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Mitochondrial complex I from Arabidopsis and rice: orthologs of mammalian and fungal components coupled with plant-specific subunits

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Abstract

The NADH:ubiquinone oxidoreductase of the mitochondrial respiratory chain is a large multisubunit complex in eukaryotes containing 30–40 different subunits. Analysis of this complex using blue-native gel electrophoresis coupled to tandem mass spectrometry (MS) has identified a series of 30 different proteins from the model dicot plant, Arabidopsis, and 24 different proteins from the model monocot plant, rice. These proteins have been linked back to genes from plant genome sequencing and comparison of this dataset made with predicted orthologs of complex I components in these plants. This analysis reveals that plants contain the series of 14 highly conserved complex I subunits found in other eukaryotic and related prokaryotic enzymes and a small set of 9 proteins widely found in eukaryotic complexes. A significant number of the proteins present in bovine complex I but absent from fungal complex I are also absent from plant complex I and are not encoded in plant genomes. A series of plant-specific nuclear-encoded complex I associated subunits were identified, including a series of ferripyochelin-binding protein-like subunits and a range of small proteins of unknown function. This represents a post-genomic and large-scale analysis of complex I composition in higher plants.

Keywords: Complex I; Plant mitochondria; Proteomics; Bioinformatics

1. Introduction

Complex I of the respiratory chain is a proton-pumping, NADH:ubiquinone oxidoreductase that oxidizes NADH in the electron transport pathway. This enzyme has been characterized in detail in mitochondrial membranes of fungi [1] and mammals [2,3]. A similar membrane-associated enzyme in bacteria appears to be related to the ancestral progenitor of mitochondrial complex I [4]. Dissection of the protein components in different species has revealed a relatively simple enzyme containing 14 subunits in bacteria, and a more complex enzyme in eukaryotes containing 32 subunits in *Neurospora crassa* [1] and 45 subunits in mammalian mitochondria [2,3,5–7]. Both the bacterial enzyme and complex I from eukaryotes have a conserved set of five iron sulfur (Fe–S) center proteins, which in combination contain eight to nine Fe–S centers [3,4]. The bacterial enzyme has a native molecular mass of approximately 550 kDa, while the eukaryotic enzymes are approximately 900 kDa. A set of seven subunits encoded in the mitochondrial genome of eukaryotes (ND1, ND2, ND3, ND4, ND4L, ND5 and ND6) form a hydrophobic core of the complex. The remaining nuclear-encoded subunits consist of both peripheral, more hydrophilic components as well as a number of hydrophobic proteins from the membrane portion of the complex [3].

In plant mitochondria, a variety of NADH:ubiquinone reductases have been identified. One enzyme is a proton pumping complex I, which is analogous to that observed in mammals and fungi, and shares with these complexes sensitivity to the inhibitor rotenone [8]. A variety of simple rotenone-insensitive nonproton pumping NADH dehydrogenases that consist of single polypeptides have also been identified in plants [9]. Complex I has been purified and analyzed from broad bean [10], red beetroot [11], potato [12,13] and wheat [14,15]. In all these species, the complex was found to consist of approximately 25–30 subunits that can be separated by electrophoresis. A small number of these subunits were found to be similar to various fungi or mammalian complex I components, based on antibody

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cross-reaction or short amino acid sequences obtained by Edman degradation. However, in many cases, the minimal N-terminal data did not provide insights into which subunit was being investigated and only a handful of the genes corresponding to these proteins have been sequenced from these diverse plant species. Mapping and sequencing of model plant mitochondrial genomes has identified six ND genes that are very similar to those identified in other eukaryotic mitochondrial genomes [8], plus two additional genes which encode subunits ND7 and ND9 [8]. The counterparts of both these latter genes are nuclear-encoded in mammals.

The complete sequencing of the *Arabidopsis thaliana* (Arabidopsis) and *Oryza sativa* (rice) nuclear genomes [16–18] now provides the opportunity for a more complete characterization of the nuclear-encoded components of complex I from plants. Two-dimensional electrophoresis has identified five nuclear-encoded Arabidopsis orthologs to known mammalian complex I components, but has not confirmed that they are physical components of an isolated Arabidopsis complex I [19,20]. Many earlier attempts to isolate complex I from plants involved complex, multistep chromatographic purification procedures [10–12]. These approaches required large amounts of plant mitochondrial material and also risked the loss of peripheral, loosely bound subunits. The immunoaffinity chromatography approach developed by Combettes and Greinenberger [14] represents an alternative approach, but still requires specialist equipment and significant antibody availability. The blue native polyacrylamide gel electrophoresis (BN-PAGE) separation of plant mitochondrial membrane complexes, perfected by Jansch et al. [13] and Werhahn and Braun [21], provides a rapid procedure for the purification of very small amounts of complex I amenable to mass spectrometry (MS) based identification of proteins using standard PAGE approaches. This type of approach allows the biochemical characterization of complex I in model plant species from which only small amounts of mitochondrial material can be routinely isolated.

In this report, we have isolated complex I by BN-PAGE from both Arabidopsis and rice and have linked proteins with genes predicted from genome sequencing. We have also searched the rice and Arabidopsis genomes for sequence orthologs of other complex I subunits identified in mammals, fungi and bacteria. In this manner, we have attempted to systematically compare the higher plant complex I to its eukaryotic and prokaryotic counterparts.

