidopsis* mitochondrial inner membrane.

The CcmB protein is predicted to contain six transmembrane helices. Its sequence is highly conserved among all flowering plants, showing 93% identity. The hydropathy profiles of plant and prokaryotic CcmB proteins are almost identical. In the wheat mitoplast fraction and in inner mitochon-

drial membrane protein extracts antibodies recognize the CcmB polypeptide (Faivre-Nitschke *et al*., 2001). The most conserved motif DEDGS/TLEL of bacterial CcmB is thought to be oriented towards the matrix and to interact with the ATP-binding domain of ABC transporter. Rayapuram *et al*. (2007) — on the basis of *in silico* topology predictions — proposed that the plant CcmB protein contains six transmembrane helices, four matrix-facing loops and three IMS loops. Using the yeast two-hybrid assay they also showed that *Arabidopsis* CcmB interacts with CCMA by means of loops oriented toward the matrix. Interestingly, the plant CcmB protein contains seven conserved cysteine residues which are absent in bacterial CcmB proteins.

The proposed topology of bacterial CcmC proteins is generally the same as that of CcmB. *E. coli* CcmC has the conserved WWD motif found in the periplasmic loop between the third and the fourth transmembrane helix. CcmC from plant mitochondria is a conserved integral inner mitochondrial membrane protein, with at least 33% hydrophobic residues (Bonnard & Grienenberger, 1995). Raczynska *et al*. (2006) showed that the CcmC protein (24 kDa) is present in *Arabidopsis* mitochondria and associated with the membrane, but not with the soluble fraction of mitochondria. These results support the localization predicted *in silico*.

The CcmE protein is one of the best characterized Ccm proteins. The soluble globular domain of this protein is anchored to the membrane by the helical region (Christensen *et al*., 2007). CcmE acts as a heme chaperone by transient covalent heme binding to the H130 residue. Stevens *et al*. (2003) and Uchida *et al*. (2004) showed that residues 30 and 134 of bacterial CcmE also play an important role in heme ligation to CcmE that is supported by structural data. The CcmE polypeptide was proposed to have a two-domain structure with a β-barrel core and an important, though not essential for the functioning, C-terminal domain which appears to be quite flexible (Enggist & Thöny-Meyer, 2003; Enggist *et al*., 2003) and in general features resembles domains of iron or heme binding proteins (Baker *et al*., 2003).

The nuclear-encoded CCME protein is transported into *Arabidopsis* mitochondria using the N-terminal cleavable targeting sequence. The localization of the CCME protein in mitochondria of different land plants, besides *Arabidopsis*, has also been confirmed in cauliflower, turnip, rapeseed and radish (Spielewoy *et al*., 2001). Additionally, it was confirmed that *Arabidopsis* CCME is exposed on the outer face of the inner mitochondrial membrane (Spielewoy *et al*., 2001). It means that this protein is oriented towards the IMS. Plant mitochondrial CCME proteins display 35–40% identity with the *Bradyrhizobium* and *E. coli* homologous polypeptides. Spielewoy *et al*. (2001) suggested that in some cases the plant and bacterial CCM proteins can be interchangeable because *E. coli* CcmC can donate heme to the *Arabidopsis* CCME. Unfortunately, due to the instability of purified *Arabidopsis* CCME protein, the heme binding to it cannot be tested easily.

So far no bacterial CcmD protein homologs have been found in plant material. Their genes have not been identified in the nuclear or mitochondrial genomes of higher plants until now. The CcmD protein of *E. coli* is a small polypeptide with 69 amino-acid residues. It strengthens the transient interactions between CcmC and CcmE, forming the CcmCDE complex (Ahuja & Thöny-Meyer, 2005). According to the recent speculations of Richard-Fogal *et al*. (2008), CcmD is also a candidate for the component of another complex CcmABCD. Those authors also provide experimental evidence for the presence of one transmembrane domain in CcmD and localization of the N-terminal domain outside and the C-terminal one inside the periplasmic membrane. Generally, one could speculate that plant CcmC and CCME proteins may compensate for the absence of CcmD making the CcmCE complex stable without the participation of a third CCM protein.

