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Review

Plant mitochondrial Complex I composition and assembly: A review☆

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ABSTRACT

In the mitochondrial inner membrane, oxidative phosphorylation generates ATP via the operation of several multimeric enzymes. The proton-pumping Complex I (NADH:ubiquinone oxidoreductase) is the first and most complicated enzyme required in this process. Complex I is an L-shaped enzyme consisting of more than 40 subunits, one FMN molecule and eight Fe–S clusters. In recent years, genetic and proteomic analyses of Complex I mutants in various model systems, including plants, have provided valuable insights into the assembly of this multimeric enzyme. Assisted by a number of key players, referred to as "assembly factors", the assembly of Complex I takes place in a sequential and modular manner. Although a number of factors have been identified, their precise function in mediating Complex I assembly still remains to be elucidated. This review summarizes our current knowledge of plant Complex I composition and assembly derived from studies in plant model systems such as *Arabidopsis thaliana* and *Chlamydomonas reinhardtii*. Plant Complex I is highly conserved and comprises a significant number of subunits also present in mammalian and fungal Complexes I. Plant Complex I also contains additional subunits absent from the mammalian and fungal counterpart, whose function in enzyme activity and assembly is not clearly understood. While 14 assembly factors have been identified for human Complex I, only two proteins, namely GLDH and INDH, have been established as *bona fide* assembly factors for plant Complex I. This article is part of a Special Issue entitled Respiratory complex I, edited by Volker Zickermann and Ulrich Brandt

1. Introduction

Mitochondrial Complex I, a proton-pumping NADH:ubiquinone oxidoreductase, is the largest protein complex (≃1 MDa) operating in oxidative phosphorylation and also the major entry point of electrons from NADH into the respiratory chain [1,2]. Complex I is a type I NADH dehydrogenase[1] (NDH-I) [3]. Included in this NADH dehydrogenase family are the type II NAD(P)H dehydrogenase (NDH-II), a non-proton pumping enzyme [4], and the sodium pumping NADH-quinone reductase (Na^+-NQR) [5,6]. Mitochondrial NDH-I is a multimeric enzyme with more than 40 subunits, one FMN molecule and eight iron–sulfur (Fe–S) clusters [7]. Bacterial Complex I has a comparatively simple composition with generally 14 subunits, one FMN molecule and eight or, in some cases, nine Fe–S clusters [7–10]. In some bacteria, Complex I contains up to three additional subunits [11,12]. The 14 subunits of bacterial Complex I are considered to be the minimal subunits required for NADH:ubiquinone oxido-reduction and proton pumping and hence, they constitute the core subunits of the enzyme [7]. In addition to the 14 conserved core subunits, mitochondrial Complex I contains 25 to 35 non-core subunits (with the number varying among species), also referred to as accessory or supernumerary subunits, which are integral components of the mitochondrial enzyme [7,13,14,197]. While 24 non-core subunits appear to be conserved in all eukaryotic lineages as gauged from sequence analysis, the occurrence of lineage-specific accessory subunits suggests additional specialized function for mitochondrial Complex I [13,15]. The finding that several disease-causing mutations in humans map to genes encoding non-core subunits underscores the role of these subunits in the assembly and activity of the mitochondrial enzyme [13].

Complex I is an L-shaped enzyme composed of two elongated domains also referred to as "arms". The membrane or hydrophobic arm is inserted into the cytoplasmic or mitochondrial inner membrane and the peripheral (soluble) or hydrophilic arm, is protruding into the

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[1] Abbreviations: EM, electron microscopy; MS, mass spectrometry; BN-PAGE, blue native polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; GLDH, L-galactone-1,4-lactone dehydrogenase; Q, quinone; FMN, flavin mono nucleotide; Fe–S, iron–sulfur; CMS, cytoplasmic male sterility; NCS, nonchromosomal stripes; NDH-I, type I NADH dehydrogenase, NDH-II, type II NADH dehydrogenase; Na^+-NQR, sodium NADH-quinone oxidoreductase; PSI, Photosystem I; ROS, reactive oxygen species; mt, mitochondrial; TCA, tricarboxylic acid; CA, carbonic anhydrase; IMS, mitochondrial intermembrane space; ACP, acyl carrier protein.

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cytoplasm or the mitochondrial matrix [16–18] (Fig. 1). The peripheral arm consists of a domain involved in NADH binding and oxidation called the N module and a domain for electron transfer to quinone, called the Q module [19]. The membrane arm harbors the proton translocation or P module, which is further divided into the proximal proton pump module (P_P module) connected to the Q module and a distal proton pump module (P_D module) [19]. The FMN and Fe–S clusters are all contained within the N and Q modules of the soluble arm [19]. The structures of Complex I of the bacterial enzyme from *Thermus thermophilus* [8,20], the fungal enzyme from *Yarrowia lipolytica* [21] and the mammalian enzyme from *Bos taurus* [22–24] were recently solved by X-ray crystallography, providing insights into the proton pumping activity of the membrane arm. The opening of proton channels located in the P module in response to conformational changes caused by quinone reduction in the Q module was postulated as a possible mechanism for proton translocation [11,25–27]. However the view that proton pumping is driven indirectly by long-range conformational changes was challenged with recent experimental evidence and the mechanism for proton transfer across the membrane arm of Complex I still remains undeciphered [28,29].

From a survey of 970 genomes in bacteria, Complex I was found to be present in the majority of Gram-negative bacteria and mostly in the phylum of Actinobacteria in Gram-positive bacteria [9,10]. With the exception of one archaeon, Complex I was found to be absent from the archaeal domain in the 88 genomes examined [10]. In eukaryotes, mitochondrial Complex I occurs in aerobic [30] and anerobic mitochondria [30,31] and in some mitochondria-related organelles (MRO) [32] recently discovered in anerobic free-living protists [33,34]. Mitochondrial NDH-I is absent in some eukaryotes such as the fungal lineages of *Schizosaccharomyces* and Saccharomycetales, and the cryptomycotan fungus *Rozella* [30,35,36]. In addition, several alveolates such as some apicomplexan parasites including *Plasmodium* spp., the chromerid *Chromera velia*, [37] and dinoflagellates [38] also lack Complex I.

In the plant kingdom, all mitochondria contain NDH-I [30] and several NDH-II [39] with the exception of one parasitic plant *Viscum scurruloideum*, which appears to be lacking Complex I [40,41]. This is in contrast to animal mitochondria, which, with the possible exception of five animal species, harbor only Complex I [30,42]. Plant Complex I was first investigated biochemically using the purified enzyme and genetically via the study of mutants deficient for Complex I in vascular plants [43,44] and Chlorophyceae [45]. This review focuses on the study of plant Complex I with emphasis on the enzyme from the vascular plant *Arabidopsis thaliana* and the unicellular green alga *Chlamydomonas reinhardtii*.

2. Architecture and subunit composition of Complex I in photosynthetic eukaryotes

2.1. A. thaliana Complex I

The architecture of plant Complex I was revealed via single-particle electron microscopy (EM) imaging of the *A. thaliana* enzyme. Similar to its bacterial, fungal and animal counterparts [23,87], plant Complex I displays an L-shaped structure consisting of one hydrophobic arm embedded within the mitochondrial inner membrane and a hydrophilic arm protruding into the mitochondrial matrix [46] (Fig. 1). However, in contrast to other Complexes I, an additional spherical domain is attached to the membrane arm at a central position on its matrix-exposed side (Fig. 1). This domain was shown to include carbonic anhydrases (CA), which are enzymes catalyzing the reversible hydration of CO_2 to HCO_3^- [88]. This domain, referred to as the CA module, is also present in Complex I of potato and maize [89,90]. Although it was initially believed to be specific to phototrophic eukaryotes, the CA domain was later also identified in the non-photosynthetic alga *Polytomella* [75]. The CAs found in the CA domain belong to the family of γ-CAs [88], whose first member was identified in the archaeon *Methanosarcina thermophila* [91].The γ-CA subunits are also detected by proteomic analysis in Complex I of *Acanthamoeba* spp., an amoebozoon lacking photosynthesis. However, their exact location in the enzyme remains to be determined [92]. In other