2. Materials and methods

2.1. Arabidopsis culture and mitochondrial isolation

A heterotrophic Arabidopsis cell culture, established from callus of ecotype Lansberg *erecta* stem explants, was maintained by weekly subculture in Murashige and Skoog basal media supplemented with 3% (w/v) sucrose, 0.5 mg/l naphthaleneacetic acid and 0.05 mg/l kinetin [22]. The cell cultures were maintained in 250 ml conical flasks in the dark at 22 °C in an orbital shaker (150 rpm). At 6–7 days, each flask (120 ml of cell culture) contained 8–10 g FW cells and growth was approximately in the middle of the log phase. Subculture of 20 ml of culture to 100 ml of fresh media initiated the cycle again. A total of 1.0–1.2 l of 7-day-old dark-grown cell suspension culture was filtered through gauze to remove media and then the cells disrupted in a Waring blender by three successive 15-s bursts. Disruption of 60 g of cells was performed in 200 ml of grinding medium (0.45 M mannitol, 50 mM sodium pyrophosphate, 0.5% (w/v) bovine serum albumin (BSA), 0.5% (w/v) PVP-40, 2 mM EGTA, 20 mM cysteine pH 8.0). Filtered cell extract was separated by differential centrifugation and mitochondria purified from the resultant organelle pellets by two Percoll gradients according to Millar et al. [19]. Approximately 5–7 mg of total mitochondrial protein is obtained using this approach.

2.2. Rice culture and mitochondrial isolation

Batches of 200 g of rice (cv Amaroo) seed were washed in 1% (w/v) bleach for 10 mins, rinsed in distilled water and grown in the dark in vermiculite slats (30 × 40 cm) at a constant 30 °C, watered daily and grown for 7 days. Rice shoots were harvested using scissors and cut into 5–10 mm lengths into a beaker on ice. Cut shoots (100 g) were ground in a precooled mortar and pestle using acid-washed sand in 300 ml of homogenization solution (0.3 M sucrose, 25 mM tetrasodiumpyrophosphate, 2 mM EDTA, 10 mM KH_2PO_4, 1% PVP-40, 1% BSA, 20 mM ascorbate, pH 7.5). After filtering homogenate through four layers of miracloth, it was centrifuged for 5 min at 1000 × *g*, then the supernatant again centrifuged at 20,000 × *g* for 20 min. The resultant organelle pellet was resuspended in wash buffer (0.3 M sucrose, 10 mM TES–KOH pH 7.5, 0.1% (w/v) BSA) and the two centrifugation steps repeated to produce a washed organelle pellet. This resuspended pellet was layered over 0–4.4% PVP-40 preformed gradient in a 28% (v/v) Percoll self-forming gradient in wash buffer and centrifuged at 30,000 × *g* for 45 min. Mitochondria formed a band towards the bottom of the gradient, the upper plastid material was discarded and the mitochondrial band removed, attempting to minimize contamination of peroxisomal material from the bottom of the gradient. Following two wash centrifugation steps at 20,000 × *g* for 15 min, the pellet was layered over a second self-forming gradient consisting of 45% Percoll in wash buffer and centrifuged for 30 min at 30,000 × *g*. Mitochondria remained near the top of the gradient while remaining peroxisomal material migrated to the bottom of the gradient. The mitochondrial layer was aspirated and concentrated by wash centrifugation steps at 20,000 × *g* for 15 min. Approximately 3–6 mg of total rice mitochondrial protein is obtained by this approach.

2.3. BN-PAGE/SDS-PAGE

BN-PAGE was performed largely according to Jansch et al. [13]. Gels consisted of a separating gel (5–15% w/v acrylamide) and a stacking gel (4% w/v acrylamide) formed in a solution of 0.25 M ε-amino-*n*-caproic acid and 25 mM Bis-Tris–HCl (pH 7.0). The anode buffer consisted of 50 mM Tricine, 15 mM Bis-Tris–HCl, 0.02% w/v Blue G250 (pH 7.0) and the cathode buffer of 50 mM Bis-Tris–HCl (pH 7.0). Mitochondrial sample pellets were suspended to approximately 2 mg ml^{-1} in 10 mM TES (pH 7.5), freeze–thawed repeatedly in liquid N_2, and centrifuged for 20 min at 20,000 × *g*. Membrane pellets were then washed with 10 mM TES (pH 7.5). Aliquots of 1–2 mg of membrane protein were suspended in 75 μl of an ACA buffer solution (containing 0.75 M ε-amino-*n*-caproic acid, 0.5 mM Na_2EDTA, 50 mM Bis-Tris–HCl pH 7.0) followed by addition of 15 μl of a freshly prepared solution of 10% (w/v) *n*-dodecylmaltoside. After 10 min centrifugation at 20,000 × *g*, 15 μl of 5% w/v Blue G250 (dissolved in ACA buffer) was added to the supernatants. Gels were run at 4 °C in a precooled apparatus with all samples and buffers precooled to 4 °C. Approximately 30 min before the run, 0.03% w/v *n*-dodecylmaltoside was added to the cathode buffer only. Electrophoresis was commenced at 100 V constant voltage for 45 min, and then increased to 15 mA without voltage limitation for 5 h. BN lanes were cut from gels, equilibrated in standard SDS-PAGE sample buffer for 30 min, laid horizontally on 12% acrylamide SDS-PAGE separating gels, sealed in with 0.5% agarose and electrophoresis continued for 5 h under standard conditions.

2.4. Quadrupole time-of-flight mass spectrometry (Q-TOF MS)

Q-TOF MS/MS was performed on an Applied Biosystems Q-STAR Pulsar (Q-TOF MS) using an IonSpray source. Protein spots to be analyzed were cut from BN-PAGE gels, destained, dried at 50 °C in a dry block heater and stored at −70 °C. For the sequencing analysis, the proteins were digested with trypsin according to Sweetlove et al. [23], injected into the electrospray source in 50% (v/v) methanol/0.1% (v/v) formic acid and selected doubly charged peptides, identified in MS-TOF mode, fragmented by N_2 collision and analyzed by MS/MS. Mass spectra and collision MS/MS data were analyzed with Analyst QS, BioAnalyst and ProID software (Applied Biosystems, Foster City).