Thiooxidation/thioreduction pathway

In the best known bacterial systems several proteins are engaged in maintaining the thiooxidation/thioreduction balance of apocytochrome *c*. These are: the periplasmic thioredoxin-like CcmG protein, disulfide reductases such as CcmH and DsbD, and components of the thiooxidation system — disulfide oxidases DsbA and DsbB (from disulfide bond formation protein). According to Reid *et al*. (2001), *E. coli* CcmG may play a role in the reduction of apocyt. *c* at the very late stage of the *c*-type cytochrome assembly, that is prior to the heme attachment by CcmF and CcmH. In contrast to the bacterial periplasmic space, the mitochondrial IMS displays generally reductive properties. No orthologs of thioredoxin CcmG or Dsb oxidases have been detected until now in plant mitochondria and CCMH remains the main important component of the reducing pathway of SI identified in plant mitochondria (Meyer *et al*., 2005). There are only speculations about other unidentified components in plant mitochondria that have captured the functions of the bacterial CcmG and Dsb proteins. One of those candidates might be a mitochondrial FAD-depended Erv1-like sulfhydryl oxidase, whose orthologs have been identified not only in yeast and human, but also in *A. thaliana* mitochondrial IMS (Levitan *et al*., 2004). The plant enzyme (*At*-Erv1) contains the CXXXXC motif in the C-terminal domain; that motif has some unique properties that

play an important role in the dimerization of the *A. thaliana* oxidase. The substrate of *At*Erv1 is not known, but the substrate of the yeast Erv1 protein appeared to be Mia40 which oxidizes target proteins and drives their import into IMS (Hell, 2008). Could an Erv1-like oxidase and Mia40-like redox-dependent receptor proteins also be necessary for the formation of disulfide bonds in apocyt. *c* in plant mitochondria, which would make CCMH important for their reduction?

E. coli CcmH is a fusion protein descending from two polypeptides. In *Rhodobacter* two separate polypeptides exist: $CcmH_{Rc}$ and CcmI; however, in *E. coli* the C-terminal part of CcmI is fused to the homolog of $CcmH_{Rc}$, forming CcmH (Sanders *et al*., 2005). Moreover, *Bradyrhizobium japonicum* proteins CycL and CycH correspond to the N- and C-terminal part of the *E. coli* CcmH protein, respectively. The only known plant homolog of the *E. coli* CcmH polypeptide — the *Arabidopsis* nuclear-encoded CCMH protein — corresponds to its N- terminal part. CcmH exposes its redox-active site — the LRCXXCQ motif — to the periplasmic space. The plant protein does not contain an N-terminal targeting sequence. Moreover, specific antibodies recognize the *Arabidopsis* CCMH protein whose molecular mass is larger than the calculated one (17.9 kDa). The topological model of this polypeptide predicts the presence of an N-terminal domain exposed to the *p*-side and the C-terminus exposed to the *n*-side of the inner mitochondrial membrane (Meyer *et al*., 2005). The plant mitochondrial CCMH protein contains a hydrophobic domain and an RCXXC motif facing IMS. Meyer *et al*. (2005) also showed that *Arabidopsis* CCMH protein homologs may reduce the CXXCH motif in a peptide that mimics apocyt. *c*. In that study, two-hybrid assays also showed interactions between *Arabidopsis* CCMH and cyt. *c*.

CCMH is an essential *Arabidopsis* housekeeping gene because the development of the homozygotic knockout embryo is stopped during the torpedo stage in insertion mutants. Interestingly, no obvious phenotype could be observed among heterozygotic plants in different stages of development; however, self-fertilized mutant plants heterozygous for the T-DNA insertion contained 25% smaller and pale seeds in their siliques, with homozygotic for the T-DNA insertion knockout embryos (Meyer *et al*., 2005). Up to the now, the plant CCMH protein could be treated as the only identified component of the putative reducing pathway. Moreover, as it will be discussed in the chapter below, plant CCMH acts not only as apocyt. *c* disulfide reductase, but most probably also assists in the process of heme ligation to the apoprotein and henceforth plays quite an important role within the plant mitochondrial SI (Meyer *et al*., 2005).

Components of system I responsible for the heme lyase activity

Very little data relates to the components of heme lyase and their activity is best described in bacterial SI. Initially, it was thought that the activity of prokaryotic HL was associated mainly with the CcmF protein containing the WWF motif. Yet recent findings indicate that the prokaryotic HL complex is also dependent on the activity of additional proteins. For instance, the 'core ligase' complex of *Rhodobacter capsulatus* is probably composed of CcmF and $CcmH_{Rc}$ polypeptides. The bacterial CcmI protein probably participates in this complex by bringing apocyt. *c* to the ligation with heme (Sanders *et al*., 2005; 2007).