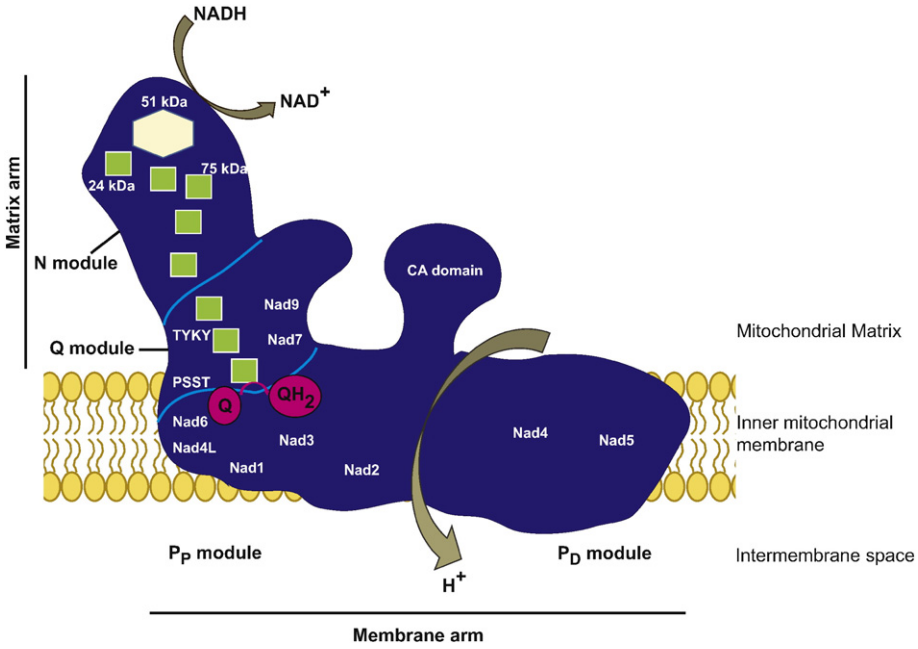

Fig. 1. Model for structure of Complex I in *A. thaliana*. Schematic representation of *Arabidopsis* Complex I structure as determined by imaging of single particle via electron microscopy [46] and subunit localization studies [47]. Complex I has an L-shaped structure with a hydrophilic arm protruding into the matrix and a hydrophobic arm embedded in the mitochondrial inner membrane. Electrons are transferred from NADH to ubiquinone (Q) via one FMN molecule (represented as a hexagon) and eight Fe–S clusters (green squares) [48,49]. Plant Complex I contains an additional carbonic anhydrase (CA) domain, attached to the membrane arm and protruding into the matrix. The function of the CA domain, which is absent from bacterial and mammalian Complex I, remains unclear. The structure of Complex I is divided into three functional modules: the NADH-dehydrogenase N-module, the ubiquinone-binding Q-module and the proton-translocating P module. The P module is further sub-divided into the proximal section (P_P) and the distal section (P_D). Only the location of core subunits within the complex is indicated on the figure.

eukaryotic supergroups with non-photosynthetic taxa such as Chromoalveolata (*e.g. Tetrahymena* spp.) and Excavata (*e.g. Reclinomonas* spp.), γ-CAs with possible mitochondrial localization are detected in the genomes [92]. However so far, γ-CAs have not been identified in Opisthokonta (fungi and animals) [92].

The composition of plant Complex I has been established via the identification of subunits from the purified enzyme. The first attempts to characterize Complex I subunits have been made using purified enzyme from *Vicia faba* (broad bean), *Solanum tuberosum* (potato) and *Triticum aestivum* (wheat) in the 1990s [93–95]. Around 30 subunits with molecular masses ranging from 6 to 75 kDa were resolved after gel electrophoresis but only a few were identified via N-terminal sequencing or by cross-reaction with antibodies recognizing the subunits of bovine Complex I. A few years later, the Blue Native Polyacrylamide Gel Electrophoresis technique (BN-PAGE) was applied to analyze respiratory enzymes in *Arabidopsis* and *Oryza sativa* purified mitochondria [50]. Complex I was subsequently electrophoretically resolved into a second dimension under denaturing conditions leading to the separation of 38 subunits, of which 30 could be identified by tandem mass spectrometry (MS) [50]. Three dimensional (3D) electrophoresis, a technique making use of BN-PAGE followed by two dimensional (2D) electrophoresis in different denaturing conditions, was then applied to determine the composition of *Arabidopsis* Complex I [96]. Using this approach, 42 subunits could be resolved and identified by MS [61]. Finally, isolated Complex I was fractionated into subcomplexes by low-concentration of detergent and the systematic analyses of subunit composition by MS led to the identification of a total of 49 subunits [47]. Out of the 49 subunits, 14 are core and 35 are non-core subunits (Table 1). Four of the mammalian accessory subunits (NDUFB6, NDUFV3, NDUFAB1 and NDUFA10) are not detected as components of plant Complex I (Table 1). NDUFB6 and NDUFV3 in mammalian Complex I have no counterpart in *Arabidopsis* or *Yarrowia* and can be considered as lineage-specific Complex I subunits. In the case of NDUFAB1 and NDUFA10, plant orthologs are predicted from genome analysis (Table 1). Of particular note is the acyl carrier protein (NDUFAB1/ACP1/SDAP), which was initially reported to be part of potato Complex I [97] but could not be detected as an integral part of *Arabidopsis* Complex I in several proteomic approaches [47,61]. Instead, ACP1 and ACP2 are matrix-resident proteins that account for the ACP activity in the *Arabidopsis* mitochondria [98]. This is in contrast to fungi and mammals where ACP is detected in association with Complex I [64,99–101]. Structural information has revealed the presence of one ACP anchored to the hydrophilic arm by subunit NDUFA6 and a second ACP anchored by NDUFB9 in the distal end of the membrane arm in bovine and human Complexes I [22,23,102].

In plants, among the 14 non-conserved accessory subunits (Table 1), five (CA1, 2, 3 and CAL1, 2) display similarity to γ-CA and nine appear to be plant-specific. Among the five γ-CA resembling subunits, two distinct γ-CA proteins (CA1 and CA2) displaying conserved active-site regions, and two γ-CA-like proteins (CAL1 and CAL2), harboring non-conservative replacements of key residues [103] were detected in the 85 kDa CA domain [47,53]. Based on the size of the CA domain and by analogy with CA from *M. thermophila*, which is functionally active as a trimer [104], it is likely that the Complex I-integrated γ-CA domain also forms a trimeric structure. However the exact composition of the CA domain remains unclear and the detection of four proteins, CA1, 2 and CAL1, 2 suggests a heterogeneous composition for this domain. Since CA proteins are more abundant than CAL proteins in proteomic investigations, it has been proposed that the trimeric CA domain may consist of a combination of any two CA and one CAL subunits [47]. Subsequently, it has been shown that yeast two hybrid experiment does not support an interaction between CA1 and CA2. Hence, it has been postulated that the CA domain is made up of either CA1 homodimer with CAL1 or CAL2, or CA2 homodimer with CAL1 or CAL2. Since both CA1 and CA2 can be found in wild-type mitochondria [47,53], different forms of Complex I must co-exist: one with CA1 and another with CA2 [105]. A third CA protein (CA3), which occurs as two isoforms of different sizes, is a *bona fide* Complex I subunit but has not been detected within the 85 kDa CA module. Additionally, no interaction was detected between CA3 and other CA/CAL subunits via yeast two-hybrid [47]. Hence, the location of CA3 within the enzyme and its function remains unknown [47,53].

Most of the nine plant-specific subunits appear to be restricted to land plants (Table 1). Among them, two subunits (At1g67350, At2g27730) are consistently detected in independent preparations of Complex I while seven subunits (At1g68680, At5g14105, At1g18320, At3g10110, At1g72170, At2g28430 and At1g72750) are not consistently found [47,53,61,62,73].

2.2. C. reinhardtii Complex I

The subunit composition of Complex I was also established from the chlorophycean *C. reinhardtii*. Complex I was separated from the other respiratory complexes by BN-PAGE of isolated mitochondria and resolved into its constitutive subunits into a second dimension via denaturing electrophoresis [52]. Twenty-nine components with molecular masses ranging from 7 to 77 kDa were subsequently identified by MS [52]. Sixteen of the twenty-nine subunits were also identified by proteomics in a Complex I assembly intermediate lacking the distal membrane arm of the enzyme [60]. Of particular note is the presence in the algal enzyme of three CA subunits (CAG1, 2, 3) and three subunits that do not have a counterpart in the *Arabidopsis* Complex I (NUOP3, NUOP4, NUOP5). A possible exception is NUOP3 for which an ortholog is predicted from protein similarity searches (Table 1), but has not yet been detected as part of *Arabidopsis* Complex I. [59]. By querying the predicted proteome for putative orthologs of fungal and mammalian Complex I subunits, the *Chlamydomonas* enzyme was proposed to comprise at least 41 proteins [52] (Table 1). Although a putative ACP ortholog is present in *Chlamydomonas*, it is yet to be identified in Complex I through proteomic analysis. An immunoreactive species can be detected in *Chlamydomonas* mitochondria with an antibody against fungal Complex I-associated ACP but it is not known if the detected protein associates with Complex I or resides only in the matrix [52].

2.3. Euglena gracilis Complex I

Outside the plant kingdom, the subunit composition of mitochondrial Complex I has also been established in another photosynthetic eukaryote, namely *E. gracilis*, a unicellular alga belonging to Euglenozoa [106,107]. In addition to euglenids, this phylum also includes kinetoplastids like *Trypanosoma* and *Leishmania*, which have a parasitic lifestyle [106,107]. Twenty of the 38 conserved core and non-core subunits of the eukaryotic Complex I were deduced from bioinformatics analysis in *E. gracilis* [108]. In addition, 14 subunits unique to kinetoplastids were also recognized in *E. gracilis*, suggesting that these additional subunits are not associated with a parasitic lifestyle [108]. Out of the 20 conserved and 14 kinetoplastid-specific subunits, eleven and five subunits, respectively could be confirmed via proteomic analysis [108]. Of particular note is the presence of γ-CA subunits, an indication that the CA domain is also present in euglenid Complex I.

3. Plant Complex I mutants

Plant Complex I mutants have been studied in maize [109–111], *Nicotiana sylvestris* [112–115], *A. thaliana* [43,71,74,86,116–118] and *C. reinhardtii* [45,60,78–85,119–121]. Mutations causing Complex I deficiency in maize and *N. sylvestris* are spontaneous lesions resulting from mitochondrial genome rearrangements [44]. In *Arabidopsis* and *Chlamydomonas*, mutations causing Complex I deficiency were isolated by reverse genetics and forward genetic screens. The majority of the mutants described carry molecular lesions in genes encoding the Complex I core subunits [45,71,86]. A few mutants are in genes encoding

non-core subunits [74,86,116] and Complex I assembly factors [117, 122]. In *Arabidopsis*, a plethora of nuclear mutants display a Complex I defect because they are specifically affected for one or several mitochondrial transcripts encoding Complex I subunits. Such mutants, referred to as surrogate mutants, map to genes encoding proteins controlling transcription of mitochondrial Complex I genes or splicing, processing and editing of the corresponding transcripts [reviewed in Refs. []].