2.5. Gene identification

Sequence orthologs were identified by BLAST analysis of *N. crassa* and bovine genes against v3.0 of the Arabidopsis protein set, a draft rice genome protein set and rice gene indices all distributed by The Institute for Genomic Research (TIGR), and the Arabidopsis and rice National Center for Biotechnology Information (NCBI) entries. The *E* (expected) values of matches were recorded to give an indication of the degree of similarity between the plant and mammalian sequences. *E* values were obtained from BLASTP and TBLASTN analyses. The most significant match was utilized for the determination of sequence orthologs, in many cases this represented a relatively stringent *E* value of less than 10^{-6}. Where low levels of identity were observed, further sequence structural analyses were undertaken to confirm them as likely matches. Predotar (http://www.inra.fr/Internet/Produits/Predotar/), TargetP (http://www.cbs.dtu.dk/services/TargetP/) and MitoProt (http://www.mips.biochem.mpg.de/cgi-bin/proj/medgen/mitofilter) targeting predictions were determined using full-length predicted protein sequences.

3. Results

3.1. BN-PAGE separation of complex I from plants

Separation of mitochondrial membranes by BN-PAGE allows the separation of intact electron transport chain complexes on the basis of their apparent molecular mass [13]. Subsequent second-dimension separation of the subunits within each complex using reducing SDS-PAGE displays these subunits for analysis. Our separation of Arabidopsis and rice membrane proteins in this manner revealed a series of complexes. We are undertaking a comprehensive identification of the proteins in each of these complexes and in Fig. 1 present the very high molecular mass section of BN-PAGE gels from Arabidopsis and rice. Several protein complexes are evident. The identification of four of these high molecular mass complexes in Arabidopsis was confirmed by sequencing components from each by MS (data not shown). The predicted masses of the complexes were derived by comparison with masses observed in potato mitochondrial preparations (Jansch et al. [13]; A.H. Millar, unpublished data).

The largest of the protein complexes contained a series of 30 kDa subunits (Fig. 1). Mass spectrometry revealed that these were products from prohibitin-like genes in both Arabidopsis (At1g03860, At2g20530, At4g28510 and At5g40770) and rice (TIGR 2952.m00055) (data not shown). This complex is likely to be an oligomer of prohibitin chaperonin proteins. A similar prohibitin complex, with a native mass of >1000 kDa, has been identified in yeast [24], where genetic studies have shown that it is essential for cell survival and is associated with proteolysis by the Clp protease system [25].

The second largest complex on the BN-PAGE strip is the NADH:ubiquinone oxidoreductase, or complex I of the respiratory chain. In Arabidopsis, this complex was present consistently in two forms of slightly different mobilities on BN-PAGE. In rice, on the other hand, only a single form of complex I was observed. The native mass of complex I has

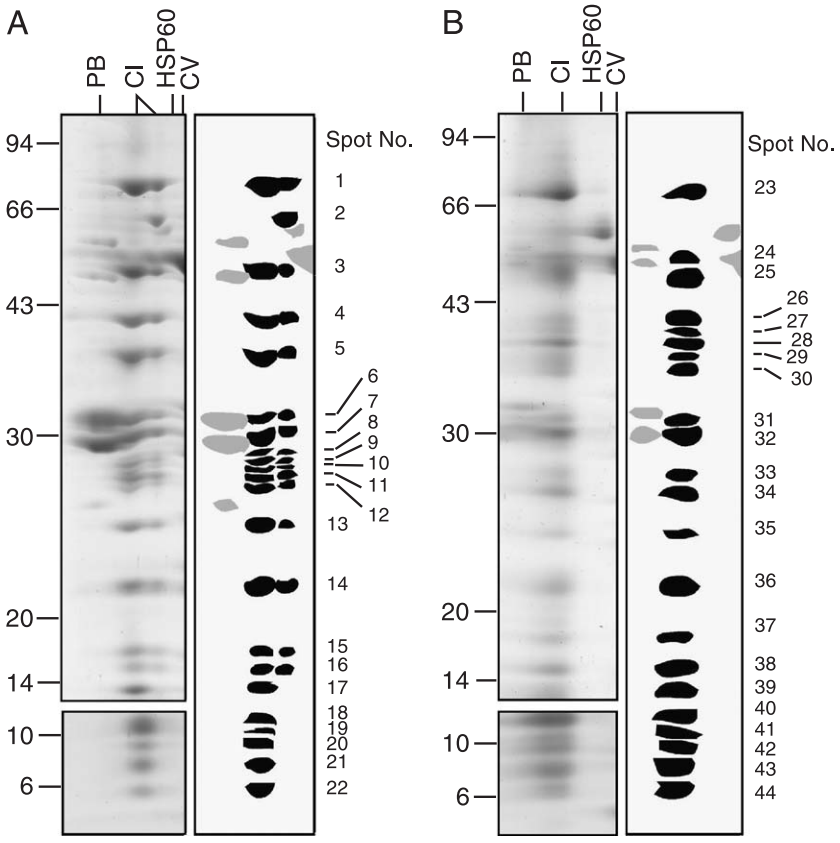

Fig. 1. BN-PAGE separation of Arabidopsis (A) and rice (B) mitochondrial complex I. Coomassie-stained image of native complex 1 separated into component subunits from plant mitochondrial membranes. Apparent molecular mass of subunits is shown in kDa. Top panels of each figure are from Tris–glycine buffer separations and lower panels the low molecular mass components separated by Tris–tricine buffer. Along side each gel image is a cartoon highlighting the protein spots identified as components of complex I by MS and spot numbers for comparison with Tables 1 and 2. PB, prohibitin complex; CI, NADH-UQ oxidoreductase (complex I); HSP60 (heat shock protein 60); CV, F_1F_O ATP synthase (complex V).

been estimated at approximately 1000 kDa in higher plants [12,13]. Following complex I, the edge of the F_1F_O ATP synthase is apparent, with a mass of approximately 550 kDa. Near the F_1F_O complex, a small amount of membrane-bound HSP60 complex (native mass approximately 750 kDa) is apparent; this protein complex is much more prominent on BN-PAGE separations of soluble plant mitochondrial proteins [13].