Recent experimental evidence indicates that the *Arabidopsis* mitochondrial CCMH protein overexpressed in *E. coli* co-immunoprecipitates with *E. coli* CcmF; on the other hand, plant CCMH could not re-

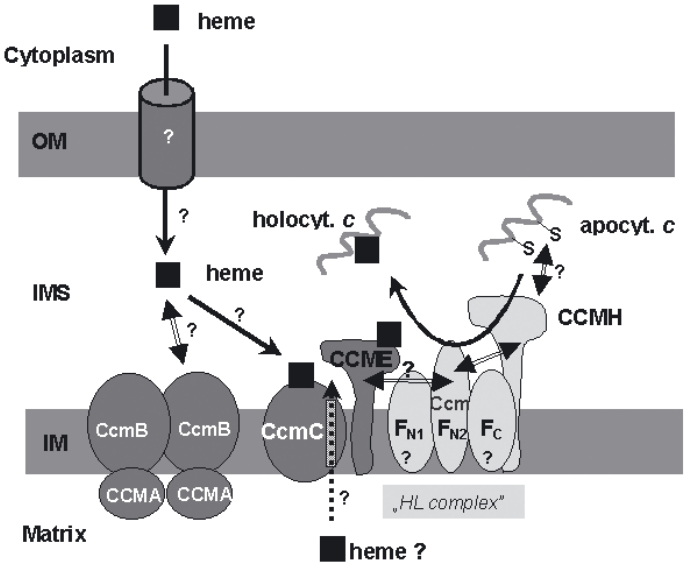

Figure 4. Model for system I of cyt. *c* biogenesis and structure of HL complex in *Arabidopsis* mitochondria.
After Giegé *et al*. (2008), modified. Details are discussed in chapters: „Components of system I responsible for the heme lyase activity" and „Protein–heme and protein–protein interactions in the prokaryotic and in the plant mitochondrial system I". Currently favored hypothetical route of transport of the plastid-synthesized heme to mitochondria and heme delivery is shown by standard arrows with question marks. Alternative hypothetical route of transport of heme by CcmC from matrix to IMS is shown by dotted arrow with question mark. Proposed protein- protein interactions are shown as double-headed arrows. Some of them and the presence of $CcmF_{N1}$ and $CcmF_C$ in the putative HL complex need to be verified experimentally (question marks).

place its bacterial ortholog for holocyt. *c* maturation in a complementation assay (Meyer *et al*., 2005).

It is worth noting that in *Arabidopsis* mitochondria, contrary to the situation in *Brassica*, two mitochondrial genes, $ccmF_{N1}$ and $ccmF_{N2}$, encode distinct $CcmF_{N1}$ and $CcmF_{N2}$ proteins. Moreover, the CCMH and $CcmF_{N2}$ proteins which form a large 500-kDa membrane complex in *Arabidopsis* mitochondria, were proposed to hold the HL activity (Meyer *et al*., 2005). The *Arabidopsis* $CcmF_{N2}$ protein is also present in an even larger complex (700 kDa; Meyer *et al*., 2005). The identity and structure of the putative plant HL is still under debate. The proposed current model of plant HL is shown in Fig. 4.

Another protein that may be involved in HL activity in plant mitochondria, the $CcmF_C$ protein (50 kDa in wheat), is highly basic and hydrophobic (Giegé *et al*., 2004). In general the sequence of $CcmF_C$ is highly conserved among plants species; nevertheless, it displays quite a low similarity to the C-terminal domain of CcmF protein of *Rhodobacter*, *Escherichia* and *Bradyrhizobium*.

PROTEIN–HEME AND PROTEIN–PROTEIN INTERACTIONS IN THE PROKARYOTIC AND IN THE PLANT MITOCHONDRIAL SYSTEM I

As it was mentioned previously, the vast majority of data concerning SI comes from the study of prokaryotic models. The knowledge about how the proteins involved in this system operate in *Prokaryota* may be useful in the elucidation of the function of similar proteins in plant mitochondria. It is known that at least two *E. coli* proteins, CcmC and CcmE, can interact directly with heme (Ren & Thöny-Meyer, 2001). Due to the fact that CcmC displays high affinity for heme, direct transfer of heme to apocyt. *c* may not be thermodynamically favored. It should be emphasized that a crucial step during the maturation of *c*-type cytochromes involves the transfer and the covalent attachment of heme to CcmE — the heme chaperone (Schulz *et al*., 1998). This step is followed by the heme transfer from CcmE to apocyt. *c*.

Ren and Thöny-Meyer (2001) showed that the direct interaction of CcmC with heme is not dependent on the presence of the WWD motif in CcmC. This motif is necessary for the formation of the CcmCE complex and for the subsequent heme transfer from CcmC to CcmE. Additionally, Ahuja and Thöny-Meyer (2005) showed that the transient interactions between CcmC and CcmE could be significantly enhanced by CcmD.

The key role during initial steps of the maturation of bacterial *c*-type cytochromes may be played by ABC transporters with the proposed stoichiometry $CcmA_2B_1C_1$ (Feissner *et al*., 2006a; Fig. 5a). In the absence of CcmA or CcmB, CcmC may form a stable complex with the heme–CcmE complex. The CcmE protein may be released from CcmC in an ATP-dependent manner only when the expression of CcmA and CcmB proteins facilitates the formation of the $CcmA_2B_1C_1$ complex (Feissner *et al*., 2006a). Those authors proposed that this complex may be treated as member of a novel subgroup of the ABC transporter family — the ABC heme release complex.