Plants are attractive experimental systems to study Complex I because complete loss of Complex I is viable, presumably due to the

Table 1
Subunits of Complex I in *Arabidopsis thaliana* and *Chlamydomonas reinhardtii*.

A. thaliana		*C. reinhardtii*		*H. sapiens*	Localization	Module	Cofactor (s)
Name	Accession number	Name	Accession number				
Core subunits							
51 kDa	At5g08530[A]	NUO6	Cre10.g422600	NDUFV1	matrix	N	FMN, N3
24 kDa	At4g02580[A]	NUO5	Cre10.g450400[J]	NDUFV2	matrix	N	N1a
75 kDa	At5g37510[A]	NUOS1	Cre12.g535950	NDUFS1	matrix	N	N1b, N4, N5
TYKY/23 kDa	At1g16700, At1g79010	NUO8	Cre12.g496750	NDUFS8	matrix	Q	N6a, N6b
PSST/20 kDa	At5g11770[A]	NUO10	Cre12.g492300	NDUFS7	matrix	Q	N2
Nad9[m]/30 kDa	AtMg00070	NUO9/ND9	Cre07.g327400[K,L]	NDUFS3	matrix	Q	
Nad7[m]/49 kDa	AtMg00510	NUO7/ND7	Cre09.g405850[K]	NDUFS2	matrix	Q	
Nad1[m]	AtMg00516/AtMg01120/AtMg01275	ND1[m,*]	AAB93446[N]	ND1[m]	mb	P_P	
Nad2[m]	AtMg00285/AtMg01320	ND2[m,*]	AAB93444	ND2[m]	mb	P_P	
Nad3[m]	AtMg00990	ND3/NUO3	Cre08.g378900[O]	ND3[m]	mb	P_P	
Nad4L[m]	AtMg00650	NUO11/ND4L*	Cre09.g402552[O]	ND4L[m]	mb	P_P	
Nad4[m]	AtMg00580	ND4[m,*]	AAB93441[Q,P]	ND4[m]	mb	P_D	
Nad5[m]	AtMg00513/AtMg0060/AtMg00665	ND5[m,*]	AAB93442[R,S]	ND5[m]	mb	P_D	
Nad6[m]	AtMg00270	ND6[m,*]	AAB93445[S]	ND6[m]	mb	P_P	
Conserved non-core subunits							
18 kDa	At5g67590[A,B,C,D,U]	NUOS4	Cre03.g146247	NDUFS4	matrix	N	
13 kDa	At3g03070	NUOS6	Cre03.g178250	NDUFS6	matrix	N	
MWFE	At3g08610[A,B,U]	NUOA1*	Cre10.g459750	NDUFA1	mb	P_P	
B8	At5g47890	NUOB8	Cre16.g679500	NDUFA2	matrix	N	
DAP13/B17.2	At3g03100	NUO13	Cre11.g467767	NDUFA12	matrix	N	
SGDH	At1g67785	–	–	NDUFB5	matrix	N	
B13	At5g52840	NUOB13	Cre13.g568800	NDUFA5	matrix	Q	
B14.5a	At5g08060	Cre12.g484700*	Cre12.g484700*	NDUFA7	matrix	N/Q	
39 kDa	At2g20360[A,B]	NUOA9	Cre10.g434450[L]	NDUFA9	matrix	Q	
ATDNK	At1g72040*	DNK1**	Cre02.g095350**	NDUFA10	?	Q/P_P	
B9	At2g46540	Cre12.g537050*	Cre12.g537050*	NDUFA3	mb	P_P	
B14	At3g12260	NUOB14	Cre12.g555250	NDUFA6	mb	P_P	
PGIV	At5g18800, At3g06310	NUOA8*	Cre07.g333900	NDUFA8	mb peripheral	P_P	
B14.7	At2g42210[C]	TIM17/TIM22	Cre14.g617826	NDUFA11	mb	P_D	
B16.6/GRIM-19	At1g04630, At2g33220	NUOB16	Cre16.g664600	NDUFA13	mb	P_P	
20.9 kDa/MNLL	At4g16450	NUO21	Cre06.g267200	NDUFB1	mb	P_D	
AGGG	At1g76200	–	–	NDUFB2	mb	P_D	
B12	At2g02510, At1g14450[B]	NUOB12*	Cre05.g244850	NDUFB3	mb	P_D	
NDU8/B15	At2g31490	NUOB4	Cre03.g204650	NDUFB4	mb	P_D	
B18	At2g02050	NUOB18	Cre06.g278188	NDUFB7	mb	P_D	
ASHI	At5g47570	TEF29*	Cre01.g007850	NDUFB8	mb	P_D	
B22	At4g34700	NUOB22*	Cre11.g467668	NDUFB9	mb	P_D	
PDSW	At1g49140, At3g18410	NUOB10	Cre12.g555150[T]	NDUFB10	mb peripheral	P_D	
NDU12/ESSS	At3g57785, At2g42310	NUO17	Cre05.g240800	NDUFB11	mb	P_D	
NDU10/KFYI	At4g00585	Cre17.g725400*	Cre17.g725400*	NDUFC1	mb peripheral	P_D	
NDU9/B14.5b	At4g20150	NUOP1	Cre13.g571150	NDUFC2	mb	P_D	
15 kDa	At3g62790, At2g47690	NUOS5*	Cre12.g511200	NDUFS5	mb	P_D	
–	–	ACP1*	Cre16.g673109*	NDUFAB1	?	Q and P_D	
Non-conserved non-core subunits							
CA1	At1g19580[E]			–	mb peripheral		
CA2	At1g47260[A,B,E,F,G,H]		Cre12.g516450,	–	mb peripheral		
CA3	At5g66510[E,F]	CAG1, CAG2, CAG3	Cre06.g293850,	–	mb peripheral		
CAL1	At5g63510[E,H,I]		Cre09.g415850	–	mb peripheral		
CAL2	At3g48680[B,E,H,I]			–	mb peripheral		
P1/11 kDa	At1g67350	–	–	–	mb		
P2/16 kDa	At2g27730	–	–	–	mb		
TIM22	At1g18320	TIM22A**	Cre01.g021050**		mb		
At3g07480*	At3g07480*	NUOP3	Cre02.g100200	–	?		
	–	NUOP4	Cre08.g378550[L]	–	?		
	–	NUOP5	Cre08.g378050	–	?		[P]
Candidate plant-specific subunits							
P3	At5g14105	–	–	–	mb		
unknown protein	At1g68680	–	–	–	Likely mb		
TIM22	At3g10110	TIM22A**	Cre01.g021050**	NP_037469.2**	?		
DUF543	At1g72170	–	–	–	?		
unknown protein	At2g28430	–	–	–	?		
TIM23-2	At1g72750[C]	TIM23**	Cre10.g434250**	–	?		

operation of metabolic by-pass for Complex I [124,125]. This is in contrast to animals where the absence of Complex I leads to premature death because there is no natural metabolic by-pass for lack of Complex I [126,127], with the possible exception of five animal species [42]. Plant mitochondria contain bypasses of Complex I in the form of type II NADH and NADPH dehydrogenases, which are located on both sides of the inner membrane [39]. Plant type II dehydrogenases are monomeric, non-proton pumping enzymes that deliver electrons to ubiquinone from the matrix or from the intermembrane space NAD(P)H pools [39]. In the sections below, we describe the different Complex I deficient mutants in plants, the phenotypic and physiological consequences due to loss of Complex I and the impact on enzyme assembly and activity.

3.1. Complex I mutants in genes encoding core and conserved non-core subunits

3.1.1. Mutants in vascular plants

3.1.1.1. Mutants in N. sylvestris and maize. The first plant Complex I mutants described are mitochondrial mutants in maize and *N. sylvestris* lacking one of the Complex I-subunit encoding genes (*nad*) as a result of mitochondrial genome rearrangements [44]. In vascular plants, seven Complex I subunits (Nad1, 2, 3, 4, 4L, 5 and 6) localized in the P module and two subunits (Nad9, Nad7) in the Q module of the enzyme are mitochondrially encoded [43] (Fig. 1). In *N. sylvestris*, the cytoplasmic male sterile II (CMSII) mutant has no detectable Complex I due to a deletion in the mitochondrial *nad7* gene encoding the Nad7 Complex I subunit [112,113,115]. The CMSII mutant is characterized by a delay in seed germination, development and a light-dependent male sterile phenotype. Loss of Complex I results in a decrease in the efficiency of photosynthetic electron transfer, highlighting the importance of mitochondrial function in controlling photosynthesis [128,129]. Metabolic studies revealed an alteration of nitrogen metabolism characterized by augmented pools of nitrogen-rich amino acids, an indication that nitrate assimilation is stimulated in the absence of Complex I [130]. The expression of many stress-related genes is up-regulated resulting in increased resistance to a variety of biotic and abiotic stresses [131].