Complex I from both plant species separated into a large number of individual bands following SDS-PAGE. A small number of these proteins have been identified from other plant species by N-terminal sequencing and the use of cross-reacting antibodies [10,12,13]. In order to systematically identify the Arabidopsis and rice proteins, bands were excised, trypsin digested and the resultant peptides analyzed by collision MS/MS and database matching. A set of 21 bands from the low-mobility complex I and 17 bands from the high-mobility form of complex I from Arabidopsis were analyzed along with 22 bands from the rice complex I (Fig. 1). This full range of separated protein bands was obtained using Tris–glycine SDS-PAGE to separate protein bands in the >14 kDa range, and Tris–tricine SDS-PAGE to separate in the <14 kDa range.

3.2. Mass spectrometry identification

Thirty different proteins were identified in the 38 gel bands of complex I from Arabidopsis and 24 different proteins in the 22 gel bands in rice (Tables 1 and 2). Database searching revealed that some of these proteins were orthologs of known complex I components from other eukaryotes (Table 1). In Arabidopsis, the products of four mitochondrial-encoded subunits (ND1, ND5, ND7 and ND9) were detected along with products of 17 nuclear-encoded known complex I gene orthologs. In rice, the products of ND1, ND7 and ND9 were detected, but the absence of the gene sequences for ND1 forced matching of these peptides to the homologous gene from other plants. The high degree of identity of these mitochondrial NAD genes across plants allowed accurate matching of the rice peptides across species. The products of 14 nuclear-encoded known complex I genes from rice were also identified.

In addition to these counterparts of well-known complex I proteins (Table 1), we identified a further set of proteins, largely of unknown function, from both Arabidopsis and rice (Table 2). Both species contained proteins at 25–32 kDa that comprise a gene family related to bacterial side-

Table 1
Identification of orthologs of known complex I components in the purified Arabidopsis and rice complex I preparations

Spot	kDa	MP	MOWSE	ProID	Arabidopsis Gene	MM	Description	Spot	kDa	MP	MOWSE	ProID	Rice Gene	MM	Description
1	76	14	483	99	At5g37510	82	76 kDa subunit	23	74	4	164	ND	AAL58200	81	76 kDa subunit
3	50	9	301	99	At5g08530	53	51 kDa subunit	24,25	53	11	486	99	BAC16718	55.2	51 kDa subunit
3	50	1	ND	99	NP_085478	44.5	ND5								
4	40	9	180	99	P93306	45	ND7	26,27	40	4	204	99	BAC19866	44.2	ND7
5	35	13	289	99	At2g20360	44	39 kDa subunit	28,30	37	3	ND	99	2451.m00084	44.3	39 kDa subunit
11		2	29	99	NP_085565	35.9	ND1	33	27	2	ND	99	NP_085565*	35.9	ND1
9	28	12	176	99	At4g02580	28	24 kDa subunit								
11	26	9	178	99	At1g79010	26	23 kDa subunit	34	25	2	ND	99	3550.m00121	23.4	23 kDa subunit
13	24	9	256	99	Q95748	23	ND9	35	24	2	100	ND	Q35322	23	ND9
14	22	5	135	99	At5g52840	19	B13 subunit	36,37,38	14-22	1	ND	99	4381.m00118	21.8	B13 subunit
14	23	3	29	99	At5g11770	24	20 kDa subunit	37	17	4	104	99	BAB84415	21.8	20 kDa subunit
15	17	7	152	99	At5g67590	17	18/21 kDa subunit	37	17	3	65	99	BAC20889	15.7	18/21 kDa subunit
17	14	6	90	99	At1g49140	12	PDSW subunit								
16	16	4	60	99	At2g33220	16	B16.6 kDa subunit	39	13	3	ND	99	4342.m00113	15.9	B16.6 kDa subunit
15	16	2	47	ND	At3g03100	18.3	B17.2 kDa subunit	37	17	3	35	99	AAG46149	18.5	B17.2 kDa subunit
19	10	5	110	99	At3g12260	15	B14 kDa subunit	40	12	4	215	99	BAC16465	14.9	B14 kDa subunit
18	11	4	118	99	At2g02050	12	B18 kDa subunit	40	12	2	53	99	AAN17398	12.1	B18 kDa subunit
20	9	1	53	ND	At4g16450	12	20.9 kDa subunit	42	10	1	ND	99	3031.m00132	11	20.9 kDa subunit
19	10	2	27	98	At2g47690	14	15 kDa subunit								
22	6	1	36	99	At3g08610	7	MWFE subunit								
								43	8	2	59	99	CAD37116	8	B8 kDa subunit

MS/MS spectra derived from trypsinated peptides of proteins (MP) were matched at Mascot against a translated NCBI database yielding a MOWSE score or against custom databases using ProID yielding a probability of match (0–100).

Table headings: Spot, protein spot number from Fig. 1; MP, number of peptides matching to predicted protein sequence; kDa, observed molecular mass; MM, predicted molecular mass of matched sequence in kilo-Daltons. Arabidopsis and rice genes are accession numbers from TIGR or NCBI.

(*) no rice gene was found but matches were 100% to the Arabidopsis gene for this subunit. Orthologous subunits between the two species are matched on the same line. Shaded regions indicate the absence of an identified ortholog.