A different model of the participation of the CcmAB proteins in the maturation of *c*-type cytochromes in *E. coli* was suggested recently by Christensen *et al*. (2007; Fig. 5b). They argued with the results of Feissner *et al*. (2006a) concerning the composition and the proposed functional role of the ABC complex. In their opinion, CcmC may not represent a necessary component of this complex because CcmA is located in the cell membrane of *E. coli* only when CcmB is there. They presented experimental data that argue against the possible interaction of CcmA with both CcmB and CcmC within one complex. They pointed out that in fact no examples of complexes like $CcmA_2B_1C_1$ are known. The WWD motif, present in CcmC, faces the periplasmic space; in contrast, highly conserved residues of CcmB are predicted to be present in cytoplasmic loops or within transmembrane domains. Moreover, CcmC could compensate for the lack of CcmA or CcmB and there is no detectable sequence similarity between CcmB and CcmC, which would be expected if they were to interact with CcmA. Christensen *et al*. (2007) also pointed out that CcmC seems to be generally an accesory protein in this complex, as this bacterial protein has probably additional functions, for instance in siderophore production. Additionally, CcmA cannot interact directly with CcmC.

All these findings indicate that the composition of the ABC transporter and its participation in heme transport need to be further explored experimentally.

It is assumed that in *E. coli*, CcmE acts as a shuttle between the CcmA–CcmB–CcmC complex and a putative HL composed of two proteins: CcmH and CcmF (Ahuja & Thöny-Meyer, 2003). Ren *et al*. (2002) also suggested that in *E. coli* SI, a stable interaction between CcmF, CcmE and CcmH may take place without any involvement of apocyt. *c*.

It is still not clear how SI operates in the maturation of *c*-type cytochromes in plant mitochondria, as the way in which it causes the heme transport and delivery process is still not supported experimentally (Giegé *et al*., 2008). Moreover, the cellular localization of plant enzymes responsible for the last steps of heme biosynthesis is still under discussion (Tanaka & Tanaka, 2007). For instance, there are strong suggestions for plastid (not mitochondrial) localization of coproporphyrinogen III oxidase

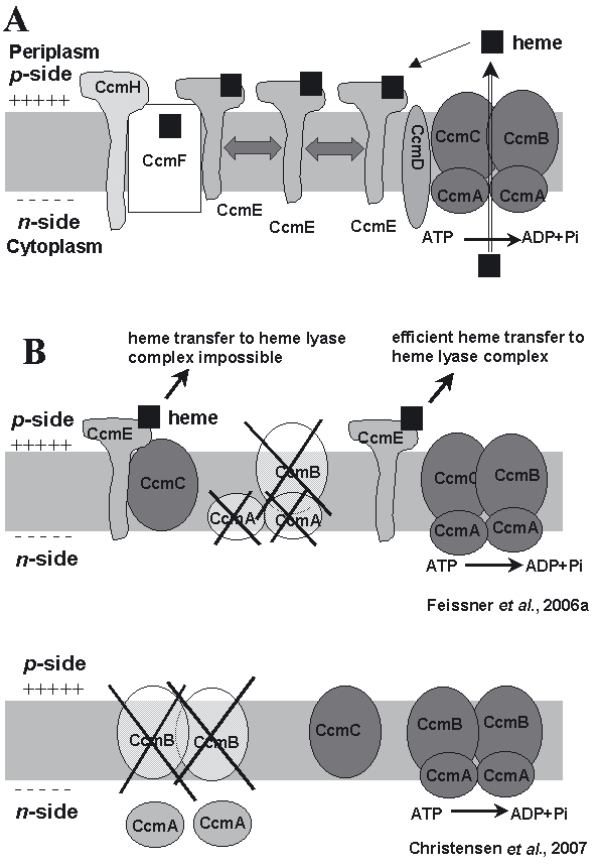

Figure 5. A. Transient interactions between *E. coli* CcmE, the ABC transporter complex and the putative HL. After Feissner *et al.* (2006a), modified. B. Models for the structure of the ABC transporter in *E. coli* maturation system of *c*-type cytochromes. The models are based on the data of Feissner *et al.* (2006a) and Christensen *et al.* (2007). Details concerning these two models are also described in chapter „Protein–heme and protein–protein interactions in the prokaryotic and in the plant mitochondrial SI".
According to Feissner *et al.* (2006a), the ABC transporter operating in heme transfer is composed of $CcmA_2B_1C_1$. When CcmA (containing ATP-binding cassette) and CcmB (containing the hexahelical transmembrane domain of the ABC transporter) proteins are present, they interact with CcmC (containing six transmembrane helices); therefore it is possible for heme to be transferred to the HL complex by CcmE. However, in the absence of CcmA and CcmB, interactions of free CcmC with CcmE make the subsequent heme transfer impossible. The existence of such ABC transporter was challenged by Christensen *et al.* (2007). In their model CcmC acts independently of the transporter because in the absence of CcmB, CcmA cannot be anchored to the membrane. Therefore, Christensen *et al.* (2007) conclude that CcmC cannot be a part of the ABC transporter, which is not involved in heme export.