In maize, the non-chromosomal stripe II (NCSII) mutant is deficient for Complex I, but assembly intermediates of the enzyme are detected [132]. This defect is caused by a deletion at the 3′ end of the mitochondrial *nad4* gene [110]. Leaf morphology is abnormal and kernel development is defective [133]. Specifically, the NCSII mutant displays striped sectors of pale-green tissues on the leaves, a phenotype attributed to the impact of Complex I defect on chloroplast function [133]. This phenotype is also observed in several other mitochondrial mutants including the NCS6 mutant deficient for Complex IV, suggesting that mitochondrial dysfunction causes pleiotropic effects on chloroplast biogenesis [44]. In the NCSII mutant, the chloroplast ultrastructure is abnormal, CO_2 fixation is decreased and photosynthetic electron transfer is impaired due to defect in the accumulation of Photosystem I (PSI) [134]. The NCS6 mutant also showed a decrease in PSI accumulation while other photosynthetic complexes were unaffected, further underscoring the long-range impact of respiratory deficiency on thylakoid membrane biogenesis [135].

3.1.1.2. Mutants in Arabidopsis. In *Arabidopsis*, the phenotypic consequence due to loss of Complex I was documented in nuclear mutants affected for the core subunits NDUFV1, NDUFV2, NDUFS1, and NDUFS7 and the non-core subunits NDUFS4, NDUFA1, NDUFA9, and NDUFB3 [51,71,86,122] (Table 1, human nomenclature for Complex I subunits). Unlike *N. sylvestris* and maize, *Arabidopsis* mutants lacking Complex I are not male sterile [58]. However, they are characterized by abnormally shaped leaves and a delay in growth and development at all stages of the plant life cycle [51,71,73]. The severity of these phenotypes appears to correlate with the amount of fully assembled Complex I in the mutant lines. Mutants with complete lack of Complex I display the most severe phenotype where the seedlings are arrested at the cotyledon stage, an indication that transition to photoautotrophic growth is compromised [71].This process, also referred to as seedling establishment, is dependent upon the provision of energy from the mobilization of stored reserves in the seed. Because this block can be alleviated by exogenous sucrose, it is likely that loss of Complex I yields seeds with reduced reserves [51,71].

Detailed comparative study was conducted on two *Arabidopsis* knockout mutants for *NDUFV1* and *NDUFS4*. While no holoenzyme accumulates in the absence of NDUFV1 subunit, trace amounts of active Complex I are detected in the absence of NDUFS4 [71]. In addition, *ndufv1* and *ndufs4* accumulate four membrane arm assembly intermediates containing the CA domain (200 kDa, 400 kDa, 450 kDa and 650 kDa subcomplexes) (see also Fig. 2 and Section 4.1.1 for a description of the assembly intermediates). Specifically, these mutants are defective in the assembly of the matrix arm and cannot form the 850 kDa subcomplex and the mature 1 MDa holoenzyme. Such a phenotype is comparable to mammalian Complex I assembly, where *NDUFS4* knockout mutants accumulate a membrane-bound subcomplex lacking a 200 kDa matrix-arm subcomplex [136].

The physiological effects linked to Complex I deficiency in *Arabidopsis* have been further investigated in detail by comparing the *ndufv1* and *ndufs4* mutants [71]. The traces of Complex I retained in the *ndufs4* mutant do not lead to an increased capacity of mitochondrial respiration and oxidative phosphorylation, compared to *ndufv1*. The main changes observed concern fluxes through glycolysis and the

Notes to Table 1:

- Complex I subunits are listed based on proteomic and bioinformatic analyses conducted by Heazlewood et al. (2003) [50], Klodmann et al. (2010) [47], Meyer et al. (2011) [51], Cardol et al. (2004) [52], Cardol P. (2011) [15], Salinas et al. (2014) [45] and Peters et al. (2013) [53].
- Accession numbers for *Arabidopsis* proteins are provided as in TAIR10 (www.arabidopsis.org). Accession numbers for *Chlamydomonas* nuclear-encoded proteins are provided as their locus name from the *Chlamydomonas* genome database JGI v5.5 and as NCBI reference sequence ID for mitochondrially-encoded proteins. Accession numbers for human proteins can be found in Hirst et al. (2003) and Caroll et al. (2006) [54,55].
- [m:] indicates mitochondrially-encoded core subunits. Multiple accession numbers are provided for *Arabidopsis* Nad1, Nad2 and Nad5 because they are encoded as gene fragments whose transcripts are trans-spliced to generate the mature transcript encoding the full-length protein [56–58].
- Plant orthologs of mammalian subunits, yet to be confirmed as part of Complex I by proteomic analyses [52,53,59,60], are indicated by a single asterisk symbol (*). Candidate orthologs newly reported in this study, identified via similarity searches using NCBI BLAST (http://blast.ncbi.nlm.nih.gov/), TAIR BLAST (https://www.arabidopsis.org/Blast/) and Phytozome BLAST (http://phytozome.jgi.doe.gov/) with default parameters, are indicated by a double asterisk (**).
- NDUFB6 (B17) and NDUFV3 (10 kDa) are two mammalian-specific subunits for which no orthologs have been found in plants.
- Six candidate plant-specific subunits are shown [53]. Although these subunits have been identified in *Arabidopsis* Complex I [47,61–63], they are not consistently detected in every study.
- Localization of subunits in the membrane or matrix arm of Complex I is provided based on previous topological studies from various model systems: matrix, matrix; mb, membrane-associated; mb peripheral, membrane-associated, but lacking predicted transmembrane domains [15,25,47,51,53,61,64–66]. The localization of a few subunits, marked as "?", remains uncertain due to lack of biochemical data or conflicting experimental data.
- Complex I can also be classified into three functional modules: the NADH dehydrogenase N-module, the quinone-binding Q-module and the proton-translocating P-module. The subunits have been tentatively assigned to each of these modules based on previous analyses in various model systems [1,19,23,47,66–69].
- Mitochondrial Complex I consists of eight Fe–S clusters, wherein N1a and N1b are binuclear (2Fe–2S) clusters and, N2, N3, N4, N5, N6a and N6b are tetranuclear (4Fe–4S) clusters [8,48,70].
- The Complex I subunits for which *Arabidopsis* or *Chlamydomonas* mutants have been characterized are represented as: ([A]) [71], ([B]) [51], ([C]) [63], ([D]) [72], ([E]) [73], ([F]) [74], ([G]) [75], ([H]) [76], ([I]) [77], ([J]) Subrahmanian et al., unpublished, ([K]) [78], ([L]) [79], ([N]) [80], ([O]) [81], ([P]) [82], ([Q]) [83], ([R]) [84], ([S]) [60], ([T]) [85], ([U]) [86].