Table 2
Identification of unknown complex I components in the purified Arabidopsis and rice complex I preparations

Species	Spot	kDa	MP	MOWSE	ProID	ID	MM	Description	PR	TP	MP
Arabidopsis	2	60	14	281	99	At3g47930	68	L-galactono-1,4-lactone dehydrogenase	M	M	M
	6	32	10	231	99	At5g66510	28	ferripyochelin-binding protein-like	–	M	–
	7	30	15	335	99	At1g47260	30	ferripyochelin-binding protein-like	M	M	M
	10	27	6	207	99	At3g48680	28	ferripyochelin binding protein-like	M	M	M
	12	25	7	136	99	At5g63510	28	ferripyochelin-binding protein-like	M	M	M
	20	9	1	48	99	At3g57785, At2g42310	13	unknown protein (like 3605.m00182)	M	M	M
	17	12	1	39	99	At2g27730	12	NADH dehydrogenase 16 kDa	–	M	M
	21	8	2	53	99	At2g31490	8	unknown protein (like BAB67906)	–	–	–
	22	6	3	65	99	At4g20150	9	unknown protein	M	M	M
Rice	31	32	2	170	99	BAB39954	28	ferripyochelin-binding protein-like			
	32	30	2	105	99	BAC16488	30	ferripyochelin-binding protein-like	M	M	M
	40	12	3	31	98	BAA81662	13	NADH dehydrogenase 16 kDa	M	M	M
	42	10	2	ND	99	3605.m00182	13	unknown protein (like At2g42310, At3g57785)	M	M	–
	43	8	1	38	96	BAB67906	11	unknown protein (like At2g31490)	–	–	–
	41	11	3	ND	99	3310.m00085, 3558.m00104	11	NADH dehydrogenase 11 kDa	–	–	–
	44	6	1	ND	99	2468.m00122	7	unknown protein	M	M	–

MS/MS spectra derived from trypsinated peptides of proteins (MP) were matched at Mascot against a translated NCBI database yielding a MOWSE score or against custom databases using ProID yielding a probability of match (0–100).
Table headings: Spot, protein spot number from Fig. 1; MP, number of peptides matching to predicted protein sequence; kDa, observed molecular mass from gel; MM, predicted molecular mass of matched sequence. The sequence paralogs At3g57785/At2g42310 and 3310.m00085/3558.m00104 could not be differentiated by MS/MS spectra obtained. Predicted protein sequences were analyzed by three targeting prediction programs, Predotar (PR), TargetP (TP) and MitoProtII (MP).
M indicates predicted mitochondrial targeting; (–) indicates no prediction of organellar targeting.

rophores known as ferripyochelin-binding proteins (Table 2). These proteins have not been shown to be associated with complex I from other eukaryotes to our knowledge, and no clear orthologs are present in mammalian genomes or the *N. crassa* genome. Both rice and Arabidopsis contain orthologs with 80–90% identity to Swiss-Prot P80730, a 22 amino acid N-terminal sequence from potato complex I, submitted by U. Herz and L. Grohmann in 1996 as the "16 kDa subunit of NADH dehydrogenase". An identical potato protein has been claimed to be the plant ATP synthase inhibitor protein (12, 26) despite the very low homology of these proteins to the inhibitor protein from other eukaryotes [26]. Whatever the possible involvement with the ATP synthase, it seems clear that this protein is found prominently in complex I preparations from potato, Arabidopsis and rice. Rice also contains two proteins annotated as "NADH dehydrogenase 11 kDa proteins" based on 80–90% N-terminal identity to a 20 amino acid N-terminal sequence from a potato complex I subunit purified by U. Herz and entered in the Swiss-Prot database (P80729). An Arabidopsis ortholog of this protein also exists and is annotated at the Munich Information Centre for Protein Sequences (MIPS) as At1g67350, but the TIGR locus entry of the same name is a frame-shifted protein sequence in the same region. We have no evidence to date for this later protein's presence in our Arabidopsis complex I preparation.

A series of small molecular mass proteins were also discovered in both Arabidopsis and rice complex I. The very similar products of the At2g57785 and At2g42310 genes, and the rice ortholog 3605.m00182, were found. These proteins are predicted to contain mitochondrial targeting presequences by both Predotar and TargetP, but their sequences do not match to proteins of known or predicted function (Table 2). There is some similarity of the rice sequence to a region near the N-terminus of the mammalian and *N. crassa* mitochondrial complex I component, ND6, but this similarity is not very evident in the Arabidopsis sequences and may be a coincidence. The Arabidopsis protein encoded by At2g31490 and the rice ortholog BAB67906 were also found, but neither were predicted to be mitochondrial by the prediction programs used here. The presence of these proteins in both species provides good initial evidence for the future investigation of these proteins as possible complex I components in plants.

Finally, there are a series of proteins identified that require extra research to more clearly define their role as potential complex I components. In Arabidopsis, the higher mobility complex I form contained a distinct protein band at 60 kDa. Sequencing revealed this protein to be galactono-lactone dehydrogenase (At3g47930). This enzyme is responsible for the final step of ascorbate biosynthesis that occurs on the inner mitochondrial membrane in plants [27]. The location and association of this protein with complex I was previously unknown, although a biochemical link with the electron transport chain had been established [27]. We were not able to identify this protein in our rice complex I samples from dark-grown rice shoots, perhaps due to very low expression of genes encoding ascorbate-synthesizing

proteins under such conditions or the lack of co-migration of this enzyme with complex I in rice. The plant complex I preparations also contain two additional small proteins (products of At4g20150 and 2468.m00122) that do not contain any obvious sequence identity to either each other or to proteins of known function from other eukaryotes. Both proteins contain mitochondrial targeting presequences according to multiple prediction programs. This provides some further bioinformatic evidence that they are likely to represent legitimate MS identifications of mitochondrial proteins. However, these single identifications need to be further investigated to substantiate claims of their association with complex I.

It should be noted that these identifications are based on purification of complex I by a one-step process, blue native gel electrophoresis, largely due to the low yields of mitochondria from these model plant species. While this technique has produced impressive separation of native complexes in its application to mitochondrial samples [13,20,21], the possibility that co-elution of complexes or nonspecific association of protein to complexes during this procedure must be entertained. In it's defense, we have not found known components of other major electron transport chain complexes or carrier proteins identified by MS in our complex I lane (Tables 1 and 2), but we cannot discount the possibility that some of the plant-specific proteins observed in our complex I preparations could be due to such co-elution or nonspecific associations.