(Santana *et al.*, 2003) and protoporphyrin IX ferrochelatase (Lister *et al.*, 2001; Cornah *et al.*, 2002; Matsuda *et al.*, 2003) coming from enzyme activity studies and targeting assays. However, there is still a possibility that some fraction of ferrochelatase or an unknown protein show low activity in plant mitochondria (Tanaka & Tanaka, 2007). If ferrochelatase is present in plant mitochondrial matrix, one could imagine heme export to IMS by CcmC, which — as was discussed earlier in the case of *E. coli* — could bind heme. If enzymes for the last steps of heme synthesis are absent from plant mitochondria, how is heme transported from plastids? No heme transporters have been identified in the plant mitochondrial OM. In that case CcmC and CcmAB are not necessary for the heme import to IMS, but rather for the heme delivery to the putative HL (Giegé *et al.*, 2008; Fig. 4).

The results of yeast two-hybrid analyses suggest that *Arabidopsis* mitochondrial CCMA protein, the product of the nuclear *NAP10* gene, interacts with CcmB forming a rather distinct kind of ABC transporter. The transmembrane domains of this transporter are encoded in the mitochondrial genome, while its ATP-binding domains — in the nucleus (Rayapuram *et al.*, 2007). The function of the plant CCMA–CcmB complex in the maturation pathway of cyt. *c* remains to be elucidated.

The expression of both nuclear and mitochondrial genes coding for CCM proteins raises important questions about the key steps in coordination of the biogenesis of nuclear and mitochondrial CCM proteins as well as the formation of the CCM complexes present in plant mitochondria. The CCM complexes, assayed through the blue native electrophoresis, reveal different sizes. It has been shown for instance that *Arabidopsis* CCMA protein is present in a 480-kDa complex that does not contain the CCME, CCMH, $CcmF_{N1}$, $CcmF_{N2}$ or $CcmF_C$ proteins (Rayapuram *et al.*, 2007). On the other hand, CCMH and $CcmF_{N2}$ are present in a 500-kDa complex in *Arabidopsis* mitochondria (Meyer *et al.*, 2005) and $CcmF_{N2}$ together with $CcmF_C$ in a large 700-kDa complex in wheat mitochondria (Giegé *et al.*, 2004). No single complex containing all known CCM proteins has been detected in plant mitochondria yet and when the stoichiometry $CCMA_2$–$CcmB_2$–CcmC–CCME–$CcmF_{N1}$–$CcmF_{N2}$–CCMH is considered, its size could still be calculated at about 300 kDa only. These observations may suggest that: (1) at least some interactions between plant CCM proteins seem to be more stable than others; (2) different CCM proteins display different stoichiometry; (3) in mitochondria plant-specific CCM proteins may be present (Giegé *et al.*, 2008) .

In *Arabidopsis* mitochondria, as revealed by blue native electrophoresis, the important interactions within the proposed HL would involve at least $CcmF_{N2}$ and CCMH. As it was presented above, heterologously expressed *Arabidopsis* CCMH coimmunoprecipitates with bacterial CcmF (Meyer *et al.*,

2005). However, the interaction $CcmF_{N2}$–CCME and the presence of $CcmF_{N1}$ and $CcmF_C$ in the putative plant HL complex need to be verified (Giegé *et al*., 2008; Fig. 4).

The precise composition of complexes containing CCM proteins remains to be elucidated in detail. The way in which the different developmental stages and environmental conditions affect such interactions is completely unclear and has to be investigated, too.

MATURATION OF PLASTID *c*-TYPE CYTOCHROMES

The plant SII model has emerged from studies performed on chloroplasts of *Chlamydomonas reinhardtii* (Xie & Merchant, 1996; Xie *et al*., 1998). Plant SII for the maturation of *c*-type cytochromes in plastids is represented by at least two protein homologs in *Bacillus subtilis*, namely ResB and ResC, which are known in plants as Ccs1 (CcsB) and CcsA, respectively. These proteins and the expression products of nuclear loci *CCS2, CCS3* and *CCS4* are postulated to form a hypothetical holocyt. *c* assembly complex in *Chlamydomonas* plastids (Xie *et al*., 1998). The maturation pathway of plastid *c*-type cytochromes also requires CcdA and ResA proteins for the thiooxidation/thioreduction pathway. It should be stressed that some sequence motifs, as for example a tryptophane-rich motif, are present both in the components of SII — as the CcsA protein — and in the CcmC and CcmF proteins of SI.