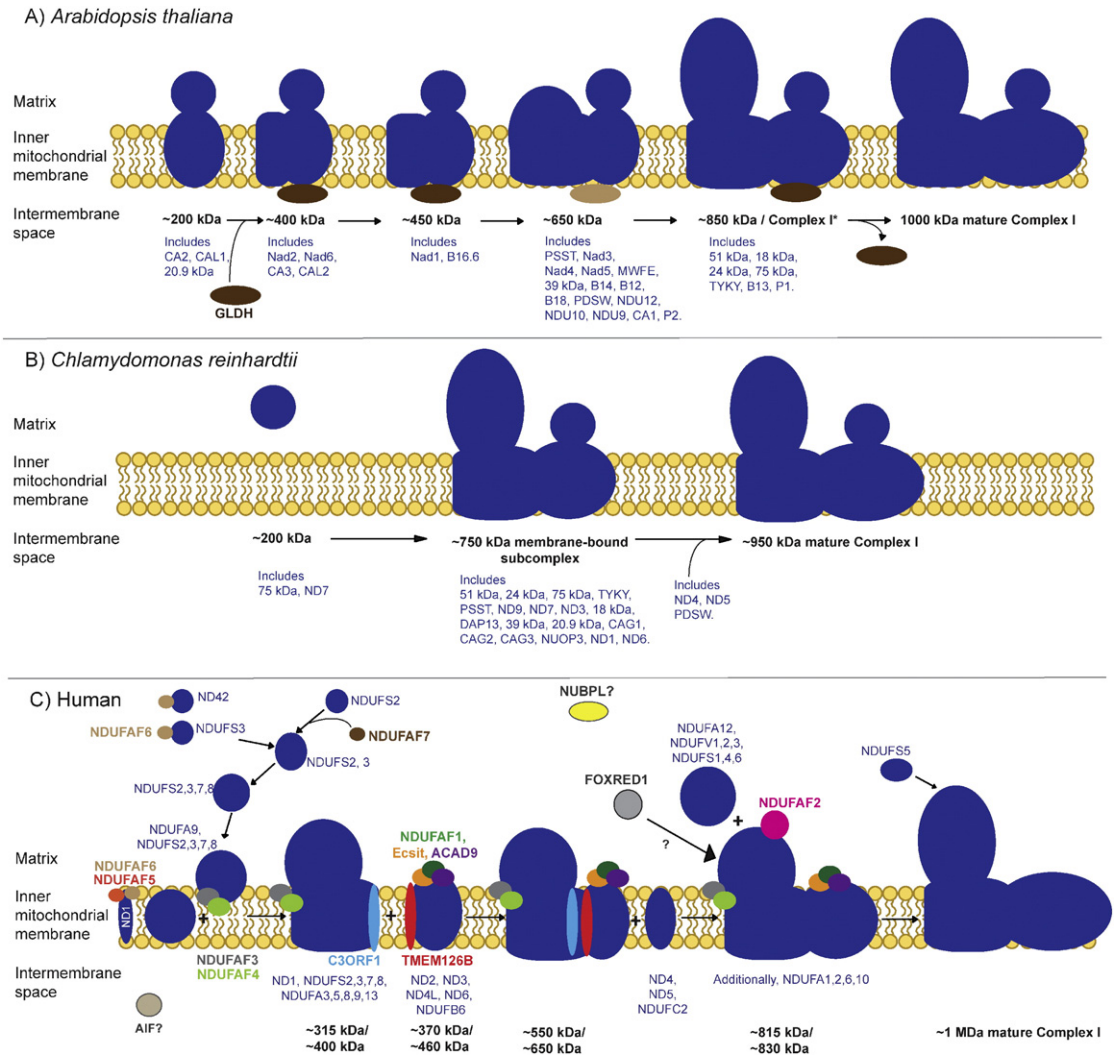

Fig. 2. Proposed model for mitochondrial Complex I assembly. The Complex I assembly intermediates are shaped in dark blue and their names are indicated below each complex. Each assembly intermediate is named according to its size. A working model for Complex I assembly is provided based on the study of assembly intermediates in wild-type and Complex I mutant strains. The minimum composition of each plant subcomplex has been listed according to proteomic investigations in *Arabidopsis* [50,51,96,144,149] and *Chlamydomonas* [52,60,84]. A) In *Arabidopsis*, the 400 kDa and 450 kDa subcomplexes are also referred to as 420 kDa and 470 kDa respectively [149]. The 850 kDa subcomplex (Complex I*) contains all the expected subunits of the peripheral arm and is proposed to form the 1000 kDa mature Complex I by the addition of membrane-bound subunits [149]. It is hypothesized that GLDH (brown oval) is an assembly factor that associates with the 400 kDa, 450 kDa, and 850 kDa subcomplexes in wild-type *Arabidopsis* [143,149,150]. However, it is not detected in the 650 kDa subcomplex that accumulates due to loss of the 18 kDa subunit [143] and hence, is expected to be released upon over-accumulation of the 650 kDa subcomplex. B) In wild-type and *nd5* mutant *Chlamydomonas* mitochondria, a 200 kDa subcomplex is detected. Immunoblotting analyses revealed the presence of the 75 kDa and ND7 matrix arm subunits in this assembly intermediate [60,84]. The *Chlamydomonas* ND1 and ND6 membrane subunits were not successfully detected by MS analysis in the 700 kDa subcomplex [60]. However, the loss of these subunits results in a failure to form the 700 kDa subcomplex [80,84], thereby indicating that ND1 and ND6 are essential for the formation of this assembly intermediate. Similarly, the loss of ND4, ND5 or PDSW subunits in *Chlamydomonas* results in the loss of a mature complex and instead causes the accumulation of the 700 kDa subcomplex [60,82,85]. Hence, ND4, ND5 and PDSW are required for the assembly of the distal membrane arm of Complex I. C) In humans, fourteen assembly factors have been identified to date for Complex I [19,144,151–154]. A simplified scheme of human Complex I assembly is shown. Subunits are indicated in blue font and assembly factors in colored font. The different subcomplex intermediates are represented in dark blue. NDUFS2 is dimethylated by the methylase NDUFAF7. ND42, NDUFS3 and ND1 are stabilized by NDUFAF6. In addition to NDUFAF6, ND1 assembly is also dependent upon NDUFAF5. Hydrophillic subunits including NDUFS2, 3, 7, 8 and hydrophobic subunits including ND1 are assembled together with the help of C3ORF1, NDUFAF3 and NDUFAF4 to form the membrane bound ~400 kDa subcomplex. Membrane bound ~460 kDa subcomplex is independently assembled with the help of TMEM126B, NDUFAF1, ACAD9 and Ecsit factors. The ~400 kDa and ~460 kDa subcomplexes combine to form the ~650 kDa assembly intermediate. The ~830 kDa intermediate is formed after subunits such as ND4 and ND5 are added to the ~650 kDa subcomplex. Soluble subunits are then added, with the help of NDUFAF2, to form the final holoenzyme. While FOXRED1 is required for the stable accumulation of the ~850 kDa subcomplex, its precise role in Complex I assembly is unknown [155]. Similarly, the exact mechanism of action for AIF and NUBPL in this assembly sequence also remains undetermined.

tricarboxylic acid cycle (TCA), which are dramatically increased in the *ndufv1* mutant compared to the *ndufs4* mutant, although the enzyme levels are similar. This suggests that complete lack of Complex I is responsible for a metabolic switch resulting in an up-regulation of the activities of glycolysis and TCA cycle that does not occur when traces of Complex I are present in the *ndufs4* mutant.

3.1.2. Mutants in the green alga Chlamydomonas

In *Chlamydomonas*, mutants deficient for Complex I display a slow growth phenotype in heterotrophic conditions. This trait was used to isolate several mitochondrial and nuclear mutants specifically deficient for Complex I activity/assembly [45,78,79]. The phenotypic consequences due to loss of *Chlamydomonas* Complex I are less severe than in vascular plants and do not cause sterility [85]. Although mitochondrial respiration and growth rate are affected, mutants defective solely for Complex I activity are the least affected with respect to their ATP content and photosynthetic electron transfer chain, compared to other respiratory-deficient mutants [137]. With respect to photosynthesis, Complex I deficient mutants display increased cyclic electron transfer around PSI, an adaptation considered to favor ATP production over CO_2 assimilation [137].

Several mutations in the mitochondrial genes encoding ND1, ND4, ND5 and ND6 subunits have been isolated and their impact on Complex I assembly and activity was analyzed in detail [45]. The lack of ND1 or ND6 leads to complete loss of the holoenzyme and Complex I activity [80,84] while loss of ND4 or ND5 yields the accumulation of a 700 kDa subcomplex, deprived of a 250 kDa membrane domain corresponding to the distal end of the membrane arm [60,81,84] (see also Section 4.1.2 for a description of the assembly intermediates). Studies have also been conducted on mutants carrying a single nucleotide deletions in the 3′UTR of *nd5*, a point mutation in *nd4* and an in-frame deletion in *nd1*. A mature enzyme is assembled in these mutants, in contrast to assembly defects due to the complete loss of the corresponding subunits. However the level of Complex I assembly and activity is dependent upon the type and the effect of the mutation [83,84]. For instance, a L157P change in ND4 has no apparent impact on the levels of assembled enzyme, although the activity is severely decreased [83]. On the other hand, a single-base deletion in the 3′UTR of the *nd5* gene results in a significant decrease of *nd5* transcript levels that yields only 17% of active mature Complex I compared to wild-type [84].

Comparative proteome analysis of an *nd6*-null mutant with the corresponding wild-type strain revealed no major changes at the level of proteins involved in oxidative phosphorylation. In contrast, the major Reactive Oxygen Species (ROS) scavenging enzymes were strongly down-regulated in the mutant. Concomitantly, lower superoxide production was observed in the mutant. Thus, the loss of Complex I, which is a major source of ROS, limits ROS production and triggers the down-regulation of ROS-scavenging enzymes [78]. Similarly, a diminished ROS production was also reported in the *nad7* mutant in *N. sylvestris* [131]. On the other hand, a point mutation in the *Arabidopsis ndufs4* gene, encoding the 18 kDa subunit, results in increased ROS. However, in an alternate *ndufs4* insertional mutant, lower levels of hydrogen peroxide accompanied by increased tolerance to salt, cold and osmotic stresses were observed [86]. The reason for this discrepancy remains unclear and the contribution of Complex I to ROS production and adaptation to stress is yet to be elaborated.

In addition, genetic studies on nucleus-encoded subunits have also been conducted. For instance, the nucleus-encoded ND3, ND4L, ND7 and ND9 subunits, which are encoded in the mitochondrial genome in land plants, have been inactivated via RNA interference. As expected from their location in the module connecting the hydrophilic arm to the membrane arm, depletion of ND3 or ND4L leads to loss of Complex I assembly [81]. In the same way, depletion of ND7 or ND9 also leads to loss of mature Complex I [78]. These subunits are located in the peripheral arm (Fig. 1) and although they do not bind any cofactors (FMN or Fe–S clusters), their presence is essential for Complex I assembly, as also demonstrated in *Neurospora crassa*, *Escherichia coli* and *N. sylvestris* [112,115,138–141]. While the loss of ND9 affects Complex I assembly, a mutation in the *ND9* promoter only affects the level of the corresponding transcript, allowing the accumulation, in decreased levels, of the mature enzyme [78,79].

Five nuclear mutants deficient for Complex I were isolated via insertional mutagenesis [85], out of which three are knockout mutants and will be discussed here. First, a *nuob10*-null mutant revealed that loss of the PDSW subunit results in the accumulation of a 700 kDa subcomplex [85]. According to structural characterization of bovine Complex I, this conserved non-core subunit localizes to the distal membrane arm facing the intermembrane space (IMS) [22]. One proposed assignment for PDSW is that it spans the length of ND4 and ND5 on the IMS side. Based on this assignment, it is possible that PDSW functions in stabilizing ND4 and ND5 in the distal membrane arm of Complex I. Hence, the loss of PDSW is characterized by the accumulation of a 700 kDa subcomplex, similar to mitochondrial mutants lacking ND4 or ND5 [81,82,84,85].

Insertional mutation in the *NUO5* gene encoding the 24 kDa core subunit results in the loss of Complex I assembly. Since the 700 kDa membrane-bound subcomplex is not detected in this mutant, it can be inferred that the assembly of this Fe–S cluster containing subunit occurs in the early stages of Complex I assembly in *Chlamydomonas* (N. Subrahmanian et al., unpublished). Similarly, an insertional mutation in the *NUOA9* gene, encoding the 39 kDa subunit of the matrix arm, results in lack of mature Complex I [79]. The lack of membrane-bound assembly intermediates upon loss of these matrix arm subunits suggests that the 24 kDa and 39 kDa subunits are part of the early stage of Complex I assembly process in *Chlamydomonas*.