3.3. Searching for Arabidopsis and rice sequence orthologs of complex I subunits from mammals and fungi

Given the diversity of the proteins found in the plant complex I preparations, it seemed advisable to consider the findings of Tables 1 and 2 in the context of which counterparts to known complex I proteins had been found and whether the missing counterparts were in fact present in plant genomes. All of the mammalian genes for the 38 nuclear-encoded proteins and the 7 mitochondrial-encoded proteins of complex I have been sequenced [2,3,5–7]. A generic nomenclature for complex I genes, taking into account the structural association data, has been proposed for the human genes [2] and is used as a helpful basis for Table 3. Purified complex I can be disassociated into Iα and Iβ subcomplexes [3]. The Iα complex contains the flavoprotein subcomplex (NDUFV1-3), the Fe–S center subcomplex proteins (NDUFS1-8) and a range of proteins of unknown function (NDUFA1-13). The Iβ subcomplex contains a set of 11 proteins (NDUFB1-11), apparently originating from the membrane arm of the complex but with no known function. Several subunits are lost from the complex during Iα and Iβ complex separation (NDUFC1-2) and one protein, the SDAP protein, is localized in both subcomplexes (NDUFAB1). The mitochondrial-encoded proteins in mammals are orthologs of the bacterial NUOA and NUOJ–NUON products. The five proteins responsible for directly binding Fe–S centers and/or FMN (NDUFS1, NDUFS7, NDUFS8, NDUFV1, NDUFV2) along with two Fe–S subcomplex proteins (NDUFS2 and NDUFS3) are counterparts of the bacterial NUOB–NUOG proteins [4]. The products of these 14 orthologous genes are also found in the extensively studied complex I from the fungus *N. crassa* [1]. In addition, a series of ten *N. crassa* complex I proteins have mammalian analogs (Table 3), while a set of four *N. crassa* complex I components (14, 17.8, 20.9, 21.3a) have no known counterparts in the mammalian complex [1].

On the basis of this information, the human protein sequences were used to search genomic and EST databases from Arabidopsis and rice to identify probable plant orthologs. In Arabidopsis, orthologs of all 14 conserved subunits were identified, with only one existing as a family of two members, the NDUSF8 orthologs At1g79010 and At1g16700. In addition, orthologs for 14 other mammalian complex I subunits were identified (Table 3). In rice, the lack of a complete mitochondrial genome sequence hampered identification, although ample evidence exists for the conservation of ND genes in the rice mitochondrial genome (Table 3). Orthologs for 10 of the 14 highly conserved complex I subunits have been sequenced in rice, 6 nuclear-encoded genes and 4 mitochondrial-encoded genes. In addition, a series of 15 other genes encoding proteins with similarity to mammalian complex I subunits were identified. In higher plants, two genes encoding Fe–S subcomplex proteins that are nuclear encoded in *N. crassa* and mammalian species (NDUFS2-3) are encoded in the plant mitochondrial genome as NAD7 and NAD9. Only one of the seven *N. crassa* complex I genes that do not have mammalian counterparts appears to have plant counterparts, namely the 20.9 subunit (Swiss-Prot X60829, data not shown in Table 3, see Table 1). Neither of the newly identified mammalian components, B14.7 and ESSS [7], have clear orthologs in plant genomes (Table 3).

Looking across the dataset presented in Table 3, it appears that a significant number of the mammalian nuclear-encoded complex I subunits have no clear orthologs yet identified in the genomes of fungi and higher plants (shown shaded). Nearly all of the Iβ subcomplex components are currently missing, along with both NDUFC components and several components in the I subcomplex. It is possible that orthologous components do exist but are absent from our analysis due to very divergent sequence; this possibility awaits further experimental analysis of the plant complex I and more extensive bioinformatic analysis. A core set of nine proteins not found in the prokaryotic enzyme do appear to be conserved across all the eukaryotes analyzed. This set is currently much smaller than the set of 27 subunits that have been identified in mammals and are not present in the bacterial enzyme.

When this genomic analysis (Table 3) is linked with components definitively identified in purified plant complex I (Table 1), an interesting pattern emerges (Table 3, asterisk columns). The Fe–S subcomplex proteins and flavoprotein

Table 3
Comparison of complex I subunits from model prokaryotic and eukaryotic organisms and identification of Arabidopsis and rice orthologs