Apocyt. *f* having an N-terminal signal sequence, is synthesized on polysomes associated with the *n*-side of the thylakoid membrane. It is targeted to the *p*-side of the thylakoid membrane in a Sec-dependent way. According to Rothstein *et al*. (1985), a specific, *trans*-acting factor TCA1 is required for the translation of apocyt. *f* in *Chlamydomonas* chloroplasts. 5′ UTR of the *pet*A transcript, encoding *Chlamydomonas* apocyt. *f*, autoregulates this process (Choquet *et al*., 1998).

Cyt. c_6 is translated as a precursor with a bipartite signal in the cytosol and is imported post-translationally into the chloroplast. Its presequence does not contain the twin R motif that is characteristic for ΔpH-dependent protein import to thylakoids (Nakamoto *et al*., 2000).

Heme transporting proteins

Ccs proteins (from: cytochrome *c* synthesis) were discovered during the screening for defects in the maturation of plastid *c*-type cytochromes of *Chlamydomonas reinhardtii* non-photosynthetic and acetate-requiring mutants. It was shown that products of four nuclear *CCS* and one chloroplast (*CcsA*) loci are required for the heme attachment, but not for the apocytochrome processing during the assembly of holocyt. *f* and c_6. The expression of *Ccs* genes in *Chlamydomonas* is under strict control (Xie & Merchant, 1996; Inoue *et al*., 1997; Xie *et al*., 1998; Nakamoto *et al*., 2000). Essential roles in heme transport and in the chaperoning function in plastids were attributed to Ccs1 and CcsA proteins.

The *ccs*A gene is encoded by the chloroplast DNA of vascular plants, liverwort, red algae and cryptomonads (Hamel *et al*., 2003). According to *in silico* analyses, the *Chlamydomonas* CcsA protein (29 kDa, 320 amino acid-long) is a hydrophobic, thylakoid membrane protein with a few transmembrane regions. The CcsA protein contains three blocks of a conserved amino-acid sequence. The most conserved one is located in the C domain, specifically in the region containing the WWD motif (WGXXWX-WDXXE). A comparison of 14 plastid and bacterial CcsA-like homologous sequences allowed identification of ten absolutely conserved residues in the conserved region that might form a putative active site. Bacterial CcsA protein is known to contain three more conserved residues H212, H309 and H347, that are necessary for the protein function (Hamel *et al*., 2003).

The *ccs*A gene rescues the photosynthetic deficiency in some *Chlamydomonas* mutant strains and restore the level of holocyt. c_6. The CcsA protein was proposed to be involved in heme binding and transport, by forming a heme channel in the thylakoid membrane.

Ccs1 is a membrane protein with three predicted transmembrane regions in the N-terminal half of the protein and with hydrophilic C-terminal half of lumenal localization (Nakamoto *et al*., 2000). The C-terminal domain is important for the Ccs1 function; also a loop directed to the stroma seems to be essential for the activity of the protein. Additionally, a histidine residue located within the last transmembrane domain is absolutely required for the proper *c*-type cytochrome assembly. The predicted topology of *Chlamydomonas* Ccs1 protein resembles the topology of *Synechocystis* CcsB (Dreyfuss *et al*., 2003).

Plant Ccs1 protein could be immunodetected as a 60-kDa polypeptide only in freshly prepared thylakoid membrane fraction and its accumulation increases with the growth of the *Chlamydomonas* culture between the log and the stationary phase. Css1 is also present in non-green plastids; however, after *Chlamydomonas* exposure to light, the abundances of Ccs1 and cytochrome *f* increase in a parallel way. Although the accumulation of cyt. *f* requires the Ccs1 function, the accumulation of Ccs1 is independent of cyt. *f* during the light-initiated greening of etiolated cultures of *Chlamydomonas* (Dreyfuss *et al*., 2003).

Ccs1 is considered to be a highly divergent protein (Dreyfuss *et al*., 2003). This may have resulted from its coevolution with the highly divergent apocytochromes.

The mutations of the *Ccs1* gene in the *Chlamydomonas* mutants studied could affect the level of transcript and protein accumulation. Nevertheless, nonsens mutations did not supress totally the protein pool in plastids. Interestingly, Ccs1 does not accumulate in *ccs*A mutants; however, it is present after complementation of the same strain with the *ccs*A gene (Hamel *et al*., 2003). This phenomenon suggests the presence of a functional 'CCS' protein complex that is involved in heme transport and attachment in the thylakoid membrane. Such a complex, which was found in solubilized *Chlamydomonas* thylakoid membranes, may contain not only the Ccs1 and CcsA proteins but also other, unidentified components, as for instance products of the expression of nuclear loci *CCS2-CCS4* (Xie *et al*., 1998; Dreyfuss *et al*., 2003; Hamel *et al*., 2003).