3.2. Complex I mutants in the genes encoding for non-conserved accessory subunits

Among the non-conserved subunits, the Complex I-associated CAs in the CA domain have been studied in detail and their function remains the subject of considerable debate [103,142]. Although the CA and CAL subunits are detected in multiple assembly intermediates of the membrane arm [77,143], *Arabidopsis* single knockout mutants for *CA1*, *CA2*, *CA3*, *CAL1* and *CAL2* are not arrested for Complex I assembly and cannot be distinguished from wild-type with respect to growth and development [73,74]. However, phenotypic differences such as decreased growth rate and mitochondrial respiration could be observed in cell cultures of the *ca2* knockout mutant. CA2 is imported into wild-type mitochondria to first form the 200 kDa assembly intermediate that exhibits a high turnover rate [145]. Subsequently, CA2 is present in all membrane-arm assembly intermediates of higher molecular weight [51,143]. The loss of the CA2 subunit resulted in an 80% decrease in the level of assembled Complex I [74]. In addition, no assembly intermediates were detected in the *ca2* mutant, which confirms a role for CA2 in the stable accumulation of the subcomplexes [51,144]. In contrast to the *ca2* mutant, the single *ca3* mutant displays a less drastic Complex I defect [74] and the *cal2* mutant accumulates wild-type levels of mature holoenzyme [51].

Since single knockout mutants did not exhibit significant morphological differences, overproduction of these subunits was attempted as a means of elucidating their function. While overexpression of *CAL1* and *CAL2* caused no apparent phenotypic alterations [73], overexpression of *CA2* causes a defect in anther dehiscence resulting in male sterility [145]. Interestingly, the overexpressing *CA2* transgenic line also exhibits rotenone-insensitive mitochondrial oxygen consumption, similar to the *ca2* knockout mutant, implying a defect in Complex I activity [145]. However, NADH dehydrogenase activity and Complex I assembly were not directly assessed in these *CA2* overexpressing lines.

The lack of morphological alterations and the presence of mature Complex I in single *ca* or *cal* mutants could be due to the redundant functions of the CA and CAL subunits. Indeed, single particle EM imaging shows that the CA spherical domain is still present in Complex I of the *ca2* single mutant [75]. In order to dissect the role of the CA domain in Complex I, double mutants were generated. Homozygous *ca1ca2* double mutants exhibited delayed embryogenesis and germination, and are unable to survive. In addition, the *ca1ca2* embryos also displayed increased levels of ROS, a phenotype which may account for seedling lethality. Although level of Complex I assembly was not assessed in these double mutants, knockdown of both *CA1* and *CA2* caused a drastic reduction in assembled Complex I, compared to the single *ca2* mutant [105].

Similarly, *cal1cal2* double mutants could not be isolated due to delayed embryogenesis and a failure to germinate, indicating that CAL1 and CAL2 are also important for plant development. Hence, *cal2* knockdown lines were generated to facilitate functional analysis. The Δ*cal1*/*cal2i* lines, carrying a *cal1* knockout and a *cal2* knockdown, displayed delayed germination, retarded growth, smaller rosette size and hypocotyls, delayed flowering and increased anthocyanin accumulation [73,77]. These lines displayed a 90–95% decrease in assembled Complex I. The residual Complex I displays only 30% NADH dehydrogenase activity compared to wild-type [77]. No assembly intermediates were detected, and the decrease in Complex I assembly was reflected in the

low abundance of several Complex I subunits, emphasizing the requirement of the CAL subunits for Complex I assembly.

While the enzymatic activity of the Complex I-integrated CA proteins could not be experimentally demonstrated [73,146], support for a possible carbonic anhydrase activity comes from the finding that a recombinant homotrimeric form of CA2 can bind CO_2/HCO_3^- [147]. Because the CA domain is not found in animal and fungal Complexes I, this domain was postulated to participate in CO_2 recycling between mitochondria and chloroplast, a process that is unique to phototrophic eukaryotes. A direct link between CO_2 recycling and Complex I-associated CA domain was observed in the double mutants Δ*ca2*Δ*cal1* and Δ*ca2cal2i*. These double mutants displayed significantly retarded growth and smaller organ development, although their mitochondrial respiration rate and Complex I defects were similar to that of the single *ca2* mutant [76]. These growth phenotypes could be completely rescued in high CO_2 atmospheres, an indication that these double mutants suffer from impaired carbon assimilation [76]. This phenotype was attributed to inefficient CO_2 cycling between mitochondria and chloroplast, under normal atmospheric conditions. When RuBisCO binds to O_2 instead of CO_2, this leads to the formation of a 2-phosphoglycolate, a toxic product which is eliminated by the operation of the photorespiratory pathway (photorespiration). Photorespiration is defined as the light-dependent consumption of O_2 and production of CO_2 [148]. In photorespiration, 2-phosphoglycolate formed in the chloroplast is catabolized to glycine in the peroxisome and decarboxylated to serine with release of CO_2 in the mitochondria [148]. Some of this CO_2 is recycled by conversion to HCO_3^- and transported to the chloroplast for efficient carbon assimilation. Hence photorespiration can be viewed as a way to recapture carbon potentially lost due to the oxygenase activity of RuBisCO. A defective CO_2 recycling mechanism can be rescued in the presence of excessive atmospheric CO_2, a condition that favors the carboxylase reaction of RuBisCO [76]. If the Complex I-associated CA domain is a component of the photorespiratory pathway, it is expected that loss of CA/CAL impacts the metabolic intermediates in this pathway. In the Δ*ca2*Δ*cal1* and Δ*ca2cal2i* double mutants, an increase in glycine was observed whereas a decrease in glycine/serine ratio was documented in Δ*cal1*/*cal2i* lines, both implying an imbalance in photorespiration [76,77]. These results support the implication of Complex I-associated CAs in photorespiration and underscore the importance of mitochondrial function for efficient carbon fixation.

4. Plant Complex I assembly

The assembly of Complex I is an intricate process due to the large number and dual genetic origin of the subunits. Moreover, the presence of prosthetic groups (one FMN and eight Fe–S clusters) adds another level of complexity in this process. Complex I assembly involves the sequential addition of hydrophobic and hydrophilic modules, initially formed independently by recruitment of subunits, and then combined to yield the final holoenzyme [151,164]. Although mammals and fungi have been the focus of study for Complex I assembly, studies in *Arabidopsis* and *Chlamydomonas* have provided insight into the sequence of assembly in plants [19,51]. Similar to mammalian Complex I, an assembly model for the plant enzyme was inferred from the identification of subcomplexes defining assembly intermediates (Fig. 2).

4.1. Stages of assembly

4.1.1. Assembly intermediates in A. thaliana

In *Arabidopsis* five membrane-bound subcomplexes with molecular masses of 200 kDa, 400 kDa, 450 kDa, 650 kDa and 850 kDa are detected in wild-type mitochondria. While the 850 kDa subcomplex is well detected, the steady-state level of the other subcomplexes is very low [50,51,71,144,150]. The subcomplexes are believed to be intermediates defining a sequence for holoenzyme assembly (Fig. 2). However this model is incomplete because the sequence of the matrix-arm assembly still remains unknown as no assembly intermediates have been identified to date.

The 200 kDa, 400 kDa, 450 kDa, 650 kDa subcomplexes are predominantly composed of membrane arm subunits [51]. In contrast, the 850 kDa subcomplex likely comprises all the matrix arm subunits evidenced by the same size of the peripheral arm in the 1 MDa and 850 kDa complexes. Conceivably, the 1 MDa holoenzyme is formed by the addition of hydrophobic subunits to the 850 kDa intermediate at the distal end of the membrane arm [149].

Some mutants blocked for Complex I assembly are characterized by the accumulation of several subcomplexes. Using such mutants, the entry point of certain subunits in the assembly process was deduced via immunodetection in the various intermediates [51]. The CA2 subunit is required at the early stages of membrane arm assembly as evidenced by its incorporation into the 200 kDa subcomplex and its detection in subsequent 400 kDa, 450 kDa, 650 kDa and 850 kDa intermediates [51,144]. The Nad6 membrane subunit is detected from the 400 kDa subcomplex onwards [51]. The MWFE, PSST and NDU9 subunits are only detected in the 650 kDa and 850 kDa membrane-bound intermediates [51,71]. Because the 650 kDa subcomplex is detected in the absence of the matrix arm 18 kDa or 51 kDa subunits [51,71,143], this further corroborates that the majority of the peripheral subunits are incorporated only in the 850 kDa subcomplex. Additional information about the partial composition of the subcomplexes was provided by proteomic analyses. For instance, CA2, CAL1 and 20.9 kDa are incorporated into the 200 kDa subcomplex; CA2, CA3, CAL2 and Nad2 are found in the 400 kDa subcomplex; CA2, CAL2 and GRIM-19 in the 450 kDa subcomplex; 24 subunits in the 650 kDa subcomplex [51,144]; and 13 subunits in the 850 kDa subcomplex [51,143,144,149] (Fig. 2).

4.1.2. Assembly intermediates in C. reinhardtii

In *Chlamydomonas*, the proposed sequence for holoenzyme assembly is comparatively simple because only two subcomplexes, the 200 kDa subcomplex and the 700 kDa subcomplex, have been detected so far (Fig. 2).