Human name	*E. coli* name	Bovine name	MW	Neurospora entry	MW	BLAST *E* value	Arabidopsis entry	MW	BN-PAGE Present	BLAST *E* value	Rice entry	MW	BN-PAGE Present
NDUFA1		MWFE	7.5	[a]	9.8	0.077	At3g08610	7.3	*	[c] 1E-12	TC70453	7.5	
NDUFA2		B8	8	X69929	10.5	3E-20	At5g47890	10.8		1E-14	CAD37116	8	*
NDUFA3		B9	9	S49807	9.3								
NDUFA4		MLRQ	9										
NDUFA5		B13	13	X56237	29.9	2E-14	At5g52840	19.2	*	3E-17	4381.m00118	21.8	*
NDUFA6		B14	14	X76344	14.8	2E-10	At3g12260	15	*	1E-08	BAC16465	14.9	*
NDUFA7		B14.5a	14.5										
NDUFA8		PGIV	19	M55323	20.8	1E-11	At3g06310/At5g18800	12		[c] 3E-44	TC69434	12	
NDUFA9		39 kDa	39	X56238	40	4E-56	At2g20360	44	*	2E-56	2451.m00084	44	*
NDUFA10		42 kDa	42										
DAP13		B17.2	17.2			2E-12	At3g03100	18	*	4E-14	AAG46149	18.5	*
GRIM-19		B16.6	16.6			2E-24	At2g33220	16.1	*	3E-19	4342.m00113	15.9	*
NDUFA11		B14.7	14.7	X56612	21.3								
NDUFAB1		SDAP	8	P11943	9.6	3E-17	At1g65290/At2g44620/At5g47630	14		0.001	3080.m00116	14	
NDUFB1		MNLL	7										
NDUFB2		AGGG	8										
NDUFB3		B12	12										
NDUFB4		B15	15										
NDUFB5		SGDH	16										
NDUFB6		B17	17										
NDUFB7		B18	18			2E-08	At2g02050	12	*	7E-07	AAN17398	12.1	*
NDUFB8		ASHI	19										
NDUFB9		B22	22			5E-08	At4g34700	13.6		[c] 2E-47	3640.m00126	12	
NDUFB10		PDSW	22	X68965	12.3	[b] 7E-08	At1g49140/At3g18410	12.5	*	[b] 5E-41	2396.m00160	12	
NP17.3		ESSS	14.5										
NDUFC1		KFYI	6										
NDUFC2		B14.5b	14.5										
NDUFS1	NUOG	75 kDa	75	X57602	78	0.0	At5g37510	81.5	*	0.0	2187.m00131	81	*
NDUFS2	NUOD	49 kDa	49	X54508	49	0.0	P93306	44.6	*	1E-160	BAC19866	44	*
NDUFS3	NUOC	30 kDa	30	A35936	31	2E-51	Q95748	22.6	*	5E-51	BAA08794	23	*
NDUFV1	NUOF	51 kDa	51	X56227	51	0.0	At5g08530	53.5	*	0.0	BAC16718	55.2	*
NDUFS4		18 kDa	18	X78082	21	9E-25	At5g67590	17.1	*	8E-21	BAC20889	15.7	*
NDUFS5		15 kDa	15			6E-04	At3g62790/At2g47690	14	*	0.017	3084.m00164	11	
NDUFS6		13 kDa	13			4E-07	At3g03070	12.2		2E-07	5177.m00204	13	
NDUFS7	NUOB	PSST 20 kDa	20	AJ001520	19.3	4E-66	At5g11770	24	*	1E-65	BAB84415	21.8	*
NDUFS8	NUOI	TYKY 23 kDa	23	X95547	21.3c	5E-68	At1g79010/At1g16700	25.5	*	7E-61	3550.m00121	23	*
NDUFV2	NUOE	24 kDa	24	X78083	24	2E-57	At4g02580	28.3	*	4E-52	AF358773	11.8	
NDUFV3		10 kDa	10										
ND1	NUOH	ND1	36	ND1	42	6E-63	NP_085565	36	*		n.a.		*
ND2	NUON	ND2	39	ND2	66	0.23	NP_085584	55			n.a.		
ND3	NUOA	ND3	13	ND3	-	1E-04	NP_085553	14		1E-03	AAA70313.1	18	
ND4	NUOM	ND4	52	ND4	-	1E-45	NP_085518	55		5E-15	BAA23429.1	28	
ND4L	NUOK	ND4L	11	ND4L	10	0.014	NP_085525	11		0.005	CAC79145.1	11	
ND5	NUOL	ND5	67	ND5	80	3E-75	NP_085478	74	*		n.a.		
ND6	NUOJ	ND6	19	ND6	-	-	NP_085495	23.5		-	BAC19862	23	

subcomplex components dominate those proteins positively identified by MS in the plant complexes, while products of most of the nuclear-encoded genes conserved between plants and other eukaryotes were also found to be present. However, in the case of both Arabidopsis and rice, a number of sequence orthologs of mammalian components have currently not be detected in plant genomes (shaded regions Table 3 and NDUFA3). Also, clear complex-I-like genes present in the plant genomes were observed in some cases to be consistently missing from the complexes purified by BN-PAGE from the two species (e.g. NDUFA8, NDUFAB1, NDUFB9, NDUFS6). It must be noted that hydrophobic proteins and those that do not digest with trypsin will be likely to remain undetected using these experimental approaches, and so such nondetections cannot be considered definitive evidence of absence. However, it is noteworthy that most of these proteins currently undetected in plant complexes and/or genomes represent components for which no functional role has been described in any system to date (see below).

4. Discussion

4.1. Conserved components of eukaryotic complex I

This genomic and proteomic analysis of the components within complex I from two model higher plants allows the higher plant complex to be compared with those from other eukaryotic lineages. All species considered here, and by implication their eukaryotic lineages, contain the genes for components orthologous to the 14 bacterial components that represent the core of a functional NADH-Q oxidoreductase. In addition, the eukaryotic genomes all clearly contain a set of nine widely conserved accessory components: six from the Iα subcomplex (NDUFA1-2, 5–6, 8–9), the NDUFAB1 subunit, one subunit of the Iβ subcomplex (NDUFB10), and the Fe–S subcomplex protein NDUFS4.

4.2. Function of widely conserved accessory subunits of complex I

The issue of the role of accessory subunits has long intrigued researchers after the extensive list of bovine subunits were identified by Walker et al. [28] over 10 years ago. Some progress has been made in determining the function of these widely conserved accessory proteins and/or their requirement for complex I function or assembly. Progress has been limited to a set of seven proteins to date, all of which fall into the class of nine proteins consistently found in all the eukaryotic genomes analyzed in Table 3.

Four of this set of seven have been shown in mammalian systems to have identifiable functions and deletion of each in either mammals or *N. crassa* leads to partial complex assembly phenotypes. These components are: NDUFAB1 (ACP), NDUFA9 (39 kDa), NDUFS4 (18 kDa) and NDUFA1 (MWFE). The acyl carrier protein (ACP) allows a well-defined non-complex I function associated with complex I in eukaryotic systems (see review by Schulte [29]). A putative role in fatty acid biosynthesis and/or lipid repair has been discussed. One of its clear roles is lipoic acid transfer to the acetyl and succinyl transferases of TCA cycle enzymes via lipoate transferases. Disruption of this gene in *N. crassa* leads to assembly of the membrane arm of the complex only, suggesting that it is required for the proper assembly of the peripheral arm [1]. The NDUFA8 subunit has been shown to bind NADPH, despite this nucleotide not being the major substrate for the complex [30, 31]. Disruption of this subunit leads to an assembled but inactive complex I in *N. crassa*. A role for this protein in the biosynthesis of a redox group required for electron transfer to ubiquionone has been proposed [32]. Papa et al. [33] have reported that the NDUFS4 subunit is phosphorylated and that this phosphorylation is involved in regulation of complex I function via a c-AMP dependent protein kinase. Disruption of NDUFS4 leads to an assembled complex I with altered kinetics of electron transfer to artificial receptors in *N. crassa* [1]. Scheffler's group has demonstrated that a truncation mutant of NDUFA1 in a hamster cell line results in a respiratory deficiency that can be fully restored by complementation with the wild-type NDUFA1 [34]. The loss of this subunit leads to disruption of complex I assembly in the hamster cell line [35].