Summing up, the current working model of *c*-type cytochrome assembly in chloroplasts includes the chloroplast-encoded CcsA protein, the nucleus-encoded Ccs1 polypeptide and possibly other, unidentified components. Heme is transferred to the thylakoid lumen from the stromal side with the participation of histidine residues and the WWD motif of CcsA and delivered to the ligation with reduced apocytochromes that are chaperoned by Ccs1.

Thiol maintenance system in plastids

Based on the components of the bacterial SII (BdbC, BdbD thiol oxidases, and ResA and CcdA proteins for thiol reduction), a similar system for maintaining thiols has been proposed to be present in the plastid compartment. CCDA and HCF164 (high chlorophyll fluorescence phenotype) are the best described plant homologs of bacterial CcdA and ResA proteins. However, no plastid orthologs of prokaryotic BdbCD proteins have been found yet. Bdb proteins are necessary for the proper folding and stability of disulfide bond-containing proteins participating in the transformation of *B. subtilis* (Meima *et al*., 2002).

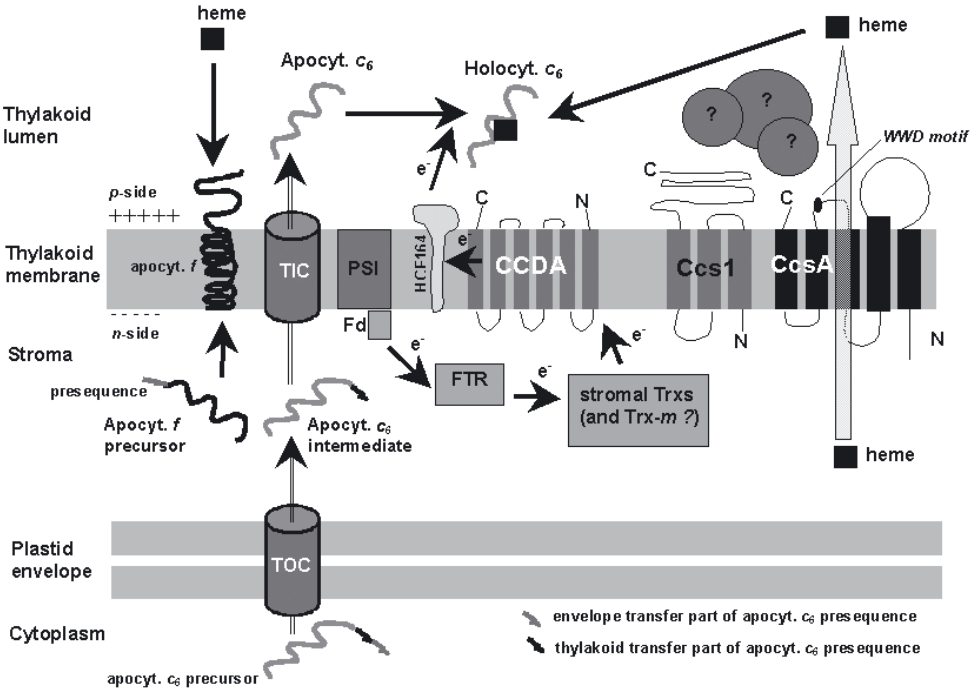

Figure 6. Model of system II for *c*-type cytochrome biogenesis in plastids based on data of Motohashi and Hisabori (2006), Page *et al*. (2004), Hamel *et al*. (2003) and Dreyfuss *et al*. (2003).

Detailed description of the system is given in chapter "Maturation of plastid *c*-type cytochromes". The model locates CcsA, Ccs1 as well as CCDA proteins in the thylakoid membrane. In addition to the channel-forming membrane proteins CcsA and Ccs1, some other factors were suggested to participate in heme transport across thylakoid membrane. The WWD motif is shown at the *p*-side domain of CcsA (black oval). Apocytochrome c_6, encoded in the nucleus, is probably transported by TOC/TIC complex, whereas apocytochrome *f*, encoded in chloroplast DNA, is directly inserted into the thylakoid membrane. Ferredoxin donates electrons to the ferredoxin-dependent thioredoxin reductase (FTR). FTR reduces stromal thioredoxins. The thioredoxin Trx-*m* is a putative source of reducing equivalent at *n*-side, whereas HCF164 plays such a role at *p*-side of the thylakoid membrane. Two presequence parts for import of apocyt. c_6 are shown.