The 200 kDa intermediate is a soluble subcomplex that displays NADH dehydrogenase activity [60,81,84]. Since the peripheral subunits ND7 and 75 kDa are detected in this subcomplex, it was considered to be an intermediate for matrix arm assembly [52]. The 200 kDa subcomplex is detected in wild-type mitochondria in addition to the 950 kDa mature enzyme [52,60,81]. It is also the only subcomplex detected upon depletion of the membrane subunits ND3 and ND4L and it co-occurs with the 700 kDa subcomplex in the *nd5* mutant [60,81]. Therefore, it is likely that the 200 kDa subcomplex is formed at the very early stages of Complex I assembly and that a pool is always maintained alongside assembly intermediates and the mature holoenzyme.

The 700 kDa subcomplex, which also bears NADH dehydrogenase activity, is detected only when assembly is blocked by the lack of distal arm subunits such as ND4, ND5 and PDSW [81,84,85,165]. In an *nd5*-null mutant, the 700 kDa subcomplex was shown to contain subunits from the matrix arm and the proximal membrane arm, but no subunits from the distal membrane arm [60]. The 700 kDa subcomplex is only attached to the inner membrane by a domain connecting the hydrophilic arm to the proximal end of the membrane arm. The 950 kDa holoenzyme is generated after the incorporation of subunits to the 750 kDa subcomplex, at the distal end of the membrane arm.

4.2. Assembly factors

It is now well acknowledged that the biogenesis of Complex I is assisted by numerous factors recruited to promote assembly of the enzyme into an active form. While Complex I assembly factors may be associated with assembly intermediates, they do not form a part of the final holoenzyme. In 1998, the first factors CIA30 and CIA84, associated with a Complex I assembly intermediate, were discovered in a

Neurospora mutant deficient for the formation of the membrane arm of the enzyme [166]. Subsequently, a patient with a mutation in a candidate assembly factor (B17.2L) was first reported in 2005. This study stimulated a quest for additional assembly factors in Complex I-deficient patients [167]. In recent years, several Complex I assembly factors have been discovered using different approaches such as biochemical identification in assembly intermediates, candidate gene approach via subtractive phylogenetic analysis and mapping of mutations via linkage analysis in families of patients with Complex I defects [167–170]. Fourteen assembly factors have been identified for mammalian Complex I: NDUFAF1–7, C3ORF1, INDH, TMEM126B, Ecsit, ACAD9, FOXRED1 and AIF (Table 2 and Fig. 2). All the Complex I assembly factors identified so far are proteins controlling post-translational steps of the enzyme biogenesis. Although the role of these factors in Complex I assembly has been experimentally validated, determination of their biochemical activity in the assembly process has proved challenging despite the presence of domains speaking to function in some assembly factors. Examples of factors for which the mechanism of action remains elusive include the methyltransferase domain containing NDUFAF5, which is necessary for early stages of ND1 assembly and NDUFAF1, Ecsit, ACAD9, NDUFAF3, NDUFAF4, C3ORF1 and TMEM126B, which are associated with various membrane-arm intermediates [19,67,152,153,164,166,168,171–178]. On the other hand recent studies have uncovered the activity and possible function of NDUFAF7 and NDUFAF6 in the assembly process.

NDUFAF7 localizes to the mitochondrial matrix [179] and is required for the stability of the ~400 kDa Complex I assembly intermediate. Yeast two hybrid experiments revealed that *Dictyostelium* NDUFAF7 interacts with NDUFS2, a subunit of the soluble arm of Complex I known to contain a dimethylated arginine residue [179]. NDUFAF7 harbors a methyltransferase domain and transient depletion of NDUFAF7 in human cells results in loss of methylation in the NDUFS2 subunit [180]. Therefore NDUFAF7 was proposed to mediate its function through dimethylation of the Arg-85 residue of NDUFS2, a chemical modification necessary for Complex I assembly [154,180].

NDUFAF6 forms a special case because its essential function appears to be dependent upon cytosolic localization despite the fact that the protein also localizes to the mitochondria. In the mitochondria, NDUFAF6 is required for the stability of the ND1 subunit [181,182] and in the cytoplasm, the protein interacts with cytosolic Hsp90 to chaperone the Complex I subunits NDUFS3 and ND42 before import into the mitochondria [183].

Table 2
Assembly factors for Complex I in mammals (*Homo sapiens* or *Bos taurus*), vascular plants (*Arabidopsis thaliana*) and unicellular green alga (*Chlamydomonas reinhardtii*).

Stage of assembly	Role in Complex I assembly	Factor	Mammalian	Functional domain	*A. thaliana* (e-value/Q/S)	*C. reinhardtii* (e-value/Q/S)
Early	Stability of ND1 subunit	NDUFAF5/C20ORF7	NDUFAF5	Methyl transferase domain	At1g22800[b,c,m] (4e-44/92%/65%)	XP_001693605/Cre13.g584750[M] (5e-32/87%/57%)
Early	Methylation of NDUFS2 subunit	NDUFAF7/MIDA	NDUFAF7	Methyl transferase domain; AdoMet Mtases superfamily	At3g28700[c,m] (4e-85/79%/59%)	XP_001700056.1/Cre02.g096400 (2e-37/40%/53%)
Early	Stability of ND1 and chaperone for ND42, NDUFS3 subunits	NDUFAF6/C8ORF38	NDUFAF6	Isoprenoid biosynthesis superfamily	At1g62730[c,m] (8e-3/82%/58%)	XP_001693265/Cre03.g194300[M,m] (1e-38/58%/54%)
Early	Factors associated with assembly intermediates	NDUFAF3/C6ORF60	NDUFAF3		At3g60150[c,m] (4e-14/67%/52%)	XP_001702394/Cre12.g496800[h] (2.4e-13/61%/52.2%)
		NDUFAF4/C6ORF66	NDUFAF4	Uncharacterized protein UPF0240	–	–
Early and intermediate	Factors associated with assembly intermediates	GLDH	XP_001253523 (1e-33)		At3g47930[a,b,c,g,m]	XP_001693696/Cre13.g567100[M,m] (2.2e-127)
		C3ORF1/TIMMDC1	C3ORF1	TIM-17 superfamily	At1g20350[c,e] (4e-32/52%/34%)	XP_001698342/Cre10.g452650[h] (4e-28/49%/34%)
		TMEM126B	TMEM126B	DUF1370	–	–
		NDUFAF1/CIA30	NDUFAF1	Complex I intermediate associated protein 30	At1g17350[a,m] (2e-10/57%/42%)	XP_001701850/Cre02.g076750/ NUOAF1[m] (1e-17/55%/49%)
		Ecsit	Ecsit		–	–
		ACAD9	ACAD9	Acyl-CoA dehydrogenase domain; Isovaryl CoA dehydrogenase	At3g45300[a,b,c,f,g,m] (2e-55/59%/53%)	XP_001691322.1/ Cre06.g296400[h,M,m] (3e-70/61%/55%)
Intermediate	Possible role in assembly of Fe–S clusters and/or mitochondrial translation	NUBPL/IND1/INDH	NUBPL	P-loop NTPase superfamily; ATP-binding protein MRP family; iron–sulfur protein	At4g19540[c,d,g,m] (4e-103/84%/56%)	XP_001702721/Cre10.g427050[M,m] (4e-87/80%/70%)
Late		NDUFAF2/B17.2L	NDUFAF2	NADH:ubiquinone oxidoreductase, 17.2 kDa subunit-like	At4g26965[g,m] (3e-04/84%/56%)	–
		FOXRED1	FOXRED1	Sarcosine oxidase family protein; FAD-dependent oxidoreductase domain	At2g24580 (0.01/81%/37%)	XP_001692123/Cre16.g671450 (2e-07/33%/47%)
	Accumulation of Complex I	AIF	AIF	Pyridine nucleotide disulfide oxidoreductase; glutathione reductase	At3g27820 (2e-23/54%/44%)	XP_001700472.1/Cre17.g712100[h] (9e-13/58%/42%)

The only plant assembly factor for Complex I that has been confirmed to be associated with assembly intermediates is *Arabidopsis* GLDH [149]. The *gldh*-null mutant has been characterized in *A. thaliana* [117,143]. The putative plant orthologs of human assembly factors were identified by similarity searches using NCBI BLAST (http://blast.ncbi.nlm.nih.gov/), TAIR BLAST (https://www.arabidopsis.org/Blast/) and Phytozome BLAST (http://phytozome.jgi.doe.gov/) using default parameters. The e-value, percentage of query coverage and percentage of sequence similarity are indicated in brackets as (e-value/Q/S). The proteins identified by similarity searches are only candidate assembly factors that need to be experimentally confirmed for their role in plant Complex I assembly.

The *Arabidopsis* accession numbers are provided from TAIR10. The mitochondrial localization of the *Arabidopsis* orthologs has been reported based on MS analysis of *Arabidopsis* mitochondrial proteome in Heazlewood et al. (2004) ([a]) [156], Taylor et al. (2011) ([b]) [157] and, Klodmann et al. (2011) ([g]) [158]; mitochondrial localization studies in Wydro et al. (2013) ([d]) [122] and Murcha et al. (2007) ([e]) [159]; Araujo et al., (2010) ([f]) [198]; subcellular localization database for *Arabidopsis* proteins (SUBA3) ([c]) [160] and target prediction algorithm TargetP ([m]) [161].

The *Chlamydomonas* accession numbers are provided as NCBI reference sequence ID and locus name from *Chlamydomonas* genome database JGI v5.5. The possible mitochondrial localization of *Chlamydomonas* orthologs has been provided based on MS analysis of *Chlamydomonas* mitochondrial proteome in Atteia et al. (2009) ([h]) [162] and, the target prediction algorithms PredAlgo ([M]) [163] and TargetP ([m]) [161].