A further three subunits of the set of seven have been shown to be required for complex I assembly in *N. crassa* through disruption experiments; NDUFA5 (B13), NDUFA8 (PGIV) and NDUFB10 (PDSW) [1]. However, no enzymatic activity of these proteins has yet been elucidated in fungal or mammalian systems. To our knowledge, there have not been deletion phenotypes or roles attributed to NDUFA2 (B8) or NDUFA6 (B14).

Notes to Table 3:
Names of human genes (NDUFXX and NDX) and counterparts in bovine, *E. coli* and *N. crassa* are based on published reviews [1–4], BLAST comparisons and database annotations. Subunits B17.2 and B16.6 are encoded by the previously characterized human genes DAP13 and GRIM-19, due to their presence in the 1α subcomplex they are placed in the NDUFA section of the table. The ESSS subunit encoded by the previously characterized human gene NP17.3 is a component of 1β and thus has been placed with the NDUFBs in the table. Similarity of Arabidopsis and rice predicted protein entries from genome sequencing programs are presented as *E* values from BLASTP and TBLASTN analysis against the set of 45 human complex I protein sequences.
[a] *N. crassa* MWFE ortholog identified by Videira [1]; [b] *E* value and matches to plant genes based on *N. crassa* sequence; and [c] *E* value and match to rice sequences using the Arabidopsis ortholog in a tBLASTn search of TIGR rice Gene Indices sequences.
Asterisks in final columns (BN-PAGE present) indicate the presence of the noted protein in the identifications recorded in Table 1 and 2. Shaded regions indicate the absence of an ortholog for a mammalian component in both fungi and plant genomes to date.

4.3. B16.6 orthologs in plants

The 43rd subunit of complex I in mammals was identified only very recently as the B16.6 subunit. This protein has a known function as GRIM-19, the product of a cell death regulatory gene induced by interferon-b and retinoic acid [6]. This provides a new, but as yet entirely unresearched link between the mitochondrial electron transport chain and apoptotic cell death in mammals. Interestingly, no *N. crassa* ortholog has been identified to date, despite the near completion of the genome sequencing program and the extensive work on complex I in this species. The subunits encoded by plant B16.6 orthologs are clearly present in both the rice and Arabidopsis complex I (Tables 1 and 3). Some evidence for a plant apoptotic/programmed cell death pathway involving mitochondria has been found, including permeability transition pore opening, cytochrome *c* release and cyt *c* dependent induction of DNA laddering [36–38]. However, other known mitochondrially located apoptosis components such as the BCL2 and BAX family of proteins, AIF and cyt *c* dependent CASPASEs, have not been found in plants [39]. Further work on the GRIM-19 homolog in plants may not only reveal its role in complex I but provides another molecular handle for the analysis of programmed cell death in plants and the role of mitochondria in this process.

4.4. The nonconserved accessory proteins of complex I

The eukaryotes analyzed in Table 3 currently vary in the presence or absence of subunits outside of the 14 subunit prokaryotic core and the 9 conserved accessory components. Each species contains a set of seemingly unique proteins that have not yet been found in divergent eukaryotic lineages. These unique classes of components number 14 in mammals (Table 3), 3 in *N. crassa* (14, 17.8, 21.3a) and maybe up to 6 in higher plants (Table 2). Whether some of these proteins actually represent structural orthologs of other nonconserved components in other species is difficult to determine until structural information is available for complex I from multiple species. But based on present knowledge, we conclude that while these lineage-specific proteins in eukaryotes may be tethered to complex I, they may not be true accessory components required for electron transport in the complex but rather may perform other functions.

4.5. Ferripyochelin binding proteins in plant complex I

The abundance of ferripyochelin-binding protein-like subunits in plant complex I remains a mystery. These proteins may have the ability to bind Fe in a chelated form, acting as Fe sensors in the mitochondrion, or even Fe stores for later assembly of Fe–S centers [40], but their actual function awaits further analysis. A 29-kDa protein in the potato complex I preparation by Herz et al. [12] contained an N-terminal sequence of ATEAQAAINEXPDRVKKDYFYGR, which did not match any known proteins at the time of publication. We have found that this sequence is a 100% match to a longer potato TIGR EST (TC24339) and BLAST analysis clearly shows this EST encodes a putative ferripyochelin-binding protein family member. A 30-kDa protein from purified broad bean complex I [10] with a reported N-terminal sequence of ATEAKKHITP may also be a ferripyochelin-binding protein, based on similarity with the potato sequence. These data suggest that the ferripyochelin-binding protein-like family of proteins are genuine members of plant complex I preparations obtained by a variety of different chromatographic and electrophoretic methods and are not an artifact of our purifications. Interestingly, Zabaleta et al. have annotated one of the genes encoding these proteins (At1g47260) in GenBank (AAK28403) as a "novel transcription factor involved in the anther-specific expression of nuclear-encoded mitochondrial complex I genes". Our discovery of the same gene product as a physical component of complex I raises some intriguing questions about the regulation of complex I expression in plants. This needs to be investigated once the full basis for this gene annotation (AAK28403) has been published.

5. Concluding remarks

Extensive analysis of *N. crassa* genetics and mammalian protein biochemistry has identified key eukaryotic components of respiratory complex I. We consider that comparative analysis of other eukaryotic lineages, as exemplified here, will also have a valuable role to play in revealing the likely purpose of the many ancillary components of complex I in higher organisms.

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