Generally, in the thiooxidation/thioreduction pathway in plastids (Fig. 6) the thiol-reducing equivalents are proposed to be transferred from the *n*-side to the *p*-side of the thylakoid membrane. For the reduction of *c*-type apocytochrome both thioredoxins and thioredoxin-like proteins are necessary. A currently preferred candidate for the source of reducing equivalents at the *n*-side is an *m*-type thioredoxin (Motohashi & Hisabori, 2006). This type of thioredoxins can transfer electrons to the lumenal HCF164 protein across the thylakoid membrane. The thiooxidation/thioreduction pathway also contains CCDA and the HCF164 protein, a homolog of the *B. subtilis* ResA protein, a putative candidate for the *p*-side transducer of reducing equivalents (Page *et al*., 2004). Plastid CCDA, being the homolog of the bacterial protein CcdA, resembles the central part of DipZ/DsbD proteins which are necessary for transmembrane thiol redox metabolism in bacterial systems (Katzen *et al*., 2002). CCDA is a polytopic membrane protein delivering the reducing equivalents to apocyt. *f* prior to its conversion to the holocytochrome. It contains six transmembrane regions and a 141 amino acid-long N-terminal extension functioning as a targeting sequence. Both the N- and C-terminal protein domains face the lumenal side of plastids (Page *et al*., 2004). The bacterial CcdA protein also contains in its transmembrane domain two conservative cysteines that are indispensable for functioning. In the chloroplast genome of *Porphyra purpurea* CCDA is encoded by the single gene that is linked to the ORF of a Ccs1-like protein. Page *et al*. (2004) showed that *Arabidopsis* CCDA could be targeted to pea chloroplasts. Other CcdA-like protein genes and their respective cDNAs have been identified in a variety of plants (rhodophyte algae *Porphyra purpurea* and angiosperms: *Descurainia sophia, Glycine max, Hordeum vulgare, Lotus japonicus, Medicago truncatula*) — without experimental determination of the cellular localization of their protein products, though.

HCF164 is a membrane-anchored 29 kDa protein (261 amino acid-long) with a disulfide-reductase activity and its active site facing the *p*-side of the membrane. HCF164 displays some similarity to the eubacterial CcsX and CcmG proteins. It appeared that recombinant HCF164 protein depleted of the first 115 amino-acid residues reduces disulfide bonds between insulin chains; yet this reduction activity is twice lower compared to the action of the purified thioredoxin protein (Lennartz *et al*., 2001).

The plastid HCF164 protein contains at least three domains; the second domain is characterized as hydrophobic, while the third, large domain is hydrophilic. The first two domains contain regions sharing some features of plastid transit peptides; therefore, it is believed that the HCF164 protein is anchored in the thylakoid membrane by the N-terminal domain (Lennartz *et al*., 2001). The C-terminal domain, which faces the lumenal side of thylakoid, as it was shown in a protease protection assay carried out by Motohashi and Hisabori (2006), contains a WCXXC motif typical of the thioredoxin active site.

Recently, Lennartz *et al*. (2006) also showed that another protein — HCF153 (15 kDa), containing a chloroplast transit peptide, is tightly bound to the thylakoid membrane. HCF153 probably participates in a post-translational step of biogenesis of cytochrome b_6f complex. Hence, HCF153 may be regarded as another protein necessary for the biogenesis of *c*-type cytochromes in plastids.

Plant nuclear genomes encode numerous isoforms of thioredoxins which may also be active in plastids (Balmer *et al*., 2003). To identify the activity of redox-linked proteins in thylakoids, Balmer *et al*. (2006) applied a new experimental approach based on the monobromobimane fluorescent thiol probe. They discovered fourteen potential thioredoxins, which were bound to the membrane. Summarizing, it is tempting to speculate that in the future new data might help in the discovery of other thioredoxins or disulfide reductases required for the maturation of plastid *c*-type cytochromes.

CONCLUDING REMARKS

In the light of the data presented above the experimental validation of the functioning of the *c*-type cytochrome maturation process in plant mitochondria and in higher-plant plastids is still insufficient. The progress in characterization of plant organellar proteomes may be helpful in the identification of novel proteins that assist in the biogenesis of *c*-type cytochromes. The growing understanding among researches of how bacterial system I was "adopted" by plant mitochondria could also accelerate the identification of unknown components of this maturation pathway (Giegé *et al*., 2004).

The plant mitochondrial system I appears to be functionally more complex than any other system known. System II, which seems to be designed for the biogenesis of plastid *c*-type cytochromes, may also contain — as yet — unidentified components. It should be added that the general view about the maturation pathways of cytochrome *c* in plant organelles may in the future depend on the recognition and characterization of proteins involved in other biological processes; moreover, it has been suggested lately that some CCM proteins may indeed perform different biological functions

being directly or indirectly involved in them (Cianciotto *et al*., 2005).

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