Similarity searches revealed the presence of candidate orthologs for many of these assembly factors (Table 2). However, their roles in plant Complex I assembly await experimental validation.

4.2.1. INDH, an assembly factor with a role in mitochondrial translation

INDH, a mitochondrial matrix-resident member of the P-loop NTPases [73,122,184,185], is the only plant ortholog of a mammalian assembly factor known to function in Complex I biogenesis [184]. A phylogenetic link between INDH and Complex I was suggested based on their co-occurrence in 53 species and a simultaneous absence in 18 species including *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* [184]. A functional link was established by the study of INDH orthologs in *Yarrowia* (referred to as IND1), humans (referred to as NUBPL) and *Arabidopsis* (referred to as INDH). The loss of IND1 results in an 80% decrease of mature Complex I assembly and activity [184]. In humans, patients with mutations in NUBPL exhibit decreased levels of fully assembled Complex I [186]. In addition, RNAi-based depletion of the human IND1 ortholog is characterized by a low abundance of Complex I-associated supercomplexes and the accumulation of a ~460 kDa membrane-bound subcomplex, lacking the peripheral arm subunits [185]. The IND1 orthologs contain a conserved CXXC motif [184]. Mutations of these conserved cysteines in the human and *Yarrowia* orthologs result in a Complex I deficient phenotype similar to the loss of IND1 [184,185]. This CXXC motif is a characteristic of Fe–S cluster binding proteins. Consequently, the recombinant forms of the human and *Yarrowia* IND1 proteins can be reconstituted *in vitro* with a 4Fe–4S cluster, which can be then transferred to an acceptor protein [184,185]. Additional proof of Fe–S binding was obtained through the *in vivo* incorporation of ^{55}Fe into recombinant *Yarrowia* IND1 expressed in *S. cerevisiae* [184]. Furthermore, this *in vivo* incorporation was dependent upon the presence of proteins from the Iron-sulfur cluster (ISC) assembly machinery involved in mitochondrial Fe–S cluster assembly [184]. Based on these findings, a role for IND1 in Fe–S cluster delivery to one or several core subunits of the soluble arm was postulated. Since the loss of IND1 does not affect other Fe–S binding respiratory complexes and mitochondrial enzymes, it has been proposed that IND1 is specifically required for Fe–S cluster assembly in Complex I [184,185].

Studies of INDH in *Arabidopsis* led to the proposal for another function [122]. Homozygous *indh* mutants can germinate only upon sucrose supplementation and exhibit retarded growth, a trait reminiscent of Complex I deficient mutants. Indeed, the loss of *Arabidopsis* INDH results in severe Complex I deficiency with the accumulation of a 650 kDa subcomplex and trace amounts of 400 kDa and 450 kDa intermediates. Intriguingly, heterozygous *indh*/+ mutants displayed unusual phenotypes, also observed in mutants deficient for the mitochondrial translation machinery, such as aberrant segregation ratios and aborted ovules/pollen grains. Subsequently, *in-organello* translation studies revealed that the mitochondrial translation rate was severely decreased upon INDH depletion with differential effects on specific polypeptides. Specifically, the steady state levels of mitochondrially-encoded Nad6 and Nad7 were strongly diminished, although the transcript levels were normal. Defects in translation of mitochondrially-encoded Complex I subunits could account for the block in Complex I assembly observed in *indh* mutants. Additional evidence for a function in translation has been obtained by human *IND1* overexpression in bacteria, which causes an increased expression of a reporter gene encoding β-lactamase [187]. This has been attributed to enhanced translation of the β-lactamase encoding transcript by overexpressed *IND1*. Together, these studies postulate an alternative role for INDH in RNA binding and translation. The two proposed functions of Fe–S cluster delivery and mitochondrial translation are not mutually exclusive. While studies in *Arabidopsis* suggests a role for INDH in translation, a role in Fe–S cluster delivery for *Arabidopsis* INDH cannot be excluded but experimental evidence is lacking at the present time. A detailed analysis of the effect of IND1 depletion on *Yarrowia* and human mitochondrial gene expression needs to be conducted to test if the role of IND1 in mitochondrial translation is conserved.

4.2.2. Plant specific assembly factors

GLDH (L-galactano,1,4-lactone dehydrogenase) is the only plant-specific assembly factor currently confirmed for mitochondrial Complex I. GLDH is a FAD-containing enzyme [188] which catalyzes the conversion of L-galactano-γ-lactone (GL) to L-ascorbate [189,190]. GLDH was localized to the mitochondria in several vascular plants including white potato, sweet potato, cauliflower, spinach, fava bean, maize, kidney bean, *Nicotiana* and *Arabidopsis* [50,188–194]. Sub-localization experiments show that GLDH is an IMS-facing integral protein at the inner membrane [143,190]. This localization is in accord with the fact that cytochrome *c*, an IMS-resident protein is the electron acceptor for the GLDH-dependent ascorbate formation [150,190].

In *Arabidopsis*, Complex I is consistently resolved by BN-PAGE as two complexes with apparent molecular mass of approximately 1 MDa and 850 kDa [50]. Through 2D-BN/SDS PAGE and MS analysis, GLDH was identified in the 850 kDa complex, which constitutes an assembly intermediate. GLDH was absent from the 1 MDa complex, the prominent form of Complex I corresponding to the holoenzyme [50,150]. Similarly, in *N. sylvestris*, a GLDH-containing 800 kDa subcomplex was detected but no GLDH was associated with the mature enzyme [117]. In *Arabidopsis*, although GLDH was not immunodetected in the mature Complex I, two proteomic studies have identified GLDH in the holoenzyme [47,53]. In these cases, a possible contamination of the 1 MDa form, which was the target of analysis, with the 850 kDa GLDH-bound form has been proposed to explain the presence of GLDH. In addition to the 850 kDa, GLDH was found to be present in two other assembly intermediates, the 450 kDa and 400 kDa subcomplexes in a separate study [149].

The association of GLDH to membrane-arm assembly intermediates but not the mature complex suggests a role for GLDH in Complex I assembly. The observation that the *Arabidopsis gldh* mutant is deficient for holoenzyme assembly and accumulates only the 200 kDa subcomplex solidifies the involvement of GLDH in Complex I assembly [117,143]. The accumulation of the 200 kDa subcomplex suggests that GLDH is necessary for the formation of the 400 kDa subcomplex from the 200 kDa assembly intermediate [143]. In the *ndufs4* mutant lacking the 18 kDa subunit, the 200 kDa, 400 kDa, 450 kDa and 650 kDa subcomplexes accumulate [195]. While GLDH co-localized with the 400 kDa and 450 kDa subcomplexes, it was absent from the 650 kDa subcomplex. This observation led to a model where GLDH associates with the membrane arm of Complex I and allows the transition from the 200 kDa to 400 kDa subcomplex, remains associated to the 450 kDa subcomplex but is released from the membrane arm upon over-accumulation of the 650 kDa subcomplex in the *ndufs4* mutant [143]. In wild-type, GLDH stays associated to assembly intermediates until the 850 kDa subcomplex and dissociates upon formation of the mature 1 MDa holoenzyme [143]. The exact localization of GLDH in the membrane arm of Complex I intermediates remains unknown. In *Chlamydomonas*, a GLDH ortholog has been identified but its role in Complex I assembly is yet to be experimentally tested.

GLDH produces ascorbate, an essential anti-oxidant that regulates several aspects of plant growth and development [196] and expectedly *Arabidopsis gldh* mutants are viable only if supplemented with ascorbate [117]. The finding that ascorbate-deficient *vtc2-1* mutant accumulates wild-type levels of Complex I was taken as evidence that ascorbate is not essential for Complex I assembly. [143]. However, it is conceivable that the residual amount of ascorbate accumulated in *vtc2-1* is sufficient to sustain wild-type level of Complex I assembly. On the other hand, GLDH-dependent synthesis of ascorbate is sensitive to rotenone, a Complex I inhibitor [150,190], an intriguing finding considering that GLDH does not associate with the mature enzyme. In addition, GLDH *in-gel* activity can be detected in the 400 kDa, 450 kDa and 850 kDa subcomplexes [149]. Taken together, these results imply a link between

ascorbate production by GLDH and Complex I function. However, the role of Complex I in regulating ascorbate synthesis remains obscure.

5. Outlook

In recent years, application of advanced biochemical and proteomic approaches has increased our understanding of plant Complex I. From initial studies in maize and *N. sylvestris*, the knowledge of plant Complex I has further expanded using *Arabidopsis* and *Chlamydomonas*. The subunit composition of the plant enzyme has been established, leading to the discovery of several CA accessory subunits, which form a domain absent from the mammalian and fungal enzymes. While the CA subunits appear crucial for Complex I assembly, studies in *Arabidopsis* suggest the CA domain is part of a metabolic network for optimal CO_2 fixation in the chloroplast. Further experimental studies are needed to decipher the functional relevance of the association of CAs to Complex I and the role of the Complex I-associated CAs in non-photosynthetic organisms. In addition, several candidate subunits have also been identified but their identity as *bona fide* Complex I subunits and role in the enzyme assembly/activity remain unexplored. While 14 assembly factors are known for mammalian Complex I, only GLDH and INDH have been demonstrated to function in *Arabidopsis* Complex I biogenesis. Indeed, proteins displaying sequence similarity to known assembly factors can be identified in plant genomes. However, their role in Complex I assembly remains elusive and awaits experimental validation. It is likely that Complex I assembly in plants also relies on additional yet-to-be-discovered factors.

Transparency Document

The Transparency document associated with this article can be found, in the online version.

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